Additional file 1: Assessment of Measurement Uncertainty (*MU*)

ddPCR measurements

A total of 12 measurements was performed for each material (3 replicates × 4 runs). Samples were consistently placed in the same positions on the 96-well plate across all measurement days to assess the repeatability of the measurement independently of inter-well variability.

Target gene concentrations were calculated using the following mathematical model:

$$C_{Target} = -\ln\left(\frac{N_{neg}}{N}\right) \cdot D_f \cdot \mathrm{V}_d^{-1} \qquad [\mu L^{-1}]$$

Where:

- C_{target} = copy number concentration of the target genes in the sample material [μL^{-1}]
- D_f = dilution factor
- N_{neg}/N = ratio of negative droplets and accepted droplets
- V_d = droplet volume [µL]

The following tables (Table 1 and 2) report the N_{neg}/N ratios obtained from all measurements.

MET	**Sample 1**				**Sample 2**				**Sample 3**				**Sample 4**			
	d1	**d2**	**d3**	**d4**	**d1**	**d2**	**d3**	**d4**	**d1**	**d2**	**d3**	**d4**	**d1**	**d2**	**d3**	**d4**
Rep 1	0.8212	0.8069	0.8069	0.8057	0.7500	0.7266	0.7062	0.5952	0.2638	0.2427	0.2602	0.1786	0.8477	0.8576	0.8537	0.8245
Rep 2	0.8104	0.8094	0.8133	0.7996	0.7082	0.7305	0.7035	0.5950	0.2823	0.2402	0.2811	0.1857	0.8504	0.8551	0.8529	0.8171
Rep 3	0.8159	0.8119	0.8174	0.8075	0.7075	0.7282	0.7157	0.6157	0.2623	0.2522	0.2640	0.1901	0.8530	0.8534	0.8548	0.8245

Table 1. *MET* N_{neg}/N ratios obtained from all measurements.

RPPH1	**Sample 1**				**Sample 2**				**Sample 3**				**Sample 4**			
	d1	**d2**	**d3**	**d4**	**d1**	**d2**	**d3**	**d4**	**d1**	**d2**	**d3**	**d4**	**d1**	**d2**	**d3**	**d4**
Rep 1	0.8778	0.8799	0.8834	0.8779	0.9040	0.8972	0.8896	0.8351	0.8801	0.8699	0.8720	0.8368	0.8742	0.8875	0.8854	0.8567
Rep 2	0.8835	0.8783	0.8770	0.8766	0.8868	0.8960	0.8894	0.8449	0.8842	0.8682	0.8796	0.8481	0.8838	0.8829	0.8838	0.8670
Rep 3	0.8827	0.8778	0.8838	0.8860	0.8881	0.8918	0.8881	0.8386	0.8673	0.8688	0.8732	0.8434	0.8754	0.8832	0.8816	0.8639

Table 2. *RPPH1* N_{neg}/N ratios obtained from all measurements.

Where d1, d2, d3, and d4 indicate the different run; Rep 1, Rep 2, and Rep 3 indicate the technical replicate in the same run; 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.

ddPCR data elaboration

The overall mean N_{neg}/N ratio can be determined by calculating the average of all values obtained for each target.

To estimate the contribution to the standard uncertainty (u) and the relative standard uncertainty (u_{Rel}) of the N_{neg}/N ratio (Table 3), repeatability and reproducibility components were combined using the following formulas:

$$u\left(\frac{N_{neg}}{N}\right) = \sqrt{repeatability^2 + reproducibility^2}$$

$$u_{Rel}\left(\frac{N_{neg}}{N}\right) = \frac{u\left(\frac{N_{neg}}{N}\right)}{\left(\frac{N_{neg}}{N}\right)} \cdot 100 \ [\%]$$

Where:

- $\left(\frac{N_{neg}}{N}\right)$ = mean of all values
- $repeat$ability = sum of squared standard deviations of technical replicates of each measurement day
- $reproducibility$ = standard deviation of all data

These parameters were used to calculate the final results and to evaluate the *MU*, based on the mathematical model described above.

Target	N_{neg}/N	Mean	u	u_{Rel} [%]
MET	**Sample 1**	0.8105	0.0074	0.9
	Sample 2	0.6902	0.0568	8
	Sample 3	0.2419	0.0378	16
	Sample 4	0.8454	0.0147	1.7
RPPH1	**Sample 1**	0.8804	0.0048	0.5
	Sample 2	0.8791	0.0251	3
	Sample 3	0.8660	0.0162	1.9
	Sample 4	0.8771	0.0106	1.2

Table 3. Overall mean N_{neg}/N ratio and standard (u) and the relative standard uncertainty (u_{Rel}) for *MET* and *RPPH1.* 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

Droplet volume

The mean droplet volume was determined as follows. Approximately 40 µL of droplets were generated by pooling the PCR mixes (including DNA) from all 4 samples for each run. The resulting emulsion was transferred to a multi-well plate and imaged. Around 30 images were acquired across the four different runs and analyzed using ImageJ software. Images were acquired using a Zeiss Axio Observer Z1/7 inverted microscope (Carl Zeiss. Oberkochen) with a 10× objective in phase contrast.

The image analysis was performed according to the method described by Košir et al. [1]. using a pixel-to-micron conversion factor of 1.606 ± 0.003 $[\mu m \cdot pixel^{-1}]$. Droplet diameters (in pixels) were measured for approximately 350 individual droplets.

Assuming droplets have a perfect spherical shape, the droplet volume (in nanoliters) was calculated from the measured diameters. Particularly, the mean of all the measured diameters (D_{mean}), along with the corresponding standard deviation (SD), was used to calculate the final V_d and the propagated uncertainty associated with it following these formulas:

$$R = \frac{D_{mean}}{2}\ [pixel] \qquad u(R) = \frac{SD}{2}\ [pixel]$$

$$r = R \cdot 1.606\ [\mu m] \qquad u(r) = r \cdot \sqrt{\left(\frac{u(R)}{R}\right)^2 + \left(\frac{0.003}{1.606}\right)^2}\ [\mu m]$$

$$V_d = \left(\frac{4}{3} \cdot \pi \cdot r^3\right) \cdot 10^{-6}\ [nL] \qquad u(V_d) = [(4 \cdot \pi \cdot r^2) \cdot u(r)] \cdot 10^{-6}\ [nL]$$

Where:

- R = radius in pixel
- $u(R) =$ uncertainty associated with radius in pixel
- r = radius in micrometers
- $u(r) =$ uncertainty associated with radius in micrometers
- $V_d =$ droplet volume in nanoliters
- $u(V_d) =$ uncertainty associated with droplet volume

The formulas used to propagate uncertainty are derived in accord with LPU (Law of Propagation of Uncertainty) general formula:

$$u = \sqrt{\sum \left[\left(\frac{\delta f}{\delta x_i}\right)^2 \cdot u_{x_i}^2\right]}$$

Where $\left(\frac{\delta f}{\delta x_i}\right)$ is the partial derivative of the function with respect to variables and u_{x_i} is the standard uncertainty associated with each input measurement.

The calculated final V_d was $(7.5 \pm 0.3) \cdot 10^{-4}$ µL, and the corresponding relative standard uncertainty is 4.3%.

Dilution factors (D_f)

The D_f was calculated by accounting for all dilution steps introduced during the measurement procedure. The empirically determined D_f was subsequently corrected using the experimentally measured dispensed volumes of the micropipettes employed at the selected nominal volumes [2]. The dilution factor was assessed through a continuous series of measurements of the theoretical volume. A total of 10 continuous weights in grams were taken, with at least 10 values collected for each measurement. The mean (m_i) and standard deviation for each measurement were calculated, taking into account that the measurements were repeated. Therefore, both the mean and standard deviation were divided by the number of nominal volumes added. The average of the means of the individual measurements was calculated, and the associated uncertainty was propagated as the uncertainty of the mean using the following formula:

$$u(m) = \sqrt{\frac{\sum_{i=1}^{n} m_i^2}{n}}\ [g]$$

Where *n* is the number of measurements performed. Additionally, the repeatability was calculated as the standard deviation of all measurement means. The measured volume was calculated by dividing the average weight means by the density of water. The density value was selected by considering the temperature range between 20-25 °C, starting from tabulated values [3]. The average value and the associated uncertainty were calculated using the following formulas:

$$\rho_{H_2O} = \frac{\rho_{20℃} + \rho_{25℃}}{2}\ [g \cdot L^{-1}] \qquad u(\rho_{H_2O}) = \frac{\rho_{20℃} - \rho_{25℃}}{2}\ [g \cdot L^{-1}]$$

The measured dispensed volumes and their uncertainty were calculated with the formulas reported hereafter:

$$V = \left(\frac{m}{\rho_{H_2O}}\right) \cdot 10^{-6}\ [\mu L]$$

$$u(V) = V \cdot \sqrt{\left(\frac{u(m)}{m}\right)^2 + \left(\frac{u(\rho_{H_2O})}{\rho_{H_2O}}\right)^2 + repeatability^2}\ [\mu L]$$

Finally, the dilution factor was calculated, and the standard uncertainty was propagated using the following formulas:

$$D_f = \frac{V_{PCR\ Mix} + V_{DNA}}{V_{DNA}}$$

$$u(D_f) = D_f \cdot \sqrt{\left(\frac{u(V_{PCR\ Mix} + V_{DNA})}{V_{PCR\ Mix} + V_{DNA}}\right)^2 + \left(\frac{u(V_{DNA})}{V_{DNA}}\right)^2}$$

Table 4 summarizes the results obtained for the estimation of the D_f together with the theoretical and empirical pipetting volumes used for its determination and calculation of the associated standard uncertainty.

Measurand	Theoretical volume [µL]	Empirical volume [µL]	Standard uncertainty [µL]	Relative standard uncertainty [%]	Estimated D*f*
Diluent (PCR Mix)	62.4	62.80	0.04	0.06	19.11 ± 0.04
Template (DNA)	3.6	3.468	0.008	0.2	

Table 4. Theoretical and empirical pipetting volumes used to determine the D_f, with their associated uncertainty.

The final experimentally estimated D_f, along with its associated standard uncertainty (u), was 19.11 ± 0.04, and the corresponding relative standard uncertainty was 0.23%.

Results and uncertainty evaluation

Based on the previously described mathematical model and considering the sources of uncertainty outlined, the concentrations of the two targets, *MET* and *RPPH1*, were calculated for the 4 samples, along with their measurement uncertainties. The uncertainty budgets are presented below (Tables 5-12) .

1. Seraseq ctDNA Mutation Mix v4 AF0.1%

MET

	Value - X	Uncertainty - u(X)	Sensitivity coefficient - Ci	Variance - u_i^2	Weight [%]
D_f	19.11	0.04	0.3	0.00015	0.2
V_d [nL]	0.76	0.03	7	0.05	75
N_{neg}/N	0.811	0.007	18	0.017	25
			Combined variance - u_c^2 [nL^{-1}]	0.07	
	C_{target} [nL^{-1}]	5.301	**Combined standard uncertainty - u_c [nL^{-1}]**	0.3	
	C_{target} [μL^{-1}]	5301	**Combined standard uncertainty - u_c [μL^{-1}]**	264	
			Relative combined standard uncertainty [%]	4.98	

RPPH1

	Value - X	Uncertainty - u(X)	Sensitivity coefficient - Ci	Variance - u_i^2	Weight [%]
D_f	19.11	0.04	0.17	0.00005	0.2
V_d [nL]	0.76	0.03	4	0.019	75
N_{neg}/N	0.880	0.005	16	0.006	25
			Combined variance - u_c^2 [nL^{-1}]	0.03	
	C_{target} [nL^{-1}]	3.214	**Combined standard uncertainty - u_c [nL^{-1}]**	0.16	
	C_{target} [μL^{-1}]	3214	**Combined standard uncertainty - u_c [μL^{-1}]**	160	
			Relative combined standard uncertainty [%]	4.97	

2. Seraseq ctDNA Mutation Mix v4 AF0.5%

MET

	Value - X	Uncertainty - u(X)	Sensitivity coefficient - Ci	Variance - u_i^2	Weight [%]
D_f	19.11	0.04	0.5	0.0005	0.03
V_d [nL]	0.76	0.03	12	0.16	10
N_{neg}/N	0.69	0.06	21	1.4	90
			Combined variance - u_c^2 [nL^{-1}]	1.6	
	C_{target} [nL^{-1}]	9.356	**Combined standard uncertainty - u_c [nL^{-1}]**	1.3	
	C_{target} [μL^{-1}]	9356	**Combined standard uncertainty - u_c [μL^{-1}]**	1256	
			Relative combined standard uncertainty [%]	13.43	

RPPH1

	Value - X	Uncertainty - u(X)	Sensitivity coefficient - Ci	Variance - u_i^2	Weight [%]
D_f	19.11	0.04	0.17	0.00006	0.03
V_d [nL]	0.76	0.03	4	0.02	10
N_{neg}/N	0.88	0.03	16	0.17	90
			Combined variance - u_c^2 [nL^{-1}]	0.19	
	C_{target} [nL^{-1}]	3.250	**Combined standard uncertainty - u_c [nL^{-1}]**	0.44	
	C_{target} [μL^{-1}]	3250	**Combined standard uncertainty - u_c [μL^{-1}]**	436	
			Relative combined standard uncertainty [%]	13.41	

3. Seraseq ctDNA Mutation Mix v4 AF5%

MET

	Value - X	Uncertainty - u(X)	Sensitivity coefficient - Ci	Variance - u_i^2	Weight [%]
D_f	19.11	0.04	1.9	0.007	0.09
V_d [nL]	0.76	0.03	47	2	32
N_{neg}/N	0.24	0.04	60	5	68
			Combined variance - u_c^2 [nL^{-1}]	8	
	C_{target} [nL^{-1}]	35.805	**Combined standard uncertainty - u_c [nL^{-1}]**	3	
	C_{target} [μL^{-1}]	35805	**Combined standard uncertainty - u_c [μL^{-1}]**	2739	
			Relative combined standard uncertainty [%]	7.65	

RPPH1

	Value - X	Uncertainty - u(X)	Sensitivity coefficient - Ci	Variance - u_i^2	Weight [%]
D_f	19.11	0.04	0.19	0.00007	0.07
V_d [nL]	0.76	0.03	4.8	0.02	25
N_{neg}/N	0.866	0.016	17	0.07	75
			Combined variance - u_c^2 [nL^{-1}]	0.10	
	C_{target} [nL^{-1}]	3.631	**Combined standard uncertainty - u_c [nL^{-1}]**	0.3	
	C_{target} [μL^{-1}]	3631	**Combined standard uncertainty - u_c [μL^{-1}]**	312	
			Relative combined standard uncertainty [%]	8.60	

4. Seraseq ctDNA Mutation Mix v4 WT

MET

	Value - X	Uncertainty - u(X)	Sensitivity coefficient - Ci	Variance - u_i^2	Weight [%]
D_f	19.11	0.04	0.2	0.00010	0.10
V_d [nL]	0.76	0.03	6	0.03	35
N_{neg}/N	0.845	0.015	17	0.06	65
			Combined variance - u_c^2 [nL^{-1}]	0.10	
	C_{target} [nL^{-1}]	4.238	**Combined standard uncertainty - u_c [nL^{-1}]**	0.3	
	C_{target} [μL^{-1}]	4238	**Combined standard uncertainty - u_c [μL^{-1}]**	310	
			Relative combined standard uncertainty [%]	7.33	

RPPH1

	Value - X	Uncertainty - u(X)	Sensitivity coefficient - Ci	Variance - u_i^2	Weight [%]
D_f	19.11	0.04	0.17	0.00006	0.11
V_d [nL]	0.76	0.03	4	0.02	40
N_{neg}/N	0.877	0.011	16	0.03	60
			Combined variance - u_c^2 [nL^{-1}]	0.05	
	C_{target} [nL^{-1}]	3.308	**Combined standard uncertainty - u_c [nL^{-1}]**	0.2	
	C_{target} [μL^{-1}]	3308	**Combined standard uncertainty - u_c [μL^{-1}]**	226	
			Relative combined standard uncertainty [%]	6.83	

Tables 5-12. Uncertainty budgets of *MET* and *RPPH1* targets quantified for the Seraseq Reference Materials.

MET CNV was calculated by determining the ratio between *MET* (target gene) and *RPPH1* (reference gene). The *MET* CNV was obtained by dividing the concentration (μL^{-1}) of the target gene (MET_{Conc}) by the concentration of the reference gene ($RPPH1_{Conc}$).

$$MET - CNV = \frac{MET_{Conc}}{RPPH1_{Conc}}$$

And the associated uncertainty (u) is calculated as:

$$u(MET - CNV) = MET - CNV \cdot \sqrt{\left(\frac{u_c(MET_{Conc})}{MET_{Conc}}\right)^2 + \left(\frac{u_c(RPPH1_{Conc})}{RPPH1_{Conc}}\right)^2}$$

In order to ensure a 95% of confidence level, $u(MET - CNV)$ was multiplied by the coverage factor $k = 2$ and the expanded standard uncertainty ($U(MET - CNV)$) was obtained together with the relative expanded standard uncertainty ($U_{Rel}(MET - CNV)$).

Results are reported in Table 13.

	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**
MET_{Conc} **[µL^{-1}]**	5301	9356	35805	4238
$u(MET_{Conc})$ **[µL^{-1}]**	264	1256	2739	310
$RPPH1_{Conc}$ **[µL^{-1}]**	3214	3250	3631	3308
u($RPPH1_{Conc}$**) [µL^{-1}]**	160	436	312	226
MET-CNV	1.65	2.9	9.8	1.28
u(MET-CNV)	0.12	0.5	1.1	0.12
U(MET-CNV) with k=2	0.23	1.1	2.3	0.26
U_{Rel} ***(MET-CNV)*** **[%]**	14.06	37.95	23.01	20.03

Tables 13. *MET* CNV results with *MU*. MET_{Conc} and $RPPH1_{Conc}$: target genes concentration; u: standard uncertainty; U: expanded uncertainty; U_{Rel}: relative expanded uncertainty; 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.

References

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