Supplemental information

Establishment of reference measurement procedure and reference material for Treponema pallidum

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Supplemental Tables

Table S1 Quantitative results in a serial dilution in duplex dPCR format

Method A (copies/reaction)			Method B (copies/reaction)		
Input concentration	Output concentration	CV	Input concentration	Output concentration	CV
2.82×10^4	2.85×10^4	2.29%	2.81×10^4	2.85×10^4	2.16%
2816.81	2816.81	4.53%	2809.03	2809.03	4.50%
280.65	263.19	4.69%	279.88	261.95	5.12%
57.05	62.19	20.33%	56.89	61.75	20.04%
11.49	9.17	59.31%	11.46	8.73	38.24%
2.28	1.47	77.82%	2.27	1.47	77.82%

Table S2 The 15 highest values for blank values of duplex assay (copies/reaction)

No.	Method A	Method B
60	2.64	2.64
59	2.64	2.64
58	2.64	2.64
57	2.42	2.64
56	2.42	2.64
55	2.42	2.64
54	2.42	2.64
53	2.42	2.20
52	2.20	2.20
51	2.20	2.20
50	2.20	2.20
49	2.20	2.20
48	2.20	1.98
47	2.20	1.76
46	1.98	1.32

Table S3 Homogeneity assessment

	Method A (copies/μL)		Method B (copies/μL)	
Unit	Repeat 1	Repeat 2	Repeat 1	Repeat 2
1	2.27×10^6	2.57×10^6	2.30×10^6	2.59×10^6
2	2.33×10^6	2.28×10^6	2.32×10^6	2.28×10^6
3	2.07×10^6	2.14×10^6	2.07×10^6	2.14×10^6
4	2.13×10^6	2.24×10^6	2.13×10^6	2.24×10^6
5	2.21×10^6	2.10×10^6	2.22×10^6	2.10×10^6
6	2.26×106	2.19×10^6	2.27×10^6	2.19×10^6
7	2.15×10^6	2.35×10^6	2.15×10^6	2.36×10^6
8	2.17×10^6	2.20×10^6	2.16×10^6	2.21×10^6
9	2.29×10^6	2.38×10^6	2.29×10^6	2.38×10^6
10	2.29×10^6	2.35×10^6	2.28×10^6	2.35×10^6
11	2.15×10^6	2.24×10^6	2.15×10^6	2.25×10^6
Q_1	1.69×10^{11}		1.89×10^{11}	
Q_2	9.70×10^{10}		9.68×10^{10}	
v_1	10		10	
v_2	11		11	
F	1.92		2.15	
$F_{(10,11)}$	2.85		2.85	
Conclusion	$F<F_{(10,11)}$, reference material is homogeneous			

Table S4 Components of the uncertainty

Components	Values
u_1	2.95%
u_2	1.93%
u_3	3.44%
u_c^2	0.24%
u_c	4.92%
$U=u_c\times k$ (k=2)	9.9%
Reference values (copies/μL)	2.21×10^6
extended uncertainty (copies/μL)	0.22×10^6

Notes: $\boldsymbol{u_1}$ Assignment values. $\boldsymbol{u_2}$ Homogeneity. $\boldsymbol{u_3}$ Stability in long-term storage. $\boldsymbol{u_c}$ Combined uncertainty.

Four wells were randomly selected from each of two different droplet reaction plates. Droplets were photographed under a microscope (Olympus, BX51) after generation. Four separate images containing at least 25 complete droplets were captured for each well (Fig. S2) and droplet volumes were estimated by using the ImageJ software (1.48V, ImageJ). Finally, the average of the total droplet volumes for each well and its uncertainty were calculated (Table S5).

Table S5 Uncertainty in the partition volume of the Sniper platform (nL)

	Plate 1	Plate 2
Well 1	0.82	0.78
	0.73	0.77
	0.79	0.83
Well 2	0.78	0.79
	0.76	0.78
	0.74	0.77
	0.69	0.82
Well 3	0.81	0.75
	0.80	0.78
	0.79	0.77
	0.75	0.78
Well 4	0.73	0.77
	0.79	0.73
	0.78	0.77
	0.81	0.77
	0.77	0.81
Mean	0.78	
SD	0.03	
u_{rel}	1.71%	

Note：

$$u_{droplet\ volume\ rel} = \sqrt{u_{droplet\ volume}^{2} + u_{effects\ of\ the\ facal\ plane}^{2} + u_{microscope\ calibration}^{2} + u_{micrometer}^{2}}$$

The uncertainty of the droplet volume was 0.68%. The effects of the facal plane was 0.15%, the microscope calibration was 1.56%, the micrometer was 0.15%.[1] The relative uncertainty of the droplet volume was calculated to be 1.71%.

Supplemental Figures

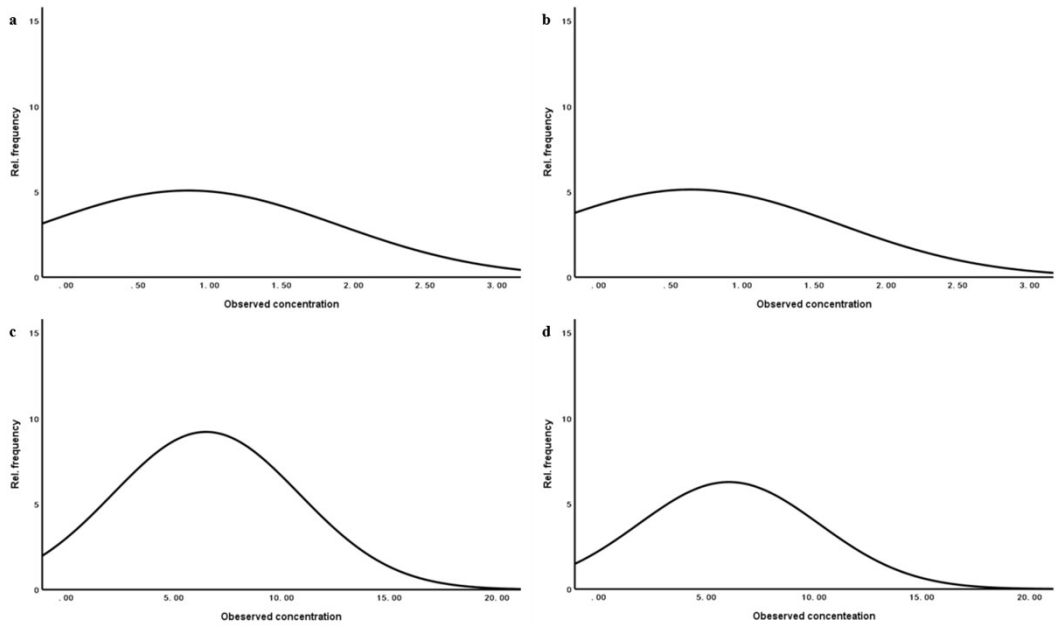

Fig S1 Distribution of the results of 60 measurements for limit of blank (LoB) and limit of detection (LoD)**. a** 60 measurements of the LoB for Method A. **b** 60 measurements of the LoB for Method B. **c** 60 measurements of the LoD for Method A. **d** 60 measurements of the LoD for Method B. The assay results for both LoB and LoD were non-Gaussian distributed

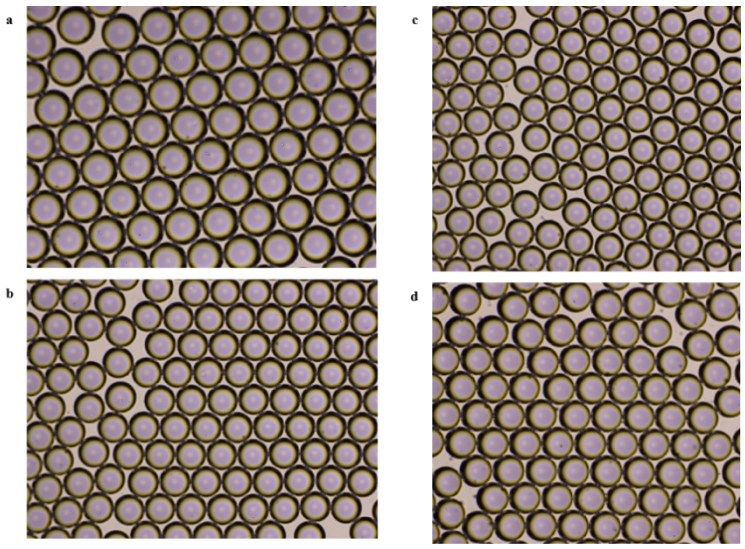

Figure S2 Droplets generated from Sniper DQ24 digital PCR as scanned by Olympus microscope with a 10× field lens.

Reference

1. L. Dong, Y. Meng, Z. Sui, J. Wang, L. Wu and B. Fu, *Scientific reports*, 2015, **5**, 13174.