Validation of droplet digital PCR for the detection and absolute quantification of *Borrelia* DNA in *Ixodes scapularis* ticks

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SUMMARY

We evaluated the QX200 Droplet Digital PCR (ddPCR™, Bio-Rad) system and protocols for the detection of the tick-borne pathogens *Borrelia burgdorferi* and *Borrelia miyamotoi* in *Ixodes scapularis* nymphs and adults collected from North Truro, Massachusetts. Preliminary screening by nested PCR determined positive infection levels of 60% for *B. burgdorferi* in these ticks. To investigate the utility of ddPCR as a screening tool and to calculate the absolute number of bacterial genome copies in an infected tick, we adapted previously reported TaqMan®-based qPCR assays for ddPCR. ddPCR proved to be a reliable means for detection and absolute quantification of control bacterial DNA with precision as low as ten spirochetes in an individual sample. Application of this method revealed the average carriage level of *B. burgdorferi* in infected *I. scapularis* nymphs to be 2291 spirochetes per nymph (range: 230–5268 spirochetes) and 51 179 spirochetes on average in infected adults (range: 5647–115 797). No ticks naturally infected with *B. miyamotoi* were detected. The ddPCR protocols were at least as sensitive to conventional qPCR assays but required fewer overall reactions and are potentially less subject to inhibition. Moreover, the approach can provide insight on carriage levels of parasites within vectors.

Key words: droplet digital PCR, *Borrelia miyamotoi*, *Borrelia burgdorferi*, pathogen detection.

INTRODUCTION

The prevalence of tick-borne illnesses and the pathogenic agents, which cause them are of growing concern. In the USA, the Centers for Disease Control estimate over 300 000 cases of Lyme disease per year, making it the most widespread arthropod-borne disease in the nation (CDC, 2015). Improved detection and diagnostic approaches for tick-borne diseases are sought for patient, public health and environmental surveillance. Droplet Digital™ PCR (ddPCR) (Bio-Rad Laboratories, Inc., Hercules, CA) is a promising technology for this task because it allows for sensitive, specific detection of single template molecules as well as precise quantification of target DNA.

Borrelia burgdorferi and *Borrelia miyamotoi* are infectious spirochetes (Tilly *et al*. 2008; Dietrich *et al*. 2010; Krause *et al*. 2013; Vayssier-Taussat *et al*. 2013) transmitted in the United States primarily by the black-legged deer tick *Ixodes scapularis* (Tilly *et al*. 2008; Vayssier-Taussat *et al*. 2013). *Borrelia burgdorferi* is the causative agent of Lyme disease, whereas *B. miyamotoi* causes a form of relapsing fever (CDC, 2013, 2014*a*). In recent years, several cases of *B. miyamotoi* infection have been found in areas where Lyme disease is endemic in the USA and other parts of the world (CDC, 2013, 2014*a*; Gugliotta *et al*. 2013; Krause *et al*. 2013; Padgett *et al*. 2014).

The molecular diagnostic method currently employed for the detection of pathogens in ticks is endpoint PCR (Ullmann *et al*. 2005; CDC, 2013, 2014*b*; Padgett *et al*. 2014) or quantitative PCR (qPCR) (Hindson *et al*. 2011; Bio-Rad Laboratories, 2014). In one iteration, real-time detection of PCR products is achieved in each qPCR reaction through the attachment of a fluorescent reporter molecule to specific TaqMan® (Life Technologies, Carlsbad, CA) probes which, upon amplification of target DNA products, becomes cleaved and fluoresces. This fluorescence can be measured and a DNA quantity interpolated from a constructed standard curve. In essence, an increased amount of fluorescence corresponds to an increased amount of target DNA product (Bio-Rad Laboratories, 2014). Extrapolating backwards can yield an estimate of original DNA concentration.

Though qPCR has proven to be a relatively successful diagnostic tool, the methods can be time-consuming, costly and imprecise (Hindson *et al*. 2011; Staff, 2012). Another drawback to using qPCR methods in diagnostics is that the accuracy of this technique is limited by the fact that amplification

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efficiencies and cycle threshold (C_T) values generated can vary greatly between runs (Hindson *et al*. 2011). Unlike the relative measurements of DNA concentration obtained through qPCR methodologies, droplet digital PCR (ddPCR) enables the absolute quantification of target DNA molecules (Hindson *et al*. 2011; Bio-Rad Laboratories, 2014; Sze *et al*. 2014). This capability is a defining characteristic of ddPCR and is potentially advantageous to a wide variety of applications including molecular diagnostic testing (Hindson *et al*. 2011; Staff, 2012; Bio-Rad Laboratories, 2014).

The ddPCR technique is based upon the partitioning of a single DNA sample into an emulsion containing tens of thousands of smaller individual reaction liposomes, known as 'droplets' (Bio-Rad Laboratories, 2014; Sze *et al*. 2014). Dilution of the sample into an appropriate range results in the majority of droplets containing zero or one target molecule. PCR is subsequently performed to amplify the target DNA template within each droplet. The droplets are then counted as either positive or negative based on the presence or absence of amplicons as determined by target-dependent fluorescence signals in individual droplets (Sze *et al*. 2014). The 'digital' aspect of the system refers to the simple readout of droplet partitions as a binary code of either 'positive' or 'negative'. Since the presence of a target molecule in a given droplet is a random event, the associated data can be fit to a Poisson distribution, which allows estimation of the number of positive droplets containing more than one target molecule (Bio-Rad Laboratories, 2014; Sze *et al*. 2014). This allows for direct calculation of DNA copy number in a given sample without the obligation of a standard curve, which is an improvement upon qPCR requirements in terms of time, money and accuracy (Hindson *et al*. 2011; Staff, 2012; Bio-Rad Laboratories, 2014; Sze *et al*. 2014).

Droplet partitioning during ddPCR reduces bias from PCR amplification efficiency and inhibitors, which reduces error rates and enables accurate quantification of DNA template (Bio-Rad Laboratories, 2014). Droplet partitioning also decreases effects of competitive amplification, allowing the sensitivity of template detection to increase by an order of magnitude when compared with qPCR under certain conditions (Hindson *et al*. 2011). Although the quality of the results yielded by ddPCR are distinct from those obtained through qPCR, the technology uses assay chemistries essentially identical to those widely used for qPCR applications (i.e. TaqMan), facilitating simple adaptation of existing qPCR protocols to ddPCR.

While ddPCR detection of certain pathogens may have notable advantages over existing technologies, its application in the detection of *Borrelia* spp. has not yet been validated in a controlled laboratory setting. In this study, we adapted previously published TaqMan-based qPCR assays for the detection of *B. burgdorferi* and *B. miyamotoi* to the ddPCR platform, in order to test the efficacy of the system (Ullmann *et al*. 2005). We first determined the accuracy and sensitivity of each assay using purified bacterial genomic DNA (gDNA). We then investigated the potential for PCR inhibition from tick DNA using total *I. scapularis* DNA extracts that had been previously tested and found to be negative for *Borrelia* spp. infection. These whole-tick extracts were then spiked with known quantities of appropriate *Borrelia* gDNA and tested. Finally, we applied the technique to other ticks collected from the same heavily *B. burgdorferi*-infected population from Massachusetts and estimated the number of *B. burgdorferi* spirochetes contained within infected nymphs and adult females.

MATERIALS AND METHODS

Sample collection

Borrelia burgdorferi B31 (35210™) gDNA was obtained from the American Type Culture Collection (Manassas, VA). *Borrelia miyamotoi* strain US178 (Rhode Island) DNA was kindly provided by the Center for Disease Control and Prevention (CDC).

Ixodes scapularis ticks were collected in late May and early June of 2014 near North Truro, MA by standard flagging techniques. Live samples were transported to UNTHSC and frozen at −80 °C prior to extraction. Details on life stage and presence of *B. burgdorferi* DNA are shown in Table 1.

DNA extraction

DNA from ticks ($n = 10$) was extracted using previously described methods (Williamson *et al*. 2010; Mitchell *et al*. 2016). All extractions were conducted in a pre-PCR clean room to limit risk of contamination. Briefly, ticks were laterally bisected with a sterile razor and pulverized by bead beating for 10 min, followed by digestion with proteinase K for 1 h. at 60 °C. Extractions were carried out using the E.Z.N.A.® Mollusc DNA Kit (Omega Bio-tek, Norcross, GA) per the manufacturer's instructions with an elution volume of 140 µL.

Conventional PCR and preliminary screening

Tick extracts were tested for amplifiable DNA using primers targeting the tick mitochondrial 16S rRNA gene as previously described (Mitchell *et al*. 2016). The presence of *Borrelia* was detected using a nested PCR assay, which targeted a portion of the *flaB* gene with *Borrelia* genus-specific primers. Reactions were performed in duplicate with positive

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Table 1. Life stages and *B. burgdorferi* infection status of ticks used in this study

Tick sample no.	Life stage	Results of *B. burgdorferi* nested PCR testing
1	Nymph	Positive
2	Nymph	Negative
3	Nymph	Negative
4	Nymph	Positive
6	Adult	Positive
7	Adult	Positive
9	Adult	Positive
10	Nymph	Positive

(plasmid DNA) and no template controls (NTC) as previously described (Williamson *et al*. 2010; Mitchell *et al*. 2016). Of the ten tick samples initially screened, two (of four total) negative and six positive ticks were utilized for later ddPCR experiments. All *I. scapularis* extracts were quantified prior to ddPCR analysis using the Qubit® dsDNA HS Assay Kit (ThermoFisher Scientific, Waltham, MA) to determine total concentration of double-stranded DNA present in each sample.

Genospecies-specific PCR primers and probes

Widely accepted qPCR primer design guidelines also apply to the design of ddPCR primers and probes (Bio-Rad Laboratories, 2014); therefore, *Borrelia* species-specific primer and probe sets used during this research were selected from a study by Ullmann *et al*. (2005). These included oligonucleotide primers and probe sequences specific for the *B. burgdorferi ospA* and the *B. miyamotoi glpQ* genes. Primer sequences specific for the *B. burgdorferi ospA* gene were MOspA-F (5′-GYAAA GTAAAATTAACART) and MOspA-R (5′-TGT TTTRCCATCTTCTTT), which yield a 74-bp amplicon. The TaqMan probe was synthesized as MBurg-P and labeled with a 5′ 6-FAM™ (blue) dye and a 3′ MGB/nonfluorescent quencher (MGBNFQ) (5′–6-FAM-GACGATCTAGGTCA AACC-MGBNFQ). Primer sequences for the hard tick relapsing fever group *Borrelia* (i.e. *B. miyamotoi*) *glpQ* gene were MglpQ-F (5′-GATAATA TTCCTGTTATAATGC) and MglpQ-R (5′-CA CTGAGATTTAGTGATTTAAGTTC), which yield a 100-bp amplicon. The TaqMan probe for relapsing fever group *Borrelia* was MglpQ-P (5′-VIC-CCCAGAAATTGACAACCAC-MGBNFQ) and labelled with a 5′ VIC® (green) dye. Sequences were evaluated for specificity, length of amplicon, secondary structuring from internal primer binding, G–C content and the melting temperature of primers and probes using the Oligonucleotide Properties Calculator (http://basic.northwestern.edu/biotools/OligoCalc.html) (Kibbe, 2007).

Droplet digital PCR

ddPCR for both *ospA* and *glpQ* assay formats was performed with a PCR reaction volume of 20 μL using the ddPCR™ Supermix for Probes (no dUTP) master mix (Bio-Rad, Hercules, CA). Each reaction included 10 μL of ddPCR Probe Supermix, forward and reverse primers at 900 nM each, probes at 250 nM and template DNA. The PCR reaction mixture was loaded into an eight-well DG8™ Cartridge (Bio-Rad) and droplets were formed with the Bio-Rad QX200™ Droplet Generator, following the manufacturer's instructions. During emulsion, the QX200 droplet generator partitions the samples into 20 000 nanolitre-sized droplets. The droplets were then transferred to a 96-well plate and sealed with a Bio-Rad PX1™ PCR Plate Sealer, as recommended by the manufacturer.

Optimal ddPCR annealing temperatures for the *ospA* and *glpQ* assays were determined by incorporating a temperature gradient from 46 to 60 °C into the annealing–extension step of the thermal cycling conditions.

Borrelia burgdorferi ospA assays were amplified using the following cycling conditions: an initial denaturation step at 95 °C for 10 min, followed by 50 cycles consisting of denaturation at 94 °C for 30 s and an annealing–extension step at 49 °C for 1 min, followed by a final extension step at 98 °C for 10 min and a 4 °C indefinite hold. The overall ramp rate was set at 2 °C s^{-1}.

For *B. miyamotoi* detection, the *glpQ* assay reactions were similarly amplified with the following cycling conditions: an initial denaturation step at 95 °C for 10 min, followed by 50 cycles of denaturation at 94 °C for 30 s and an annealing–extension step at 52 °C for 1 min, followed by a final extension step at 98 °C for 10 min and a 4 °C indefinite hold. Overall ramp rate was set at 2 °C s^{-1}. After cycling, droplets were immediately analysed or stored at 4 °C until analysis on the QX200™ Droplet Reader.

Data acquisition and analysis

The QX200 Droplet Reader analyses each droplet individually, in a single-file fashion, using a two-dye, two-channel detection system. The blue dye channel (channel 1) detected *ospA* PCR products, while the green dye channel (channel 2) detected *glpQ* amplicons. Droplets were classified as positive or negative according to a threshold manually set across all wells within a single run based upon results of the NTC sample. Positive droplets contain at least one copy of the target DNA molecule and display increased fluorescent amplitude compared with the negative controls.

The number of positive and negative droplets read in each channel is used by the QuantaSoft™

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v.1.7.4.0917 (Bio-Rad) software to calculate the concentration of the target DNA sequences, along with their Poisson-based 95% confidence intervals (Hindson *et al*. 2011). The number of template copies per unit volume was estimated from the number of positive events detected by the droplet reader in the corresponding channel (channels 1 and 2 for FAM™ and VIC® dyes, respectively), and the number of total droplets by maximum-likelihood (Strain *et al*. 2013). The distribution of templates among droplets was assumed to follow a Poisson distribution, and the number of positive droplets was assumed to follow a binomial distribution. 95% confidence intervals were estimated under the same assumptions. The droplet size was assumed to be 0·91 nL (Strain *et al*. 2013). The concentration reported by QuantaSoft™ equals copies of template per μL of the final 1 × ddPCR reaction. This value was multiplied by the reaction volume to calculate the total number of template copies detected per sample.

Limit of detection and absolute quantification

A 1:100 dilution of each control *Borrelia* spp. gDNA sample was made and total double-stranded DNA (dsDNA) concentration quantified *via* Qubit® dsDNA HS Assay Kit (ThermoFisher Scientific). From this, a 7-sample standard dilution series was generated to test detection limits and quantification accuracy *via* ddPCR. Serial dilutions of control DNA were prepared separately for each *Borrelia* spp. in order to determine the sensitivity of the ddPCR instrument and protocol for each target species. Serial dilutions ranged from 10 to 100 000 copies per reaction of control *B. burgdorferi* DNA, and 6 to 165 000 copies per reaction of control *B. miyamotoi* DNA. Fresh dilutions were prepared daily for each experiment. PCRs were prepared according to the ddPCR conditions previously detailed with an added template volume of 1 μL diluted *Borrelia* control DNA. Expected genomic copies were calculated at each sample concentration according to the equation below. Expected copies were then compared with measured copies generated by the QuantaSoft™ (Bio-Rad) software in order to evaluate the ability of the ddPCR system to provide absolute quantification of known concentrations of *Borrelia* DNA. Expected copy calculations were based upon the following equation:

$$\text{Genome copies} = (\text{ng template}) \times (6{\cdot}022 \times 10^{23}) \\ (\#\text{bp} \times 650\ \text{Da/bp}) \times (1 \times 10^{9})$$

The linear *Borrelia* chromosomes were initially estimated to be 910 724 and 907 294 bp in length for *B. burgdorferi* and *B. miyamotoi*, respectively (Fraser *et al*. 1997; Hue *et al*. 2013), 650 Da is the average molecular weight for a DNA base pair (bp), and 10^9 is the unit conversion from nanograms to grams. Later, copy number calculations for *glpQ* were made using a revised *B. miyamotoi* genome of size of 1 715 670 bp (see the Discussion).

PCR Inhibition by background host DNA

To evaluate potential inhibitory effects on the performance of ddPCR detection and absolute quantification, a series of diluted concentrations of control *Borrelia* DNA was spiked into pre-determined quantities of total DNA extracted from *I. scapularis* ticks that had previously tested negative for *Borrelia* infection. PCR assays included 10 μL of ddPCR Probe Supermix, forward and reverse primers at 900 nM each, probes at 250 nM and 4·9 μL of uninfected *I. scapularis* total DNA spiked with 1 μL of various concentrations of control *Borrelia* gDNA. The concentrations of control gDNA were identical to the template serial dilutions tested previously. Once ddPCR was completed, results of assays with and without the presence of tick DNA extract were evaluated for concordance.

Validation and estimation of B. burgdorferi *carriage levels in ticks*

Six *I. scapularis* ticks (three adults, three nymphs) previously determined to be positive for *B. burgdorferi* were selected for ddPCR testing in order to validate the *ospA* assay. Validation of developed ddPCR protocols was carried out only for the *ospA* assay because *B. miyamotoi* was not detected among available tick samples. Each tick sample was tested using the ddPCR *ospA* assay as previously described with 5·9 μL of *B. burgdorferi*-infected tick DNA extract added as template at various concentrations. Results were analysed with QuantaSoft™ (Bio-Rad) software. The *B. burgdorferi* carriage level was estimated by calculating the number of spirochetes detected per infected tick. This value was found by multiplying the elution volume of tick sample extract (140 μL) by the number of template copies detected per μL template (a product of input template amount and generated copy number), assuming 100% extraction efficiency. All experiments were conducted in replicates of three unless otherwise noted.

RESULTS

Optimization of annealing/extension temperatures in ddPCR

The resulting droplet digital data from the investigation of optimal annealing temperature for the *B. burgdorferi*-targeting assay, *ospA*, are shown in Fig. 1A. No amplification of bacterial DNA was

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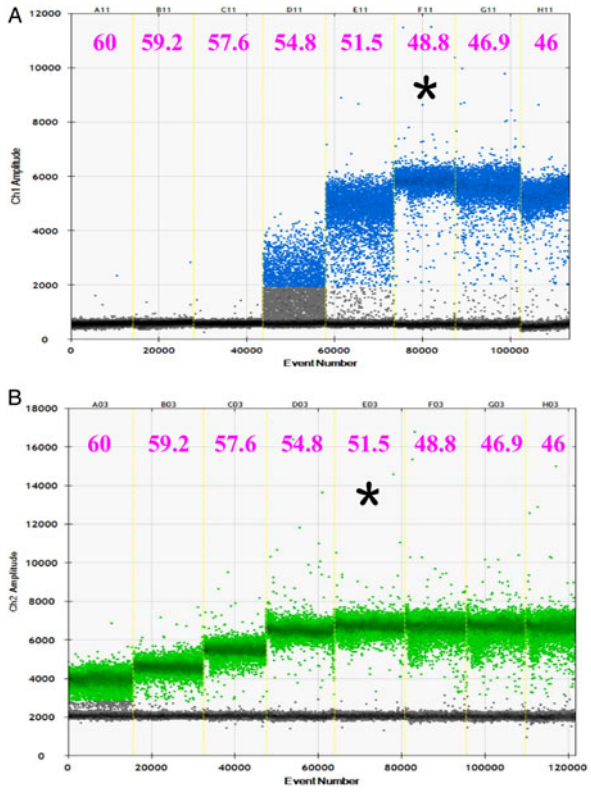

Fig. 1. Optimal annealing temperatures for *Borrelia* DNA assays. (A) ddPCR results of annealing temperature gradient for amplification of *B. burgdorferi* target *ospA* and (B) *B. miyamotoi* target *glpQ*. Event number (*X*-axis) reflects cumulative number of droplets counted per experiment. Yellow vertical lines demarcate samples from individual wells. The calculated annealing temperature tested per sample is indicated for each ddPCR well in °C. Amplitude on *Y*-axis refers to relative fluorescence of individual droplets (blue and green, positive; black, negative).

detected with the *ospA* assay above an annealing temperature of 54·8 °C. The optimal ddPCR result had a clustering of positive droplets at a fluorescent amplitude above 6000 rfu (relative fluorescent units) with minimal 'rain'-down of positive droplets from this positive-clustering corresponding to a calculated annealing/extension temperature of 48·8 °C. Subsequent experiments were therefore carried out with an annealing/extension temperature of 49 °C.

The ddPCR data from the *B. miyamotoi*-targeting *glpQ* assay are shown in Fig. 1B. Positive amplification droplets were generated at each annealing temperature investigated. However, the sample which generated the optimal ddPCR data (with a positive-droplet line around 7000 rfu and little droplet rain) had a calculated annealing temperature of 51·8 °C. Therefore, 52 °C was selected as the optimal annealing temperature for the *glpQ* ddPCR assay.

Estimation of detection limits

Target DNA could be detected for both *B. burgdorferi* and *B. miyamotoi* sample sets over the entire dilution series spanning five orders of magnitude (Fig. 2). Copy number detection for *B. burgdorferi ospA* at the lowest dilution of 10 fg of gDNA was 10·6 copies μL^{-1}, which corresponds well to the expected copy number of 10·17 copies μL^{-1}. Copy number detection for *B. miyamotoi glpQ* at 10 fg gDNA was lower than expected, giving 5·4 copies μL^{-1} compared with a calculated copy number of 10·21 copies μL^{-1}(Table 2).

Estimation of B. burgdorferi *carriage levels in* I. scapularis *nymphs and female adults*

Ixodes scapularis nymphs ($n = 3$) and female adults ($n = 3$) previously determined as positive for *B. burgdorferi* by nested PCR and sequencing of the *flaB* gene fragments were analysed for the *B. burgdorferi ospA* gene copy numbers per sample, assuming a theoretical 100% extraction efficiency of target DNA. In all cases, ticks identified as positive by nested PCR were also found to be positive by the ddPCR assay. Gene copy number in nymphs varied with an average of 2292 and range from 231 to 5268 (Table 3). Adult female *I. scapularis* ticks were found to average 51 179 with a range of 5647–115 797 copies of *ospA* per tick.

DISCUSSION

Optimal annealing temperatures determined for ddPCR were considerably lower than that employed in the multiplex qPCR by Ullmann *et al.* (55 °C). While ddPCR is theoretically more immune to suboptimal amplification conditions, we did detect increased 'rain' (i.e. droplets of intermediate intensity between the average of positive and negative droplets) in samples outside the optimal annealing temperature determined here. The presence of *Borrelia*-negative tick gDNA did not appear to interfere with detection limits or copy number calculations.

Calculation of copy numbers using dilutions of *B. burgdorferi* gDNA for *ospA* assays corresponded well with the theoretical copy number predicted, particularly at the lowest dilution (10·6 detected *vs* 10·17 expected copies μL^{-1}, Table 2); however, differences in detected *vs* theoretical copy number did increase with higher target concentration. The maximum difference was seen at the highest concentration (95·6 pg μL^{-1} gDNA, 97·3 million detected copies μL^{-1} *vs* 122 million theoretical expected copies per μL). Nevertheless, the performance of the assay was strongly linear over five orders of magnitude of gene copy abundance ($R^2 = 0{\cdot}9996$) (Fig. 3A).

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Table 2. Limits of detection for *ospA* and *glpQ* ddPCR assays. In both cases the entire dynamic range of approximately 5 orders of magnitude down to 10 fg could be detected. For *glpQ*, an alternative calculation was performed based on increased estimated genome size (see text).

B. burgdorferi ospA Assay			*B. miyamotoi glpQ* Assay			
Template Amount	Expected Copies	Total Copies Detected	Template Amount	Expected Copies	Total Copies Detected	Recalculated Theoretical Copies
95·6 pg	97,251·99	122,000·00	190 pg	194,013·96	115,000·00	103,041·71
48·4 pg	49,236·36	60,200·00	50 pg	51,056·30	29,500·00	27,116·24
23·6 pg	24,007·81	31,840·00	10 pg	10,211·26	5,360·00	5,423·25
1 pg	1,017·28	1,206·00	1 pg	1,021·13	500·00	542·32
250 pg	254·32	382	250 fg	255·28	118·00	135·58
100 fg	101·73	110	100 fg	102·11	58·00	54·23
10 fg	10·17	10·6	10 fg	10·21	5·40	5·42

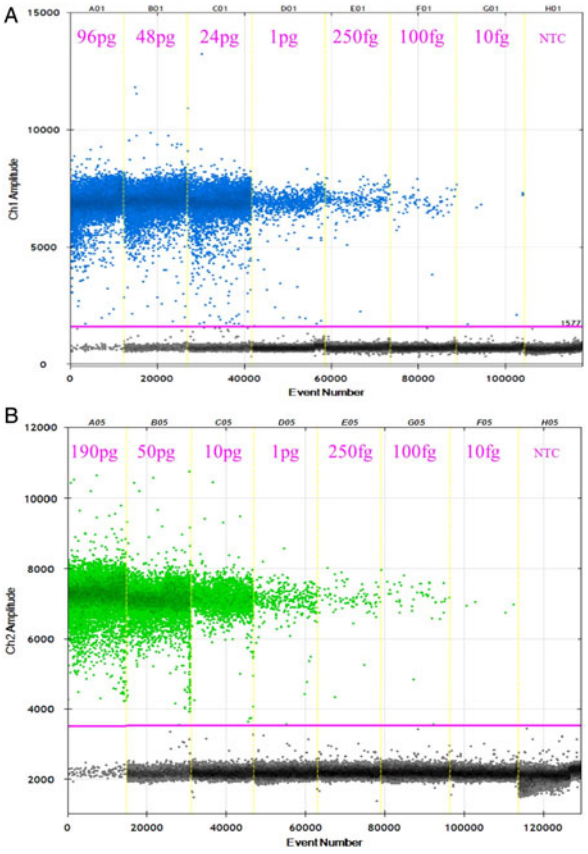

Fig. 2. Detection limit estimation by ddPCR for (A) *B. burgdorferi ospA* and (B) *B. miyamotoi glpQ* assays. Pink horizontal bar indicates manually assigned cutoff for positive droplets based on no template control (NTC). Template concentration is listed above each well.

Copy number calculations made using dilutions of *B. miyamotoi* DNA for *glpQ* assays were not as concordant with the estimated theoretical copy numbers, despite strong linearity of the assay over the dynamic range tested ($R^2 = 0{\cdot}9999$) (Table 2, Fig. 3B). Results indicated a pattern of consistent underestimation of the target numbers by approximately 50% across the entire range of concentrations. The consistency of the copy number inaccuracy suggested a potential systematic error in our process with respect to the *B. miyamotoi glpQ* assay that was not present for *B. burgdorferi ospA*. Upon further investigation it was revealed that the *B. miyamotoi* gDNA had been purified using a commercial genomic prep kit that employed a silica spin column (A. Replogle, personal communication 2015). This is in contrast to the density ultracentrifugation purification methods utilized for *B. burgdorferi* DNA acquired from ATCC (ATCC, personal communication 2015). The spin column chemistry would be expected to co-purify gDNA as well as any large plasmids. Reports in the literature on genomes of relapsing fever *Borrelia* frequently note the presence of linear megaplasmids of 160 kb or larger, in addition to numerous other plasmids (Miller *et al*. 2013). In order to adjust the estimated genome size for *B. miyamotoi* to include possible megaplasmids, we back-calculated the total genome size (i.e. chromosome plus purified plasmids) from this DNA preparation using the average detected number of gene copies over the range of dilutions and estimate the total to be 1 708 315 bp. Using this value, we then re-calculated the expected and theoretical copy numbers from the *glpQ* assays (Table 2, Fig. 3C). The re-calculated detected copy numbers were similar to the revised theoretical expected copy numbers and consistent with the performance seen in the *B. burgdorferi ospA* assay. However, it should be noted that the predicted effective genome size of 1·7 Mb based on ddPCR *glpQ* abundance as estimated here is considerably larger than that extrapolated from Miller *et al*. based on a linear chromosome size of ~950 kb and inclusion of large megaplasmids common in relapsing fever group *Borrelia* of ~160–170 kb. One possible reason that could give rise to an artificially inflated genome size prediction would be contamination by foreign DNA, which in turn could arise

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Table 3. Estimation of *Borrelia burgdorferi* genome copies per infected nymph or adult female *Ixodes scapularis* tick

			Tick sample no.	1	4	10	6	7	9
			Stage	NYMPH			ADULT		
Borrela burgdorferi carriage level (genome copies per tick)	Individual replicates			190	1471	5173	5837	29 092	114 373
				271	1281	5363	5458	33 695	112 000
						5315	5647	32 176	119 593
						4651		33 410	1 17 220
	Tick overall	Means		231	1376	5268	5647	32 093	115 797
		St. dev.		N/A	N/A	282·7	282·7	1824·4	2867
		Range				230–115 797			
	Tick stage	Means			2292			51 179	
		St. dev.			572·8			46 949	
		Range			230–5268			5647–115 797	

from components in the medium or by contamination of the culture by other microbes, or both. To address the former, we performed PCR using primers targeting the mammalian mitochondrial cytochrome B gene (Kocher *et al*. 1989). No amplicon was detected following gel electrophoresis (data not shown). Next we performed PCR of the bacterial 16S ribosomal RNA gene using universal primers 515F and 806R (Caporaso *et al*. 2011), and Sanger sequenced the resulting amplicons. While a mixture would be expected to yield multiple N's upon sequencing, the resultant sequence was clean, consistent with a pure culture (data not shown). Further investigation will be required to address this inconsistency.

Determination of bacterial load was made for *B. burgdorferi* in infected *I. scapularis* ticks. The numbers revealed infection levels spanning an approximately 20-fold range of 231–5268 gene copies per nymph, and 5647–115 797 gene copies per adult female. This indicates a wide variance in infection levels in ticks. A range of spirochete levels in nymphs and adults has been previously reported based upon microscopic methods, as well as temporal changes in spirochete number following blood feeding (Piesman *et al*. 1990). However, more conclusive estimates will require further analysis of other variables including evaluation of DNA extraction efficiencies, as well as tick host physiology and the role of time since feeding or infection. Still, these data should serve as a starting point and conservative lower estimate of carriage levels of *B. burgdorferi* in these ticks.

Concluding remarks

The ddPCR assays tested here resulted in data comparable to that reported using qPCR in terms of sensitivity, dynamic range and detections limits, and confirm the utility of ddPCR assays for the detection of *Borrelia* spp. in *I. scapularis*. One advantage of ddPCR over qPCR may include reduced need for technical replication and standard curves. The ddPCR technique also provides absolute quantification allowing for more accurate estimation of bacterial loads in infected samples. The discrepancy found between predicted and calculated genome copy numbers with *B. miyamotoi glpQ* highlights the importance of properly defining 'genome size' in relation to total DNA, as opposed to simply using published chromosome size data. It should be noted that while the *B. miyamotoi* sample was tested for the possibility of contamination, the tests conducted were not exhaustive and foreign DNA could still have led to the overestimation of the total genome size of this organism, and this value should be viewed with caution. Nevertheless, performance of the ddPCR assay led to linear output over a range of 10–100 000 copies per sample with an R^2 of 0·9999.

The ddPCR technique is still relatively new. Although not specifically tested, one limitation is the reduced ability to perform multiplex assays using a two-channel system such as the one tested here. Presumably future iterations of the platform may have additional capacity for multiplexing with other dyes, provided the dye chemistries can be made compatible with the droplet oil. Another complication with ddPCR is the inherent fragility of the droplets themselves. Careless handling can lead to rupture of the droplets, and full automation of droplet formation may prove more difficult than procedures designed for traditional PCR or qPCR. Under the current format, the process is also limited to the preparation of eight samples at a time. While the impact of this constraint is somewhat lessened by the reduced need for standard curves and large numbers of technical replicates, it necessarily limits the platform to a low-to-medium range sample number throughput. Nevertheless, the accuracy and precision generated by the technique suggests that it will find useful niches in molecular diagnostics.

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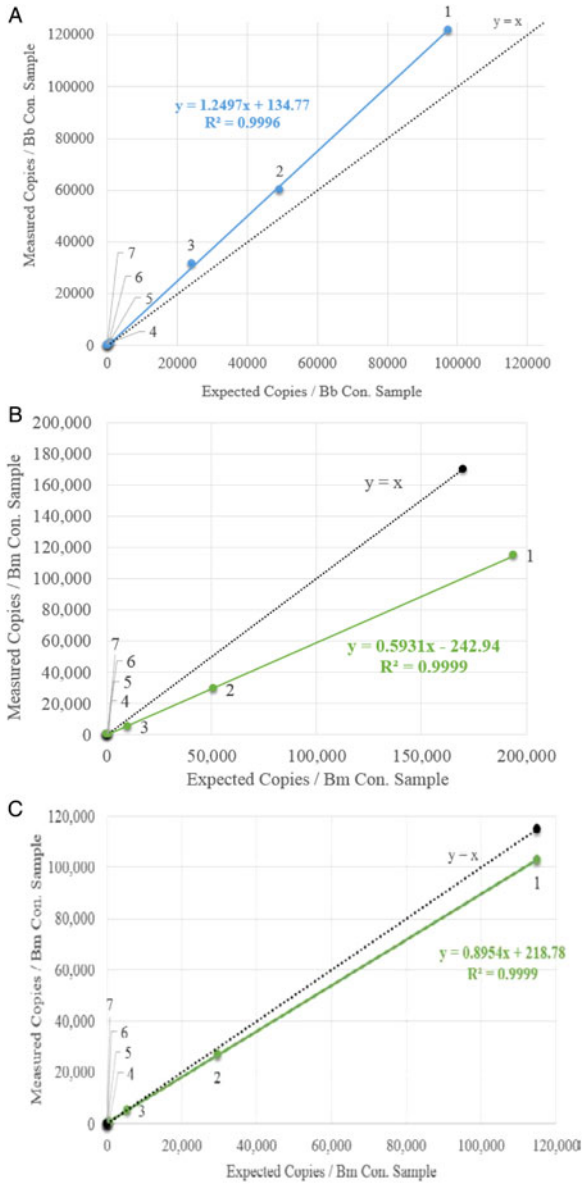

Fig. 3. Relationship of expected theoretical copy numbers to actual detected copy numbers for (A) *ospA* and (B) *glpQ* using *B. burgdorferi* (Bb) and *B. miyamotoi* (Bm) control DNA, respectively. (C) Corrected copy number calculation based on estimated *B. miyamotoi* whole genome size of 1 715 670 bp. See the text for details.

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