Evaluation of measurement uncertainty of chip-based digital PCR using DNA certified reference material with determination of partition volume by scanning electron microscopy

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1. Introduction

Currently, digital PCR (dPCR) has been increasingly used for DNA quantification in many areas. However, the the measurement uncertainty of the chip-based dPCR (cdPCR) was not well investigated. In this research, we evaluated the measurement uncertainty of the cdPCR using DNA certified reference material, including determination of the partition volume.

We evaluated the partition volume on the chip by scanning electron microscopy (SEM) and evaluated the measurement uncertainty of the cdPCR using DNA CRM, NMIJ CRM 6205-a whose DNA mass concentration is certified. We also evaluated the linearity range from 1.5 copies/μL to 450 copies/μL in dPCR reaction mixture.

2. DNA CRM (NMIJ CRM 6205-a)

NMIJ CRM 6205-a
- This CRM consists of two kinds of 600-bp DNA solution having different sequences (DNA600-G and DNA600-T).
- The certified values of total DNA mass fraction were obtained by following analytical methods;
  - ➢ Nucleobase measurement by formic acid hydrolysis/LC-IDMS
  - ➢ Phosphorous measurement by ICP-MS

This CRM is intended to be used to assign the value of DNA sample for the evaluation and control the precision of DNA analytical methods.

3. Materials & Methods

3.1 Sample preparation
NMIJ CRM 6205-a was diluted to obtain]1.5, 3, 15, 30, 75, 125, 250 and 450 copies/μL in dPCR mixture.
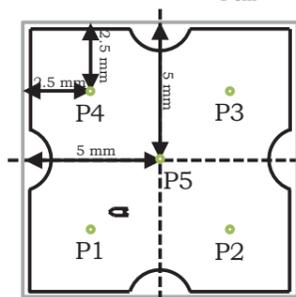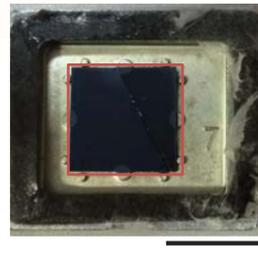
3.2 SEM for partition volume evaluation
3.2.1 Instrument
JSM-7100F (JEOL)

3.2.2 Observing condition

Parameter	Condition
Accelerating voltage	5 kV
Beam current	300 pA
Detector	BSE comp.
Magnification	× 450 for area × 250 for thickness
Calibration	With MRS-6 (Geller MicroAnalytical laboratory)

3.2.3 The observing places on chip

1 cm

P4 P3
P5
P1 P2

5 places of both front and back on the chip and thickness of the chip were observed by SEM.

3.3 Digital PCR for DNA quantification

3.3.1 Instrument
Quant Studio 3D Digital PCR System (Thermo Fisher Scientific)

3.3.2 The sequences of primer and probe

	Sequence
F-primer	5'-CACCCGTTATCTCAGCCCTAAT-3'
R-primer	5'-GGGTAGCTATGAGGCATGGATT-3'
probe	5'-VIC-TCTGCGGTTTAGTCTGG-MGB-3'

3.3.3 PCR reaction mixture

	Stock	Working
Master Mix	2×	7.5 μL
F-primer	10 pM	1.35 μL
R-primer	10 pM	1.35 μL
Probe	10 pM	0.375 μL
H$_2$O	–	1.425 μL
DNA		3 μL
Total		15 μL

3.3.4 PCR condition

Temperature	Time	Cycle
96 ºC	10 min	1
56 ºC	2 min	40
98 ºC	30 sec	
56 ºC	2 min	1
10 ºC	∞	

4. Results & Discussion

4.1 Partition volume evaluation by SEM
4.1.1 Typical SEM images of the well

Side A (Front)	Side B (Back)	Cross section

100 μm 100 μm 200 μm
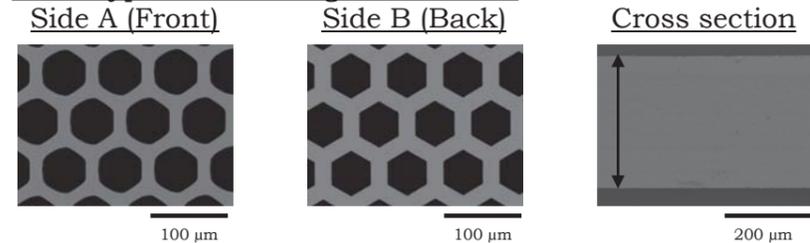
- The surface are of 45 partitions and the thickness of 3 cross sections were observed by SEM.
- The shape of partition was hexagonal prism.

4.1.2 Calculation of partition volume

$$V = \frac{t}{3}(S_A + S_B + \sqrt{S_A S_B})/1000$$

V : Partition volume (pL)
t : Thickness (μm)
S_A : Side A surface area (μm^2)
S_B : Side B surface area (μm^2)

Hexagonal prism

	Average
S_A (μm^2)	2661
S_B (μm^2)	2260
t (μm)	306
V (pL)	753

4.1.3 Uncertainty of partition volume

Uncertainty component	Relative uncertainty (%)
Side A surface area	0.37
Side B surface area	0.09
Thickness	0.24
Magnification calibration	0.45
Combined uncertainty	0.64
Expanded uncertainty (k=2)	1.27

Partition volume: **753.2 pL ± 9.6 pL**

4.2 Accuracy Validation by DNA CRM
4.2.1 Linearity

y = 0.0022x - 0.0012
R² = 0.9999

From 1.5 to 450 copies/μL in PCR mixture, good linearity was observed, but there were variations at lower concentration range.
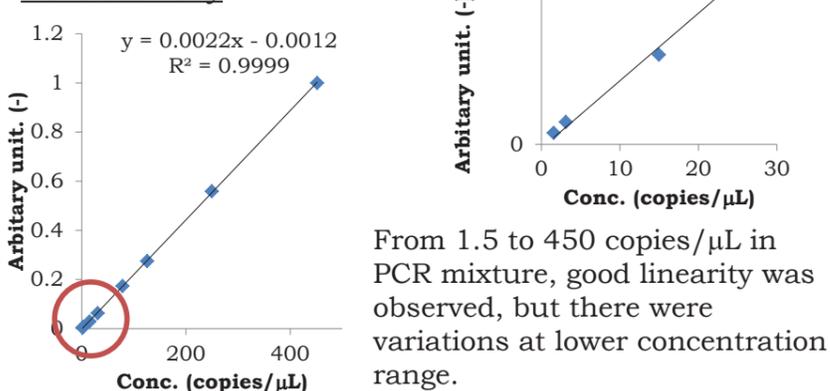
4.2.2 DNA quantification by the chip-based dPCR

Theoretical value   Result

Good agreement with the certified value!
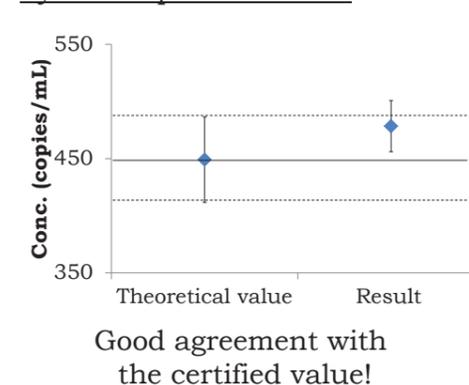
4.2.3 Measurement uncertainty of cdPCR

Uncertainty component	Relative uncertainty (%)
Sample preparation	0.00
Variation between chips	**2.24**
Weight (Sample preparation)	0.00
Weight (PCR mixture)	0.23
Partition volume	0.64
Combined uncertainty	2.34
Expanded uncertainty (k=2)	**4.69**

The main uncertainty component of was variation between chips and the expanded uncertainty was less than 5%.

5. Conclusions

- The main uncertainty component of cdPCR was variation between chips and the expanded uncertainty was less than 5 %.
- By evaluating partition volume on the chip, it was able to quantify DNA accurately and SI traceable by using cdPCR.