

## Quantitative Detection of *Chlamydia* spp. by Fluorescent PCR in the LightCycler®

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### ABSTRACT

Quantitative detection of intracellular bacteria of the genus *Chlamydia* by the standard cell culture method is cumbersome and operator dependent. As an alternative, we adapted hot-start PCR to the glass capillary quantitative PCR format of the LightCycler®. The optimized PCR was consistently more efficient than commercially available pre-assembled PCRs. Detection by quantitative PCR of as few as single copies of DNA of *Chlamydia* spp. was accomplished by SYBR® Green fluorescence of the dsDNA product and by fluorescence resonance energy transfer (FRET) hybridization probes. The PCRs were 15-fold more sensitive than the cell culture quantitative assay of *C. psittaci* B577 infectious stock. The number of chlamydial genomes detected by *C. psittaci* B577 FRET PCR correlated well with cell culture determination of inclusion forming units (IFUs) ( $r = 0.96$ ,  $P < 0.0008$ ). When infected tissue samples were analyzed by cell culture and PCR, the correlation coefficient between IFUs and chlamydial genomes was higher with *C. psittaci* B577 FRET PCR ( $r = 0.90$ ,  $P < 0.0004$ ) than with *Chlamydia omp1* SYBR Green PCR ( $r = 0.85$ ,  $P < 0.002$ ).

### INTRODUCTION

Quantitative detection of bacteria of the obligately intracellular genus *Chlamydia* relies on the standard procedure of inoculating cells grown on coverslips or microwells with the suspended specimen, fixing the cells after 24–72 h, and microscopically enumerating chlamydial intracellular inclusions stained with fluorescent monoclonal antibody (2,4,10). This method is cumbersome, requires experienced personnel, and is prone to subjective error. Few investigators have evaluated the labor-intensive competitive quantitative PCR as replacement of the cell culture method for quantification of chlamydiae (1,2).

Advances in PCR instrumentation and use of fluorescent labels for detection of amplification products have facilitated several approaches to single-tube real-time PCR quantification of input target copies. In particular, the LightCycler® (Roche Molecular Biochemicals, Indianapolis, IN, USA) format uses hot air to rapidly cycle PCRs loaded in glass capillaries while measuring fluorescence emitted from the reaction vessels (13,14). This allows monitoring of the fluorescence emitted by a number of detection formats, including direct detection of the dsDNA product with specific dsDNA binding dyes (5,13) and hybridization-based detection methods (3,8,11,13).

Here, we report the adaptation of hot-start PCR methodology to the glass capillary format of the LightCycler. This was used for amplification and quantitative detection of as few as single copies of DNA of *Chlamydia* spp. by SYBR® Green fluorescence (Molecular Probes, Eugene, OR, USA) of the dsDNA product and by fluorescence

resonance energy transfer (FRET) hybridization probes. These methods were compared to cell culture quantification of chlamydiae in concentrated chlamydial stock and in lung specimens from *Chlamydia*-infected mice. FRET quantitative PCR was 15-fold more sensitive and more accurate than quantification of chlamydiae in cell culture.

### MATERIALS AND METHODS

#### Primers and Probes

Primers and probes are shown in Table 1. The *Chlamydia omp1* genus-specific PCR targeted the single-copy gene encoding the chlamydial major outer membrane protein. It used primers 191CHOMP and CHOMP271 (6,7) to amplify a 287–293-bp DNA fragment of all chlamydial species, which was detected by the 520-nm fluorescent emission of the dsDNA binding dye SYBR Green. The *C. psittaci* B577 *omp1*-specific FRET PCR used the *Chlamydia* genus-specific primers 191CHOMP and CHOMP271 in combination with the *Chlamydia omp1* genus-specific anchor probe CHLANPR and the *C. psittaci* B577 *omp1*-specific probe B577PR (Synthegen, Houston, TX, USA). FRET PCRs specific for strains of other chlamydial species (Table 1) substituted the B577PR probe with an analogous fluorescein-labeled probe corresponding to the nucleotide sequence of the respective strain. CHLANPR was 5'-labeled with the dye LightCycler Red 640 (LCR640) and 3'-phosphorylated; B577PR was 3'-labeled with fluorescein. If both probes annealed simultaneously to the product, fluorescence energy emitted by B577PR was transferred to the adjacent LCR640 of CHLANPR,

**Table 1. *Chlamydia omp1* Amplification Targets, Primers, and Hybridization Probes**

	5'-GCYTTTGGG	ARTGYGGITG	YGCAC-3'	TGIGCRTATT	GGAAIKMWGC	IOCVAA-(Phosphate)-3'	5'-TAC
<b>191CHOMP</b>							
<b>CHLANPR*</b>			5'-(LC-RED640)-GAY				
<b>B577PR*</b>							
<b><i>C. psittaci</i> B577</b>	GCTTTATGGG	AGTGTGGTGG	TGCGACTTTA	GGAGCAGAGT	TOCAATAAGC	TCAGTCTAAT	60
<b><i>C. pneumoniae</i></b>	.C.....	.A.C.....	.A.....G	.T.A.	.T.	A.....C.A	60
<b><i>C. pecorum</i> LW613</b>	.G.....	.A.....A	.A.....	.T.A.	.T.	A.A.G.A	60
<b><i>C. trachomatis</i></b>	A...C.C....	.A.....G..	.A..G....	.TTCT.	.T.	.A.....A	60
<b>B577PR*</b>	ATTCAACATT	TCAATTTTAG	GATT-(Fluorescein)-3'				
<b><i>C. psittaci</i> B577</b>	OCTAAAATTG	AAATGTTGAA	TGTAGTCTOC	AGCCAGGCAC	AAATTTGTGT	TCACAAGCCT	120
<b><i>C. pneumoniae</i></b>	.G.....	.GAC.T..	.GA...GT	.A.GT.T.G.	.CTCT..	AA.....A.C	120
<b><i>C. pecorum</i> LW613</b>	.CGGG..C	.GAAC.C..	C.TC.T..T	.A.GT.....	.ACT..	A.....A...	120
<b><i>C. trachomatis</i> A</b>	.G.A...	.GA.....	.TC.T.GT.	ATG..T.GG	.ACTA.	.A.T.....G	120
<b><i>C. psittaci</i> B577</b>	AGAGGATACA	AGGGAACAGC	ATTTCCTTTA	CCTCTAACAG	CTGGTACT--	----GATCAG	174
<b><i>C. pneumoniae</i></b>	.AG.C.T.	.A.OGTT..	T.C.C..G	.AAC.GAC.	.OGTAGC	A-----ACA	174
<b><i>C. pecorum</i> LW613</b>	OG.....TG	TA..OCAGA.	TC.G...C.T	.A..CT.T.	.A.A..GSA	AACT...TCT	180
<b><i>C. trachomatis</i> A</b>	.A.....TG	TT..GG.G.A	.G...AC.T	GA.A.T..C.	.A.A..AGA	AGCT-----	174
<b><i>C. psittaci</i> B577</b>	GCAACTGACA	CTAAGTGGC	TACAATTA	TACCACGAAT	GGCAAGTTGG	TTTAGCCCTC	234
<b><i>C. pneumoniae</i></b>	.T....GA.	.A.....T..	G..C..C..T	.T..T....	.A...	AGCCT.T..A	234
<b><i>C. pecorum</i> LW613</b>	ACTGA.A.AT	TG..AAAT..	C..G..C..C	.T.....	.C...	.GC...AT.G	240
<b><i>C. trachomatis</i> A</b>	.G..A.GA.	.GAT..	CT.T...G.C	.T..G.	.CAA.	.C..T	234
<b>CHOMP271*</b>			5'-GCTCKI	GAOCAITKWA	CIOCAATGTA	IGG-3'	
<b><i>C. psittaci</i> B577</b>	TCATTATGGAT	TGAACATGCT	TGTTCTTAC	ATTAGCGTAA	ACTGGTCAAG	AGC	287
<b><i>C. pneumoniae</i></b>	.CA..C	.A...TCCTT.	A..G.A...	.G.A...C	.A.....T..	.T.	287
<b><i>C. pecorum</i> LW613</b>	.A...A..C	.T.....	C.....	.OG.T..TC	.G.....TA.	.T.	293
<b><i>C. trachomatis</i> A</b>	.CA....	.A.T...T.	CAC.....	.G.A..T.	.A.....TA.	.T	287

The amplified *omp1* DNA sequence is shown for the four chlamydial species. Primers and probes were combined for PCRs as described in Materials and Methods. Oligonucleotides marked with an asterisk are the reverse complement of the corresponding *omp1* sequence. I, inosine; K, G or T; M, C or A; R, G or A; W, A or T; Y, C or T.

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resulting in increased emission at 640 nm. The ratio of 640 nm:520 nm emission at the end of the annealing segment allowed for the most accurate product detection. The human COX-1 PCR used primers from a Human COX-1 Relative RT-PCR Kit, no. 5306 (Ambion, Austin, TX, USA) to amplify a 401-bp fragment of a linearized plasmid containing COX-1 cDNA, which was added in known quantity to samples before DNA extraction. This allowed for calculation of the recovery of DNA after extraction and, thus, for absolute quantitative detection of chlamydial DNA in these samples.

## DNA Amplification and Detection

All PCRs were performed in glass capillaries (Roche Molecular Biochemicals) in 20- $\mu$ L volumes, using 5  $\mu$ L sample DNA (10–250 ng total DNA) and 15  $\mu$ L 1.33  $\times$  PCR mastermixture. The PCR buffer (20 mM Tris-HCl, pH 8.4) contained 0.05% each Tween<sup>®</sup> 20 and Nonidet<sup>®</sup> P-40 (NP40), 0.03% acetylated bovine serum albumin (BSA) (Roche Molecular Biochemicals), 200  $\mu$ M each dATP, dCTP, and dGTP, 600  $\mu$ M dUTP, 3 mM MgCl<sub>2</sub>, and 10 mM KCl (SYBR Green PCRs) or 4.5 mM MgCl<sub>2</sub>, and 50 mM KCl (FRET PCRs). In SYBR Green PCRs, the SYBR Green stock solution (Molecular Probes) was used at a dilution of 1:10000 (13). All primers were used at 1  $\mu$ M concentration, and FRET probes were used at 0.5  $\mu$ M. Per 20- $\mu$ L reaction, 1 U hot-start Platinum *Taq* DNA polymerase and 0.2 U uracil-N-glycosylase (Life Technologies, Rockville, MD, USA) were used.

Standard reactions including negative controls contained 10<sup>4</sup>–0 template molecules (chlamydial genomic DNA extracted from purified chlamydial elementary bodies or COX-1 plasmid template) in a background of 100 ng re-extracted salmon sperm DNA (Life Technologies). Chlamydial DNA was quantified by the PicoGreen<sup>®</sup> fluorescent assay (Molecular Probes) and diluted according to a calculated mass of 1.37  $\times$  10<sup>-15</sup> g per *C. psittaci* or *C. pneumoniae* genome.

A 2-min incubation at 50°C for activation of uracil-N-glycosylase (to prevent PCR product carryover) was followed by denaturation at 95°C for 1

min, then 50 cycles of 95°C for 0 s, 53°C (all *Chlamydia* PCRs) or 59°C (COX-1) for 3 s, followed by fluorescence acquisition in the FRET PCRs, or for 10 s in the SYBR Green format, followed by extension at 72°C for 15 s in all PCRs. For SYBR Green PCRs, single fluorescence acquisition in each cycle was specified 2°C–3°C below the melting temperature ( $T_m$ ) of the amplification product at 80°C (*Chlamydia omp1*) or 87°C (COX-1) after a 10-s temperature equilibration. After cycling, melting curves of PCR products were acquired by stepwise increase of the temperature from 65°C to 95°C (SYBR Green PCRs) or from 45°C to 85°C (FRET PCRs).

## Quantitative Culture of *Chlamydia* and Sample DNA Extraction

Cell culture of chlamydiae on coverslips and determination of inclusion forming units (IFUs) by staining with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against chlamydial lipopolysaccharide (LPS) followed the procedure described before (2,4,10). DNA extraction from 100  $\mu$ L dilutions of purified elementary bodies of *C. psittaci* B577 or *C. pneumoniae* CDC/CWL-029 or from 20  $\mu$ L 10% mouse lung tissue suspension was performed with the *High Pure* PCR Template Preparation Kit (Roche Molecular Biochemicals), following the manufacturer's instructions. DNA was eluted in 400  $\mu$ L 10 mM Tris-HCl, pH 8.5, elution buffer.

## RESULTS AND DISCUSSION

### Optimization of Microvolume Glass Capillary PCR

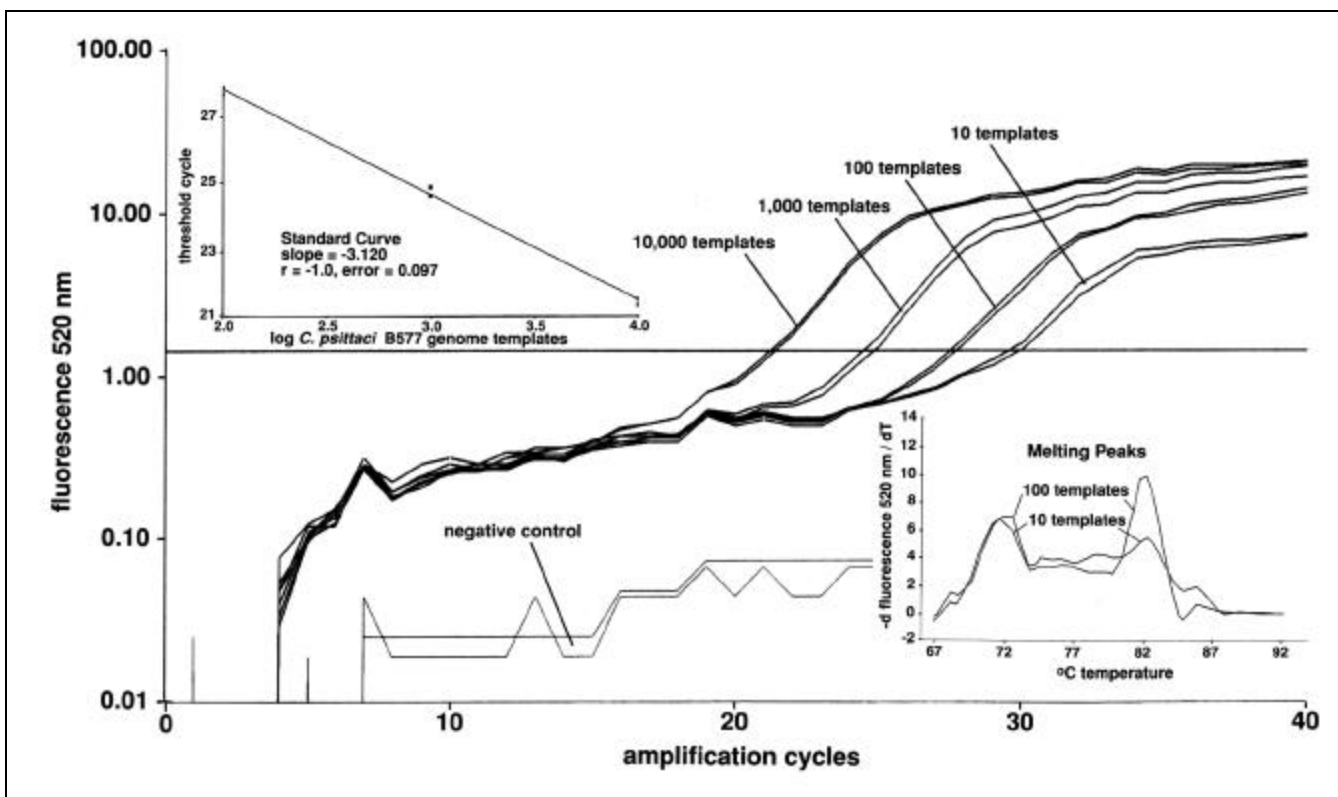
Before specific applications, we established robust generic conditions for real-time quantitative PCR with fluorescent detection in the LightCycler format, which employs glass reaction vessels and rapid temperature changes (14). The conditions indicated in Materials and Methods represent the results of the optimization of the following parameters: pH of PCR buffer, concentrations of MgCl<sub>2</sub>, KCl, nonionic detergents, BSA, nucleotides, primers,

probes, SYBR Green, Platinum *Taq* DNA polymerase, uracil-N-glycosylase, and thermal cycling and fluorescence acquisition conditions. We have used these methods in more than 20 different PCRs including RT-PCRs. If the primers/probes are designed according to accepted standard criteria (9) (Mfold program Web site: <http://www.ibc.wustl.edu/~zucker>) to amplify a target of 100–400 bp, it is typically not necessary to adjust the PCR buffer conditions, in particular magnesium concentration, aside from thermal cycling parameters.

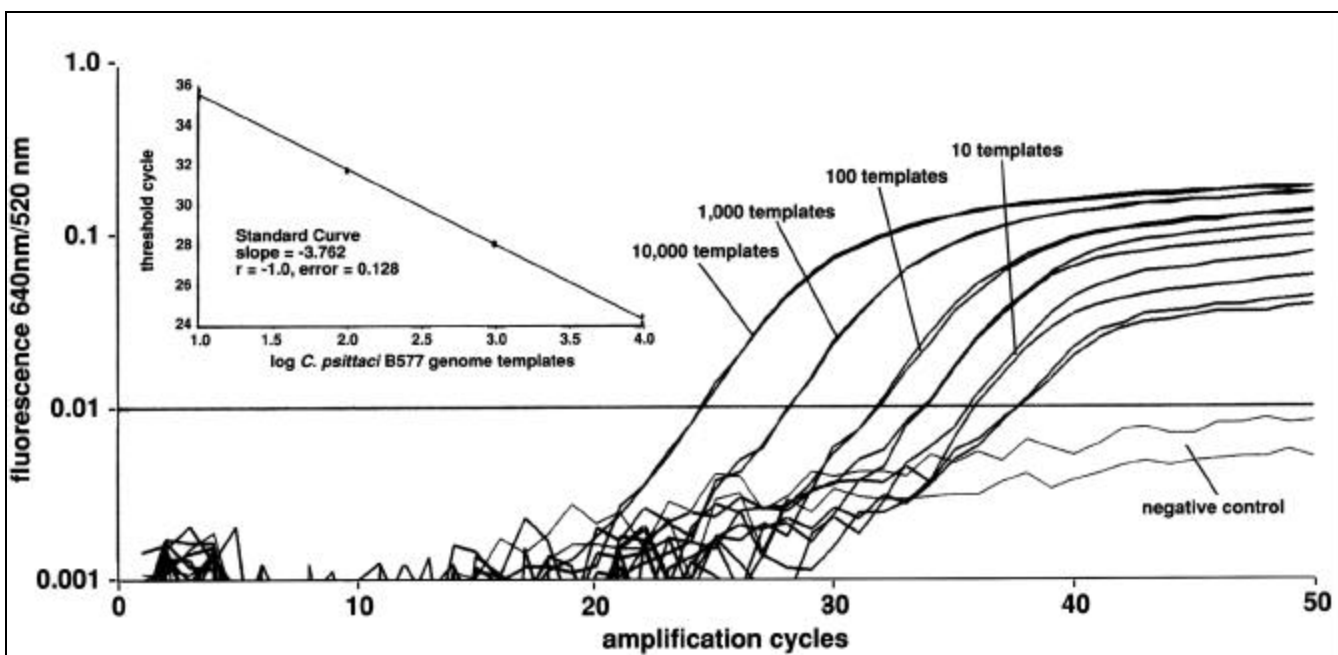
During optimization, it became clear that quantitative PCR required extremely robust reaction parameters to be reliable not only for detection but also for accurate quantification of the target. We found, in particular, that nonionic detergents and BSA were indispensable, as was a sufficient background of unrelated DNA. While the PCR was relatively insensitive to a pH higher than 8.4, a pH lower than 8.3 resulted in strongly decreased amplification efficiency. This explains the critical need for highly pure sample DNA dissolved in a buffer with a pH of approximately 8.5 and the consistent use of identical buffer and volume for samples and standards in quantitative PCR. The use of hot-start Platinum *Taq* DNA polymerase at 1 U/20  $\mu$ L reaction was also critical for reliable results. For consistent results, it was also important to prepare the master mixture just before use from separate stock solutions of enzymes, combined nucleotides, combined primers and probes, and PCR buffer combined with BSA (and SYBR Green, for SYBR Green PCR only). Frozen aliquots of mastermixtures, even without enzymes, rapidly degraded and gave poor quantitative results.

Lack of potassium resulted in increased SYBR Green fluorescence and reduced primer dimer formation, but also reduced amplification efficiency and hybridization probe fluorescence. KCl at 10 mM and MgCl<sub>2</sub> at 3 mM in SYBR Green PCR provided for an optimal balance between amplification efficiency, nonspecific product accumulation, and SYBR Green fluorescence. In the hybridization probe PCR formats, the concentration of KCl at 50 mM and of MgCl<sub>2</sub> at 4.5 mM reflect the reduced need for avoidance of non-

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**Figure 1. *Chlamydia omp1* SYBR Green PCR.** Fluorescence emission curves of the duplicate *C. psittaci* B577 standard DNAs at concentrations of  $10^4$ – $10$  template copies per PCR are displayed with the noise band. Melting curves of a 100 and a 10 template PCR in the lower right corner show the melting point of the primer dimer at approximately 72°C, of the specific product at approximately 82°C, and an additional melting peak in the 10 template reaction at approximately 86°C. At the 80°C fluorescence acquisition temperature, the signal of the 10 template PCR becomes positive too early because of the additional signal from the 86°C peak. Therefore, the standard curve in the upper left corner is constructed from the 10 000, 1000, and 100 template PCRs only, and data for lower template copies are obtained by interpolation between the 100 and 10 template standards. The intra-assay coefficient of variation is 15%.



**Figure 2. *C. psittaci* B577 FRET PCR.** The FRET format in the 640 nm:520 nm fluorescence ratio display resulted in highest accuracy of the log-linear standard curve for all template concentrations. Duplicates of DNA samples from mouse lungs are presented between the 100 and 10, and after the 10 template standards, respectively. The intra-assay coefficient of variation is 10.5%.

specific PCR products coupled with the increased probe fluorescent signal under these conditions.

It is noteworthy that a substantial amount of primer dimer could always be observed in the SYBR Green PCR format (Figure 1). This is in contrast to several commercial PCR buffers, which in our hands produced little or no primer dimer. However, conditions of the commercial buffers appeared to negatively affect specific amplification efficiency as well, because PCRs using these buffers typically required 5–10 more cycles to reach the threshold cycle for  $10^4$  target molecules than those using our optimized buffer.

### SYBR Green PCRs

Figure 1 represents a typical amplification plot of the *Chlamydia omp1* PCR using SYBR Green detection of the *C. psittaci* B577 product. Tenfold serial dilutions of purified *C. psittaci* B577 genomic DNA were used as standards, from a starting concentration of  $10^4$  copies per PCR down to 10 copies. As is evident from the noise band at approximately 1.5 fluorescence units, the negative control remained below this

cutoff even after 50 cycles of amplification. All duplicate *C. psittaci* B577 DNA standards are truly positive, each displaying a fluorescent signal clearly separated from that of the negative control. The slope of the signal increase over cycle number is virtually identical for all standards, indicating that the amplification efficiency remains unchanged during the critical first 35 PCR cycles. The threshold cycle distance between  $10^4$  and  $10^3$ , and between  $10^3$  and  $10^2$ , templates is approximately 3.2, while the distance between the  $10^2$  and 10 template threshold cycles is about 2.2. At 10 templates, the melting curve insert in Figure 1 indicates aberrant amplification products with a  $T_m$  of approximately  $86^\circ\text{C}$  aside from the specific product with a  $T_m$  of approximately  $82^\circ\text{C}$ . No such aberrant products appear in the 100 template PCR. Since the fluorescence signal of the *Chlamydia omp1* PCR is acquired at  $80^\circ\text{C}$ , such aberrant products falsely increase the signal at low template numbers, resulting in an inaccurate standard curve. To remediate this shortcoming, we constructed the standard curve for samples with more than 100 target copies from the  $10^4$ ,  $10^3$ , and  $10^2$  template stan-

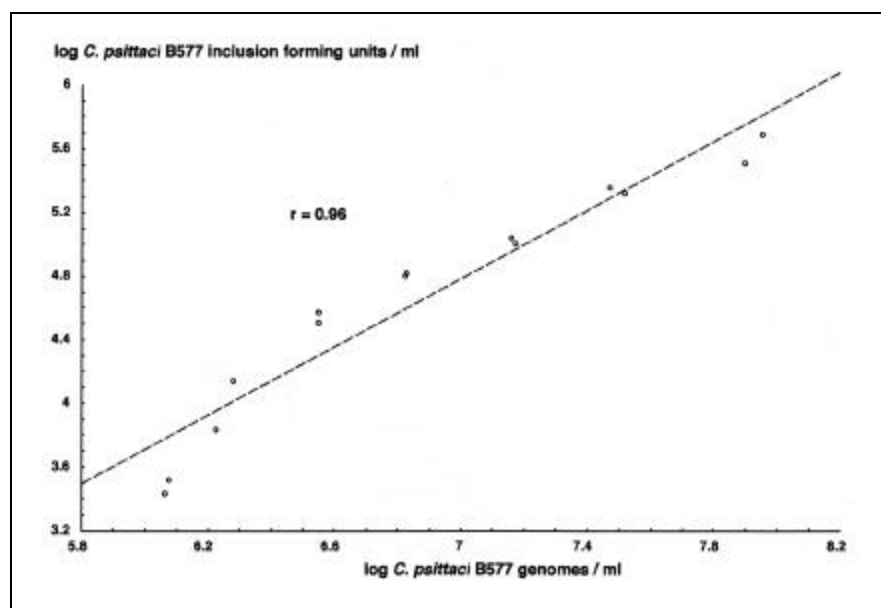
dards. This resulted in a linear threshold cycle versus log template copies standard curve (standard curve insert in Figure 1), while we interpolated between the  $10^2$  and 10 standards for samples with less than 100 target copies.

The problem with a false signal increase at low template numbers was absent in PCRs of the human COX-1 template with a product containing 58% GC. The approximately  $90^\circ\text{C}$   $T_m$  of the specific amplification product allowed the acquisition of SYBR Green fluorescence at  $87^\circ\text{C}$ . At this temperature, nonspecific products are melted and thus do not bind SYBR Green any more and do not contribute to the fluorescent signal. This results in equal spacing of the signal curves for all standards and a linear standard curve between  $10^4$  and 10 template copies (data not shown).

To confirm the *Chlamydia* genus specificity of the SYBR Green PCR, we successfully amplified and quantified with similar accuracy purified *C. pneumoniae* DNA and amplified the *omp1* locus of DNA isolated from cell culture harvests of *C. trachomatis* serovar A and *C. pecorum* LW613 cultures. Limiting dilution analysis of multiple replicate PCRs of *C. psittaci* B577 and *C. pneumoniae* CDC/CWL-029 standards exhibited a Poisson distribution of positive and negative results when the dilution of the standard approached or exceeded a theoretical number of one or less template copies per 5  $\mu\text{L}$  PCR input. These data demonstrated that the quantitative PCR format in fact detected single copies of input templates.

### FRET PCRs

Using the target of the *Chlamydia omp1* SYBR Green PCR, we established a dual probe FRET PCR, which generated the signal by FRET. The PCR as shown in Figure 2 is specific for *C. psittaci* B577. However, the degenerate LCR-640-labeled probe CHLANPR hybridizes to all known chlamydial *omp1* sequences. For detection of different chlamydial strains, it is therefore sufficient to replace the fluorescein-tagged B577PR with another cognate, similarly fluorescein-labeled probe. Using this approach, we gener-



**Figure 3. Linear regression analysis of *C. psittaci* B577 quantification by cell culture and *C. psittaci* B577 FRET PCR.** *C. psittaci* B577 stock was twofold serially diluted from 1:10 000 to 1:640 000. These dilutions were analyzed by both assays as described in Materials and Methods. Corresponding results were plotted, and the least square linear regression was calculated. The intra-assay coefficient of variation for the cell culture determination is 16.1%, as compared to the more accurate FRET PCR with 10.5% intra-assay variation. Major deviation from linearity at both extremes of the dilution series is caused mainly by the nonlinearity of the cell culture assay.

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ated equally functional FRET PCRs for strains of the three other chlamydial species (Table 1) as well. The FRET format was evaluated similar to the SYBR Green PCR. Again, the FRET PCRs allowed for linear quantification of chlamydiae down to single copies of the input template, without relative overamplification at low template numbers (Figure 2).

The FRET format in positive samples resulted in absolute enhanced LightCycler Red 640 emission (F2, 640 nm), as well as an increased ratio of 640 nm to fluorescein (F1, 520 nm) emission. This second readout was comparable to an internal reference dye format and thus rendered the result independent of variations in absolute fluorescence. In our hands, the FRET PCR format was by far the most robust and consistent method. In the FRET PCRs, it was important to place the probes at least 60 bp downstream of the primer annealing to the same DNA strand, and

to acquire fluorescence within 3 s of equilibration at the annealing temperature. Violation of these parameters resulted in delays in signal appearance of up to 10 cycles, presumably because the *Taq* DNA polymerase displaced one or both probes.

An advantage of the various formats of the PCRs is that the genus-specific *Chlamydia omp1* PCR can be used for screening of samples by the inexpensive SYBR Green detection method. If desired, the FRET PCR format can then be used to more accurately quantify chlamydiae in positive samples, particularly those with low copy numbers. Also, the increased specificity of the probe format may be used to confirm SYBR Green PCR results and for strain-specific typing. FRET is advantageous over other hybridization-based detection formats of the products because the probes carry single fluorescent labels and are easier to synthesize than TaqMan<sup>®</sup> probes (Applied Bio-

systems, Foster City, CA, USA) (3). Furthermore, the time-consuming optimization of probe folding, which is necessary for molecular beacons or scorpion primers, is typically not required for FRET probes (11,12,13).

## **Correlation between PCR and Cell Culture Quantification of *C. psittaci* B577**

Pre-PCR sample DNA extraction was the single most critical parameter for absolute quantification of chlamydiae in samples. We evaluated the percent recovery of DNA by adding known copy numbers of COX-1 template before extraction and found that glass matrix binding and elution of sample DNA resulted in high recovery of DNA without PCR inhibitors. As calculated from COX-1 PCRs, typically 85%–95% of sample DNA was recovered. If DNA was extracted in one batch per experiment, recovery was consistently high

enough that normalizing to 100% DNA recovery was unnecessary.

We first examined the correlation between chlamydial cell culture and *C. psittaci* B577 FRET PCR by analyzing serial dilutions of purified *C. psittaci* B577 elementary body stock by both methods. As evident in Figure 3, there was a linear correlation ( $r = 0.96$ ,  $P < 0.0008$ ) in this log-log regression plot between chlamydial IFUs and chlamydial genomes found for the serial dilutions (1:10000-1: 640 000) by cell culture and PCR, respectively. The regression plot reveals that the correlation is weakest at both extremes of the dilution series and that the deviations mainly result from nonlinearity of the cell culture quantification. An interesting result is the fact that these data demonstrate that only approximately 0.5% of all chlamydial elementary bodies were infectious.

We then evaluated cell culture versus PCR determination in 24 homogenized mouse lungs infected with *C. psittaci* B577. Not unexpectedly for animal infection specimens, the correlation between the methods was lower. The correlation coefficient between IFUs and *C. psittaci* B577 FRET PCR was higher ( $r = 0.90$ ,  $P < 0.0004$ ) than for the *Chlamydia omp1* SYBR Green PCR ( $r = 0.85$ ,  $P < 0.002$ ). Background interference was the actual factor limiting the sensitivity of both the *Chlamydia omp1* SYBR Green PCR and *C. psittaci* B577 FRET PCR to  $4 \times 10^3$  chlamydial genomes/100 mg lung tissue. This is, however, about 15-fold more sensitive than cell culture quantification, which has, in our hands, a practical limit of  $6 \times 10^4$  IFU/100 mg tissue.

Collectively, these results demonstrate that the FRET and SYBR Green quantitative PCR methods can successfully and reliably replace many aspects of the cell culture method for chlamydial quantification. We envision that these fluorescent techniques will make PCR the method of choice for diagnostic and quantitative detection of chlamydiae.

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