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Note

Quantification of *Legionella* DNA certified reference material by digital droplet PCR

Maud Baume[a,*], Astrid Cariou[b], Adélaïde Leveau[b], Noémie Fessy[a], Frédéric Pastori[b], Sophie Jarraud[a], Sophie Pierre[b]

[a] Hospices Civils de Lyon, Centre National de Référence des Légionelles, Lyon, France
[b] Bio-Rad, Food Science Division, Marnes-la-Coquette, France

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ABSTRACT

A value was assigned in 2009 to the *Legionella* DNA Certified Reference Material, and the stability study conducted using quantitative PCR found a low level of degradation. Herein, the Digital Droplet PCR method for *Legionella* DNA was qualified and used to provide absolute quantification of the CRM.

Legionnaire's disease (LD) is acquired by the inhalation of aerosols from *Legionella*-contaminated environmental sources. The prevention of LD is achieved through monitoring of *Legionella* levels in water. This can be done by quantifying *Legionella* DNA using PCR-based methods, and to ensure calibration of these methods a Certified Reference Material (CRM) has been available since 2009. In the present study, a novel method of absolute quantification of DNA (Pinheiro et al., 2012) was qualified for *Legionella* DNA quantification and tested on the CRM to assess its fitness to be used (Bhat and Emslie, 2016; White et al., 2015).

The value of the CRM for *Legionella* DNA was assigned by limit dilution assays performed during a ring trial in 2009; an accelerated stability study established the shelf-life of the CRM (Baume et al., 2013). Its stability has since been verified in real-time by assessment of the Ct using a quantitative PCR (qPCR) method (*Legionella* and *Legionella pneumophila* GeneDisc Duo; Pall GeneSystems, Bruz, France) analyzed on GeneDisc Cycler (Pall GeneSystems). The accepted variation is ± 0.50 log (twice the uncertainty of the qPCR method used). Since 2009, no variation > 0.15 log has been detected in the CRM samples tested, but a slight degradation is noticeable (Fig. 1). This method was chosen for stability assays because there was no direct DNA quantification method available at the time. The limit of this method is its dependence on the CRM itself for calibration, so if a variation is detected, it cannot be attributed with certainty to a variation in the DNA concentration in the CRM as it could be due to the uncertainty of the qPCR method. Furthermore, it requires multiple CRM tubes to be tested simultaneously to establish a mean value, which is time-consuming and uses a large number of CRM tubes.

In order to quantify the *Legionella* CRM by Digital Droplet PCR (ddPCR), the method linearity, limit of detection (LOD) and limit of quantification (LOQ) were first qualified. The ddPCR reactions were performed on 5 µL of CRM dilutions and ddPCR amplification mix (ddPCR™ Supermix for Probes (No dUTP); Bio-Rad) in a 25 µL final volume. The effective reaction size used to generate the droplets was 20 µL. The primers/probe* concentrations were 900 nM/250 nM respectively. The digital-PCR thermal profile was 95 °C for 10 min (enzyme activation) for 1 cycle, 95 °C for 30 s (denaturation) then 58 °C for 1 min (annealing/extension) for 59 cycles, 98 °C for 10 min (enzyme deactivation) for 1 cycle, 4 °C for 30 min (hold; slow ramp rate 2 °C/s). The droplet generation, the amplification step, and reading were performed using a QX 200™ Droplet Generator, C1000 Touch™ Thermal Cycler, and QX 200™ Droplet Reader (Bio-Rad), respectively. The target chosen for the ddPCR assay was a *Legionella pneumophila* specific gene (*LpSpGe**), and the CRM was tested on dilutions according to the ISO/TS 12869 protocol ("ISO TS 12869

* Corresponding author.
E-mail address: maud.baume@chu-lyon.fr (M. Baume).

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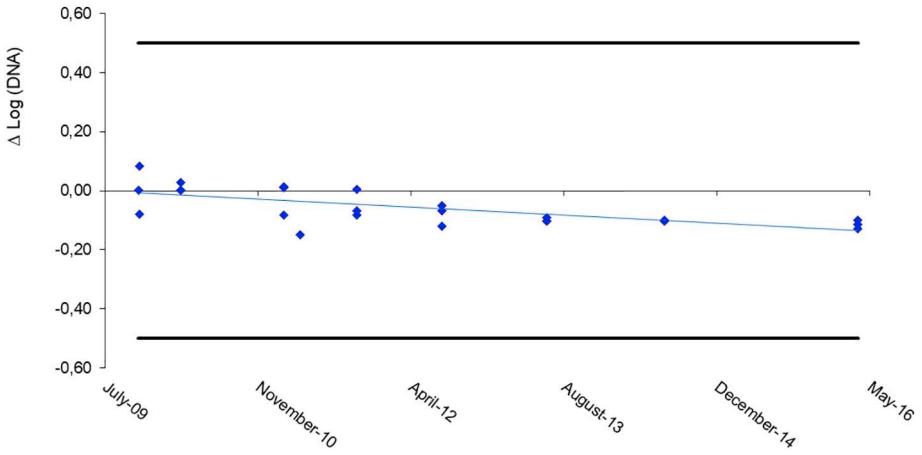

Fig. 1. Variation of DNA quantity over time. Each point represent the difference between the initial value of the CRM and the quantity estimated by qPCR experiments over time, expressed in Log of Genomic Unit. Limits (+0.50 and −0.50 Log) represented by bold lines. Linear regression parameters: y = −5E-05x + 2.1697; R^2 = 0.4664.

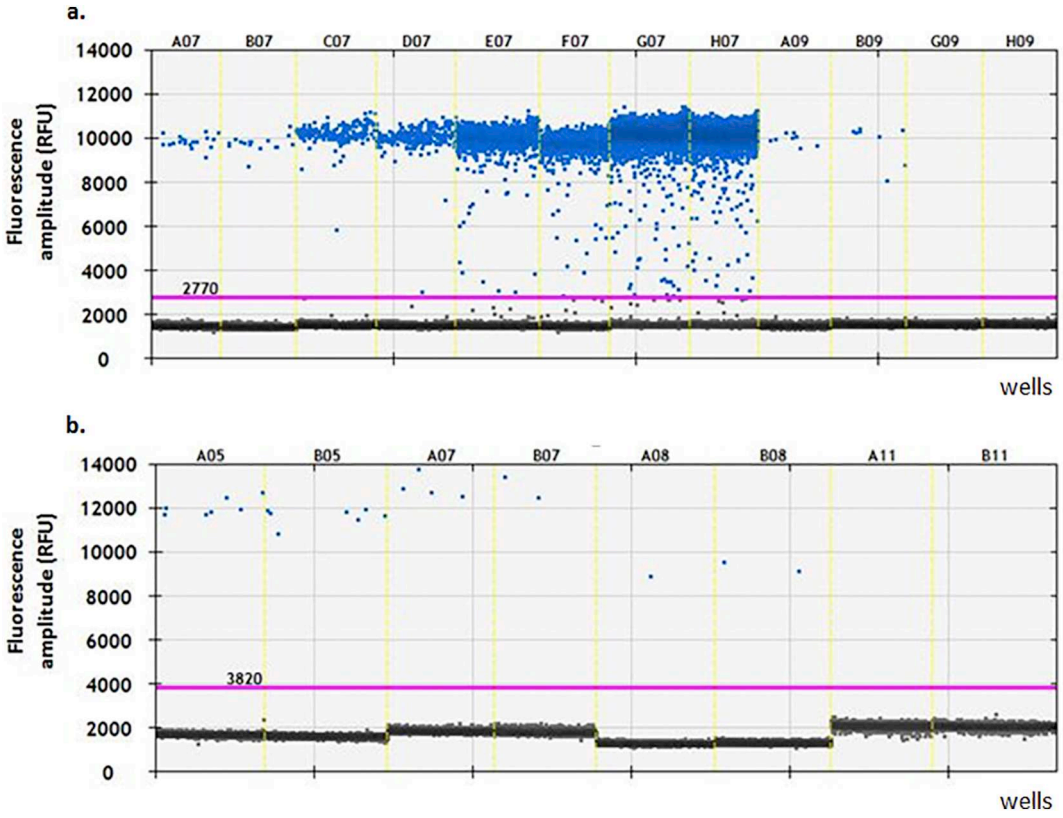

Fig. 2. ddPCR 1D profiles of linearity, LOQ and LOD. (a). Linearity-LOQ. A07-B07: 42GU/reaction; C07-D07: 420 GU/reaction; E07-F07: 4200 GU/reaction; G07-H07: 42000 GU/reaction; A09-B09: 15 GU/reaction (LOQ); G09-H09: negative control. (b). LOQ-LOD. A05-B05: LOQ at 15 GU/reaction; A07-B07: LOD at 5 GU/reaction; A08-B08: LOD at 2.5 GU/reaction; A11-B11: LOD at 1.25 GU/reaction. Duplicate wells per condition. RFU: Fluorescence Unit. Thresholds represented by thick lines at 2770 (a) and 3820 (b) RFU. Positive population: above threshold; negative population: below threshold.

Water quality – Detection and quantification of Legionella spp. and/or Legionella pneumophila by concentration and genic amplification by quantitative polymerase chain reaction (qPCR),", 2012). As negative control, TE pH 8.3 was used instead of the DNA sample. The ddPCR analyses were performed using the QuantaSoft™ Software (Bio-Rad), and only results with droplets number > 10,000 were analyzed. For linearity, 5 independent dilutions of the CRM were tested in duplicate; the concentrations tested were 42,000, 4200, 420, 42, and 15 Genome Units per reaction (GU/reaction). For LOD, 10 independent dilutions of the CRM were tested in duplicate; the concentrations tested were 5, 2.5, and 1.25 GU/reaction. For LOQ, 10 independent dilutions of the CRM were tested in simplicate and

Table 1
Results of linearity in simplicate and duplicate analysis.

Samples (GU/reaction)	Mean (log GU/reaction)	SD (log GU/reaction)	Bias (log GU/reaction)	E_{LIN} (log GU/reaction)
42,000	4.57	0.03	−0.06	0.07
	4.58	0.04	−0.04	0.05
4200	3.56	0.04	−0.06	0.07
	3.57	0.04	−0.06	0.07
420	2.56	0.05	−0.06	0.08
	2.55	0.07	−0.07	0.10
42	1.62	0.09	−0.01	0.09
	1.56	0.07	−0.06	0.09
15	1.14	0.14	−0.04	0.14
	1.15	0.10	−0.03	0.11

GU: Genomic Unit; SD: Standard Deviation; $E_{LIN:}$ Linearity Exactitude.
In mean, SD, Bias and E_{LIN} columns: first line = simplicate analysis, second line: duplicate analysis.

duplicate; the concentrations tested were 25 and 15 GU/reaction respectively.

The ddPCR 1D profile for linearity is presented in Fig. 2a, and for LOD and LOQ in Fig. 2b. For all conditions, the fluorescence ratio between the positive and negative populations is > 2, allowing a good separation of the 2 populations to quantify the samples.

According to ISO 12869, the Linearity Exactitude (E_{LIN}), expressed as $Log_{10,}$ should be ≤0.15 to be validated, which was the case for 42,000 to 15 GU/reaction (Table 1).

The LOD is validated if 90% of the results are positive, which was the case at 2.5 GU/reaction when the analyses were performed in duplicate, and at 5 GU/reaction in simplicate. The LOQ is validated if LOQ Exactitude (E_{LQ}) was ≤0.15 (expressed as Log_{10}), which was the case at 15 GU/reaction when the analyses were performed in duplicate, and 25 GU/reaction in simplicate.

Then, to estimate the CRM value by ddPCR, 10 randomly chosen samples of the CRM were each rehydrated in 255 µL of Tris buffer to an estimated concentration of 41,667 GU/µL. Each sample was diluted independently into 8 replicates to an estimated concentration of 417 GU/µL, and 5 µL of each replicate were analyzed by ddPCR (Table 2). The droplet number was between 10,000 and 20,000, but as the DNA concentration was well within the working range (10 to 120,000 GU/reaction) (Table 2), this had no significant impact on quantification results as only at the lower and the higher end of the working range does fewer droplets in a well lead to significant variation in DNA concentration (Deprez et al., 2016).For each sample tested, the mean, median, and standard deviation (SD) of the 8 replicates were calculated. A linear mixed model was used to estimate the within and between replicate variability, and to estimate the overall mean value. The estimated concentration ± SD obtained by ddPCR was 1782.3 ± 144.5 GU / 5 µL, leading to an estimated 9,089,730 ± 736,950 GU per tube.

Compared to the certified value of 2009 (10,627,646 ± 1,631,787), the estimated value is not significantly different (p-value = 0.065) and is consistent with a slight degradation of the CRM, but this cannot be confirmed owing to the difference in methods used. These results provide a new value for the CRM which can be the reference for future stability monitoring. It also shows the ddPCR method can be useful for reference materials, as it can be used to assign a value with reduced uncertainty and is less time-consuming than qPCR. The additional advantage is its independence from pre-existing standards as well as fewer number of samples to be tested, which makes it the preferred method for stability monitoring.

*Bio-Rad confidential information: Available on request.

Table 2
Results of the ddPCR assays on *Legionella* DNA CRM.

	Tube 701 n = 8	Tube 717 n = 8	Tube 725 n = 8	Tube 736 n = 8	Tube 742 n = 8
Mean number of accepted droplets	15,206	16,035	17,192	11,503	17,661
Range number of accepted droplets	13,443–17,107	13,136–17,569	13,757–18,996	14,539–18,914	15,266–18,886
Mean DNA concentration in GU/5 µL (SD)	1868.12 (49.69)	2365.31 (164.97)	1772.12 (119.38)	1728.75 (77.04)	1718.12 (158.70)
Median DNA concentration in GU/5 µL	1872.50	2348.75	1723.00	1707.50	1647.50
Range of DNA concentration in GU/5 µL	1785.00–1932.50	2157.50–2645.00	1655.00–1960.00	1682.50–1915.00	1527.50–1960.00

	Tube 868 n = 8	Tube 929 n = 8	Tube 953 n = 7[a]	Tube 969 n = 8	Tube 980 n = 8	Total n = 71	
Mean number of accepted droplets	16,201	17,028	17,655	16,116	16,428		
Range number of accepted droplets	12,823–18,633	14,178–18,887	16,552–18,558	12,923–18,982	14,521–18,316		
Mean DNA concentration in GU/5 µL (SD)	1545.62192.41)	1738.88 (68.99)	1645.36 (90.83)	1591.25 (110.80)	1848.44 (75.71)	1776.66 (258.36)	
Median DNA concentration in GU/5 µL	1583.75	1752.50	1632.50	1598.75	1816.25	1715.00	
Range of DNA concentration in GU/5 µL	1127.50–1752.50	1585.00–1805.00	1537.50–1822.50	1410.00–1760.00	1765.00–1970.00	1127.50–2645.00	

GU: Genomic Unit; SD: Standard Deviation.
[a] results of one replicate of tube 953 were discarded due to a technical issue.

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