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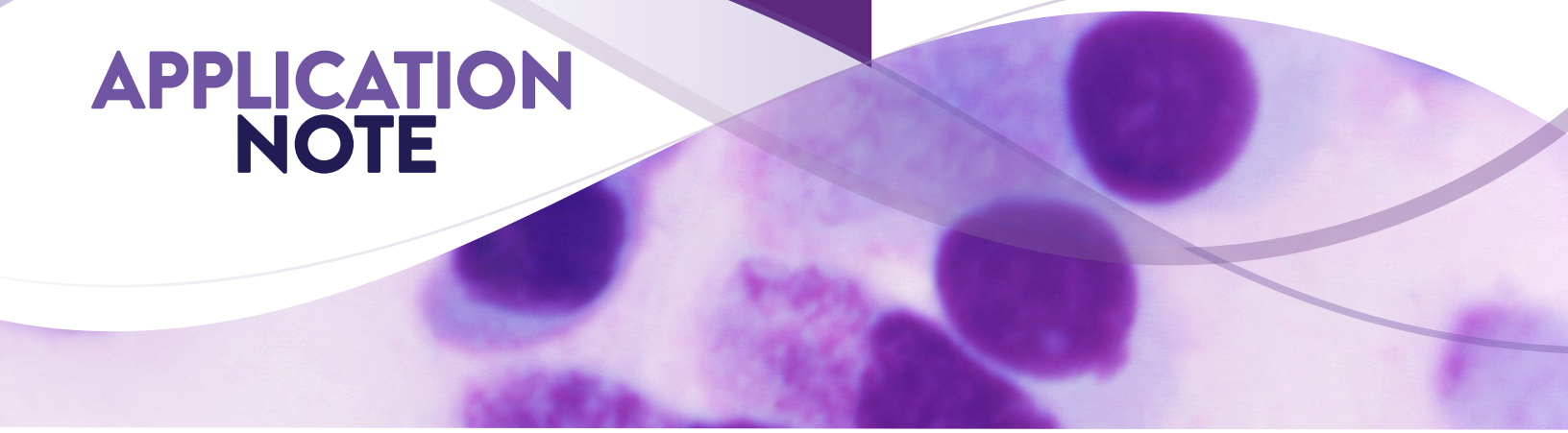
APPLICATION NOTE



PERFORMANCE ASSESSMENT OF QUANTITATIVE SYNTHETIC *CHLAMYDIA TRACHOMATIS* LGV I, II, AND III REFERENCE MATERIALS FOR QPCR APPLICATIONS

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ABSTRACT

Chlamydia trachomatis is a bacterial pathogen primarily transmitted through sexual contact, often causing conditions such as urethritis and cervicitis. If left untreated, these infections can progress to severe reproductive health complications, including infertility and pelvic inflammatory disease. Early detection is crucial for effective treatment and prevention. PCR-based diagnostics have proven to be rapid, sensitive, and specific for detecting *C. trachomatis* infections, but such methods necessitate quality control materials to ensure reliability. To support this need, ATCC® has developed analytical reference materials in the form of synthetic DNA products for the LGV I, LGV II, and LGV III serovars of *C. trachomatis*. The goal of these products, which we aim to showcase herein, is to enable accurate calibration of PCR assays and support improvements in the detection and surveillance of *C. trachomatis* LGV infections.

BACKGROUND AND INTRODUCTION

Chlamydia trachomatis is an obligate intracellular bacterium responsible for a range of human infections, including sexually transmitted infections (STIs). Its life cycle alternates between two forms: the elementary body (EB), which enters and infects host cells, and the reticulate body (RB), which replicates inside the host cell. After replication, RBs transition back into EBs, which are then released—either by extrusion or cell lysis—to seek out and infect new hosts. The *C. trachomatis* genome consists of a single circular chromosome, which contains genes for most essential metabolic functions but lacks the ability to produce ATP, making it dependent on the host cell for energy.[1]

As a pathogen, *C. trachomatis* can cause genital infections, pelvic inflammatory disease, and sometimes infertility. It can also cause ocular infections that can lead to blindness. *C. trachomatis* strains are divided into several serovars that cause different symptoms in the host during infection. To date, there are 15 known serovars, of which 3 are associated with ocular infections (A through C), 9 are associated with genital infection (D through K), and 3 are associated with lymphogranuloma venereum (LGV I through LGV III). Lymphogranuloma venereum (LGV) is a rare but aggressive infection that begins with painless genital or oral lesions. It then progresses to a second stage characterized by significant pain, aches, bleeding, and fever. If left untreated, the infection can advance to a third stage marked by severe tissue damage in which lymph nodes and anogenital regions may undergo necrosis or rupture.[2]

Early detection is crucial to prevent progression to later stages of infection. Currently, diagnosis of *C. trachomatis* begins with patient sample collection, followed by one of several approaches: molecular methods such as polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP); microscopy methods such as direct fluorescent antibody (DFA) tests; or, less commonly, culture of the patient sample. Among these options, molecular diagnostics like PCR are known for their speed, sensitivity, and specificity in detecting *C. trachomatis* infections, and they are the only method capable of confirming an LGV diagnosis.[3] To maintain accuracy and reliability, these

assays require analytical reference materials (ARMs) for quality control. ARMs also play a critical role in assay development by improving target sensitivity and specificity.

ATCC® has an extensive catalog, including authenticated biomaterials and genomic data to support STI research. To support molecular diagnostics, ATCC® developed three ARMs for *C. trachomatis* LGV serovars in the form of quantitative synthetic DNA. The goal for these ARMs, which we aim to showcase herein, is to enable accurate calibration of PCR assays and support improvement in the detection and surveillance of *C. trachomatis* LGV infections.

MATERIALS AND METHODS

When developing the *C. trachomatis* LGV synthetic DNA products (Table 1), we applied the same rigorous approach used across our quantitative synthetic nucleic acid portfolio. Extensive research was conducted to identify key genomic target regions that are compatible with published and commercial molecular assays. We analyzed several published genome sequences to inform our design. Each product is manufactured under ISO 13485 guidance and stabilized in a proprietary storage buffer to ensure consistent performance and a 5-year-long shelf life.

To demonstrate the utility of the *C. trachomatis* LGV synthetic DNA products as positive controls for PCR-based applications, we selected several qPCR assays from primary literature (Table 2), including from the U.S. Centers for Disease Control and Prevention (CDC) (Chen *et al*., 2007, Chen *et al*., 2008).[4,5] We compared the assay performance of these synthetic DNA products with genomic DNA counterparts from our catalog (Table 1). Experiments were conducted on the CFX Opus Real-Time PCR System (Bio-Rad) using the Invitrogen SuperScript III Platinum One-Step qRT-PCR Kit (Thermo Fisher Scientific). Cycling parameters and oligonucleotide concentrations followed the conditions reported in each source publication. Synthetic DNA was tested at concentrations ranging from 50-50,000 genome copies/reaction (GC/rxn) and genomic DNA at concentrations ranging from 50-5000 GC/rxn.

Table 1: Table 1: ATCC® products featured in this study.

ATCC® Number	Product Name	LGV Serovar
BAA-4001SD™	Quantitative Synthetic *Chlamydia trachomatis* LGV Type 1 DNA	I
BAA-4002SD™	Quantitative Synthetic *Chlamydia trachomatis* LGV Type 2 DNA	II
BAA-4003SD™	Quantitative Synthetic *Chlamydia trachomatis* LGV Type 3 DNA	III
VR-901BD™	Quantitative Genomic DNA from *Chlamydia trachomatis* LGV Serovar I strain 440	I
VR-902BD™	Quantitative Genomic DNA from *Chlamydia trachomatis* LGV Serovar II strain 434	II
VR-903D™	Quantitative Genomic DNA from *Chlamydia trachomatis* LGV Serovar III strain 404	III

Table 2: Published assays assessed for product compatibility in this study.

Authorship	Gene Target	Testing Range
Chen *et al*., 2007[4]	Cryptic plasmid	Synthetic DNA: 5×10^1 – 5×10^4 genome copies/reaction Genomic DNA: 5×10^1 – 5×10^3 genome copies/reaction
	pmpH	
Chen *et al*., 2008[5]	*pmpH*	
Morré *et al*., 2005[6]	*pmpH*	
Schaeffer *et al*., 2008[7]	*pmpH*	
	ompA	
Stevens *et al*., 2010[8]	*ompA*	
Verweji *et al*., 2010[9]	*pmpH*	
Zigangirova *et al*., 2013[10]	Cryptic plasmid	
	16S rRNA	

RESULTS

To evaluate the performance and utility of quantitative synthetic ARMs for *C. trachomatis* LGV I, II, and III in qPCR applications, we conducted experiments using published assays (Table 2) and verified assay compatibility (Table 3). Amplification plots were generated with serial dilutions ranging from 50-50,000 GC/rxn for synthetic and 50-5000 GC/rxn for genomic *C. trachomatis* LGV I-III DNA. Comparative amplification results for qPCR assays targeting the *pmpH* gene (Figure 1A) and the cryptic plasmid (Figure 1B), based on the assay described by Chen *et al*. 2007, demonstrate efficient and comparable performance of both synthetic and genomic DNA across all three LGV serovars, as anticipated from prior *in silico* analysis. These single-plex assays detected all three LGV serovars using the same set of primers and probes.

To demonstrate LGV-specific assay performance, we evaluated compatibility using published LGV-targeted qPCR assays (Table 2). qPCR amplification of the LGV I and LGV III products was achieved with an LGV-specific multiplex assay targeting the *ompA* gene from Schaeffer *et al*. 2008 (Figure 2A). With this assay, neither synthetic nor genomic DNA from LGV II could be amplified. *In silico* analysis revealed that

the reverse primer and probe did not bind the gDNA (ATCC® VR-902BD™) well and that primer dimerization within the LGV II assay and possibly DNA folding at the amplicon site in the synthetic DNA (ATCC® BAA-4002SD™) sequence could have contributed to the amplification issue. The LGV II synthetic DNA product was compatible with an LGV IIb-specific *pmpH* assay from Verweji *et al*. 2010 (Figure 2B). In this case, the genomic DNA counterpart (ATCC® VR-902BD™) extracted from strain 434 does not contain the LGV IIb-specific insertion sequence targeted by the Verweji *et al*. 2010 assay, so it did not amplify. All six products were experimentally verified with the assays from Chen *et al*. 2008, Morré *et al*. 2005, Stevens *et al*. 2010, and the *pmpH* assay from Schaeffer *et al*. 2008 (Table 3). In the case of the assays from Zigangirova *et al*. 2013, we experimentally verified the LGV I and LGV II products with the cryptic plasmid assay and the LGV II products with the 16S rRNA assay. These data are available in the Supplemental section. A table with *in silico* mismatch analysis of the assays with each synthetic DNA product is also available in the Supplemental section (Table S1).

Table 3: Experimentally verified assay compatibility with synthetic and genomic *C. trachomatis* LGV DNA products.

Assay Information		LGV I		LGV II		LGV III	
Source	**Target**	**BAA-4001SD™**	**VR-901BD™**	**BAA-4002SD™**	**VR-902BD™**	**BAA-4003SD™**	**VR-903D™**
Chen *et al*., 2007[4]	cryptic plasmid	✔	✔	✔	✔	✔	✔
Chen *et al*., 2008[5]	*pmpH*	✔	✔	✔	✔	✔	✔
Morré *et al*., 2005[6]	*pmpH*	✔	✔	✔	✔	✔	✔
Schaeffer *et al*., 2008[7]	*pmpH*	✔	✔	✔	✔	✔	✔
	ompA	✔	✔	✖	✖	✔	✔
Stevens *et al*., 2010[8]	*ompA*	✔	✔	✔	✔	✔	✔
Verweji *et al*., 2010[9]	*pmpH*	Not run	Not run	✔	✖*	Not run	Not run
Zigangirova *et al*., 2013[10]	16S	Not run	Not run	✔	✔	Not run	Not run
	cryptic plasmid	✔	✔	✔	✔	Not run	Not run

* The Verweji *et al*. 2010 assay targets an insertion mutation in LGV II variant, IIb, which the synthetic LGV II DNA contains, but *C. trachomatis* LGV II strain 434, from which VR-902BD™ gDNA is derived, does not; therefore, there was no amplification of VR-902BD™ with this assay.

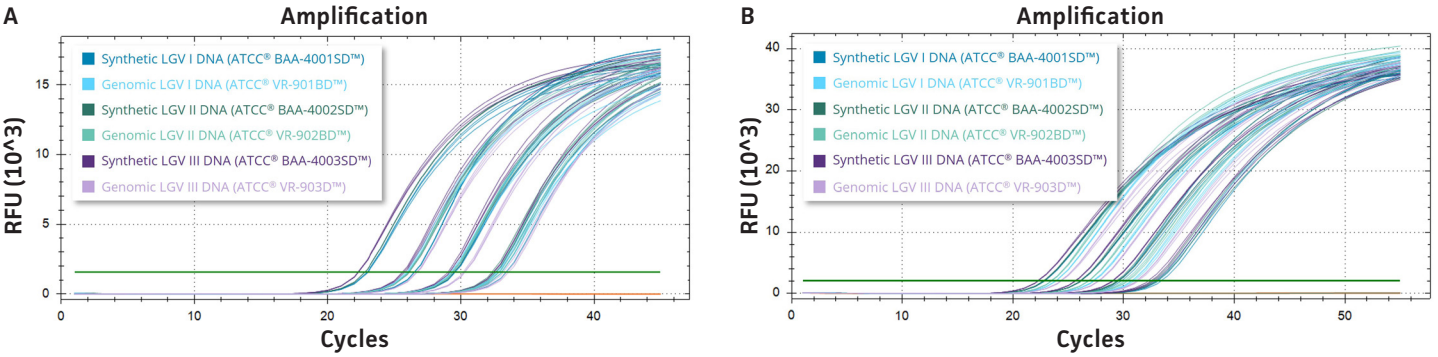

Figure 1: qPCR amplification results for assays compatible with all synthetic and genomic *C. trachomatis* products. Assays from Chen *et al*. 2007 targeting the (A) *pmpH* gene and (B) cryptic plasmid were experimentally verified for all synthetic and genomic LGV DNA products. Amplification plots were generated with serial dilutions ranging from 50-50,000 GC/rxn for each synthetic and 50-5000 GC/rxn for each genomic *C. trachomatis* LGV I-III DNA control. As expected, the synthetic DNA products performed comparably with their corresponding gDNA counterparts, with R^2 values for all templates ranging from 0.997-1.000.

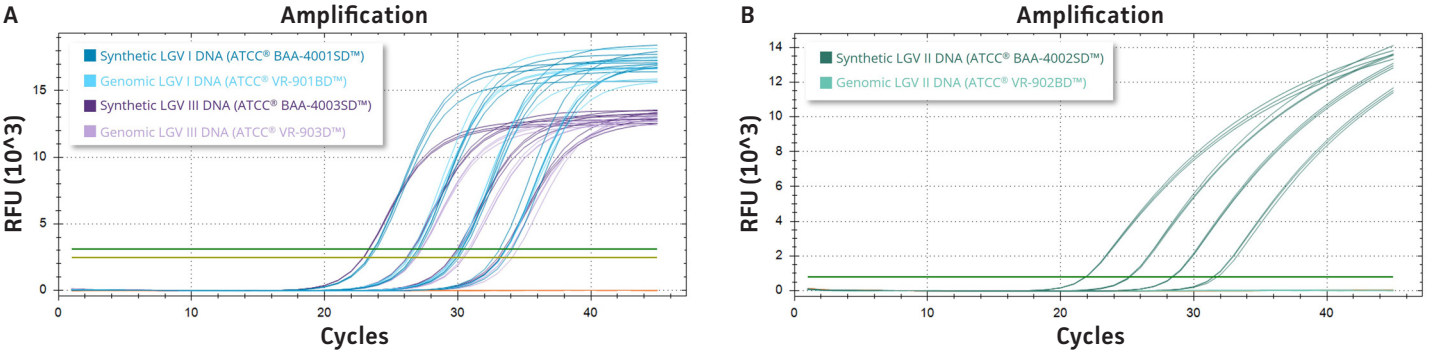

Figure 2: qPCR amplification results of LGV-specific assays. The (A) LGV-specific multiplex assay targeting the *ompA* gene from Schaeffer *et al.* 2008 and (B) a *pmpH* assay specific to LGV IIb from Verweji *et al.* 2010 were experimentally verified for a subset of products. (A) Amplification plots were generated with serial dilutions ranging from 50-50,000 GC/rxn for synthetic and 50-5000 GC/rxn for genomic *C. trachomatis* LGV I and III DNA; the LGV II assay within the multiplex was not compatible with either the synthetic or genomic DNA from LGV II. (B) We show compatibility with an LGV IIb–specific assay; although here, only the synthetic DNA is compatible due to the assay targeting an LGV IIb–specific insertion site in the genome that is not present in the genome of our gDNA template. In these assays, we observe comparable performance of synthetic DNA and gDNA, with R^2 values ranging from (A) 0.996-1.000 for all templates and (B) 0.999 for the synthetic LGV II DNA.

CONCLUSIONS

These data confirm that our quantitative synthetic DNA ARMs for *C. trachomatis* LGV I-III perform comparably to their native gDNA counterparts. We experimentally verified compatibility with multiple published assays for detection and quantification of LGV serovars. As a result, these products serve as reliable analytical reference materials and positive controls for the development, verification, and validation of qPCR tests. Most experimental data shown here demonstrated excellent linearity, supporting their use in generating standard curves for qPCR assays to determine *C. trachomatis* load in the samples. Additionally, these synthetic DNA products can be safely handled under BSL-1 conditions, offering a significant advantage over LGV gDNA, which must be generated under BSL-3 conditions. Finally, these *C. trachomatis* LGV I-III synthetic DNA products are manufactured under ISO 13485 guidance, ensuring regulatory compliance, consistent quality, and enhanced confidence in their use.

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SUPPLEMENTAL DATA

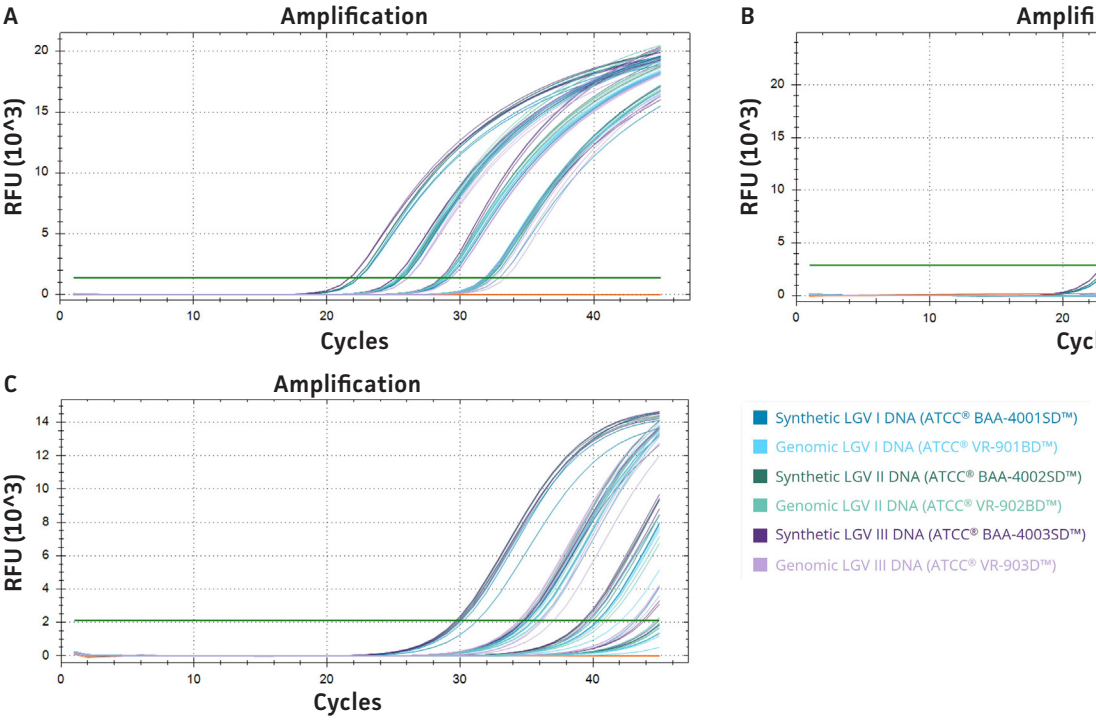

Figure S1: qPCR amplification results of *pmpH* assays. Synthetic and genomic DNA from all three LGV serovars were experimentally verified with assays from (A) Chen *et al*. 2008, (B) Morré *et al*. 2005, and (C) Schaeffer *et al*. 2008. Amplification plots were generated with serial dilutions ranging from 50-50,000 GC/rxn for synthetic and 50-5000 GC/rxn for genomic *C. trachomatis* LGV I-III DNA. As expected, the synthetic DNA products performed comparably with their corresponding gDNA counterparts. The R^2 values ranged from 0.991-1.000 for all templates across the assays from (A) Chen *et al*. 2008 and (B) Morré *et al*. 2005.

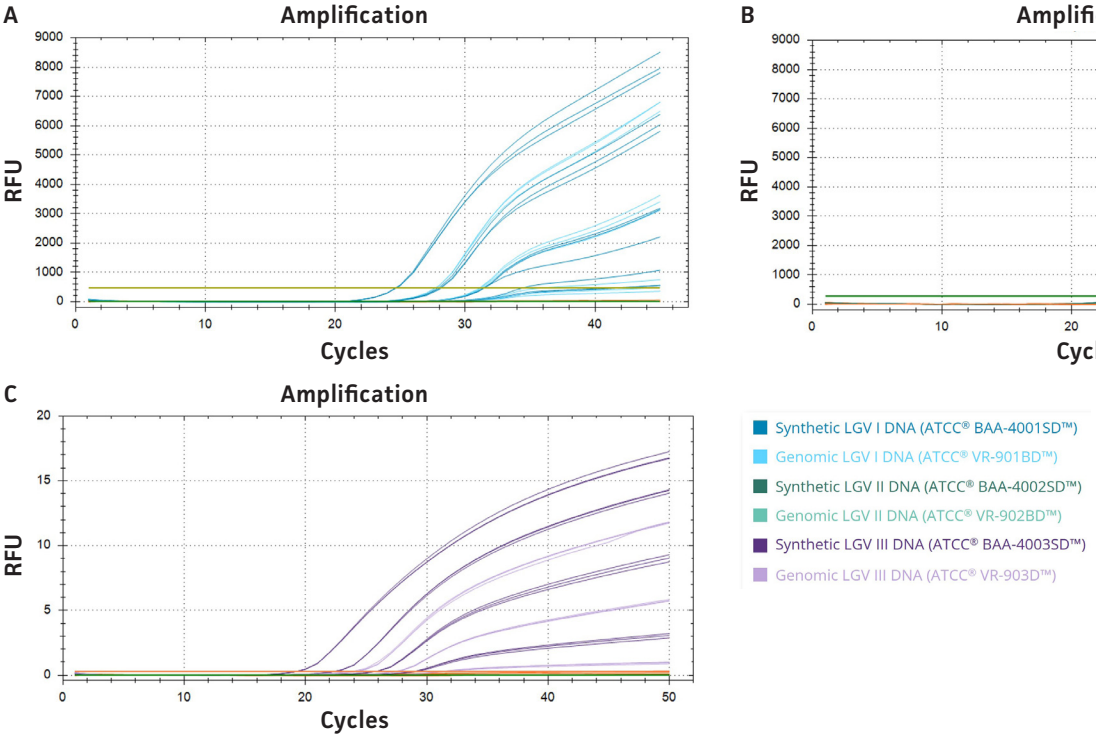

Figure S2: qPCR amplification results of the LGV-specific multiplex *ompA* assay from Stevens *et al*. 2010. (A) LGV I, (B) LGV II, and (C) LGV III synthetic and gDNA products were experimentally verified. Amplification plots were generated with serial dilutions ranging from 50-50,000 GC/rxn for synthetic and 50-5000 GC/rxn for genomic *C. trachomatis* LGV I-III DNA controls. Graphs are separated here by LGV serovar due to fluorescent signal differences. Here, the synthetic DNA products performed similarly to their corresponding gDNA counterparts; although, the LGV III gDNA amplified less efficiently than the synthetic LGV III DNA in this case.

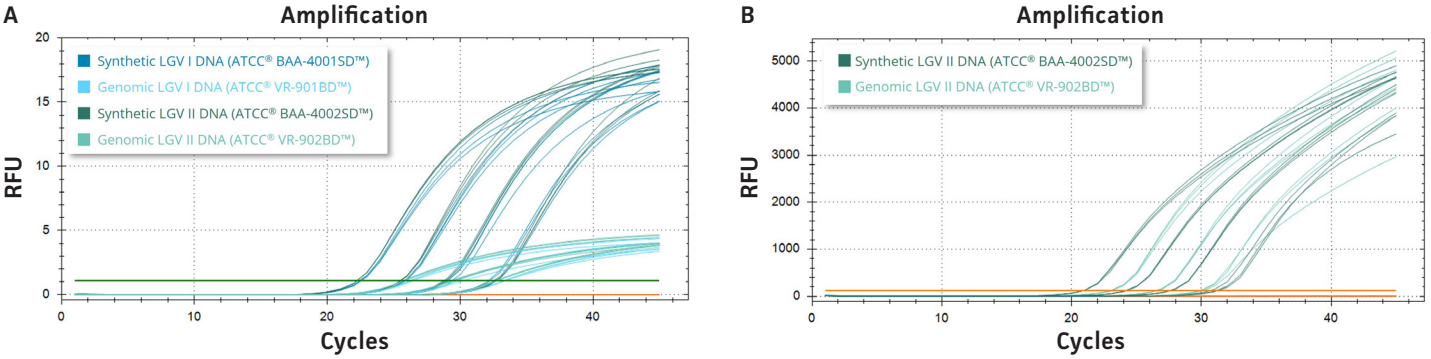

Figure S3: qPCR amplification results of the assays from Zigangirova *et al.* 2013. (A) LGV I and LGV II DNA products were experimentally verified with the cryptic plasmid assay, and (B) the LGV II DNA products were also experimentally verified with the 16S rRNA assay. Amplification plots were generated with serial dilutions ranging from 50-50,000 GC/rxn for synthetic and 50-5000 GC/rxn for genomic controls. The synthetic DNA products performed comparably with their corresponding gDNA counterparts, with R^2 values for all templates across both assays ranging from 0.998-1.000; although, in the case of the (A) cryptic plasmid assay, the synthetic DNA had stronger fluorescent signals than the gDNA counterparts for both LGV I and II.

Table S1: *In silico* assessment of assay binding to each ATCC® *C. trachomatis* LGV synthetic DNA

Publication	Gene Target	Primer or Probe	Sequence (5' to 3')	Mismatches with BAA-4001SD™	BAA-4002SD™	BAA-4003SD™
Chen *et al.*, 2007	Cryptic plasmid	Forward Primer	GGATAACTCTGTGGGGTATTCTCCT	0	0	0
		Reverse Primer	ACGCCTGAGATCTCCAAACTAGT	0	0	0
		Probe	TAGACCCTTTCCGAGCATCACTAACTGTTG	0	0	0
	pmpH	Forward Primer	GGATAACTCTGTGGGGTATTCTCCT	0	0	0
		Reverse Primer	ACGCCTGAGATCTCCAAACTAGT	0	0	0
		Probe	TAGACCCTTTCCGAGCATCACTAACTGTTG	0	0	0
Chen *et al.*, 2008	*pmpH*	Forward Primer	GGATAACTCTGTGGGGTATTCTCCT	0	0	0
		Reverse Primer	AGACCCTTTCCGAGCATCACT	0	0	0
		Probe	CCTGCTCCAACAGT	0	0	0
Morré *et al.*, 2005	*pmpH*	Forward Primer	CTGTGCCAACCTCATCATCAA	0	0	0
		Reverse Primer	AGACCCTTTCCGAGCATCACT	0	0	0
		Probe	CCTGCTCCAACAGT	0	0	0
Schaeffer *et al.*, 2008	*pmpH*	Forward Primer	CTGTGCCAACCTCATCATCAA	0	0	0
		Reverse Primer	AGACCCTTTCCGAGCATCACT	0	0	0
		Probe	CCGCCTGCTCCAACAGTTAGTGATG	0	0	0
	ompA	Forward Primer	CAGCATCTTTCAACTTAGTTGGGTTA	0	0	4
		Reverse Primer	AGCTCATATTTGGTACAGCATCCTT	0	≥6	≥6
		Probe	TCGGAGATAATGAAAATCAAAGCACGGTCA	0	≥6	≥6
		Forward Primer	CAGCATCTTTCAACTTAGTTGGGTTAT	0	0	4
		Reverse Primer	TGATCTAAGCTCATATTTGGTACAAGCTTA	≥6	0	≥6
		Probe	CGGAGATAATGAGAACCATGCTACAGTTTCAGA	≥6	0	≥6
		Forward Primer	CGCTTCCTTCAACTTAGTTGGATT	≥6	≥6	0
		Reverse Primer	TCAAAGCAGTGTTAGGAACAAGCT	≥6	≥6	0
		Probe	TTCGGAACAAAAACACAATCTACTAACTTTAATACAGCG	≥6	≥6	0
Stevens *et al.*, 2010	*ompA*	Forward Primer	GACATATGCAGGATGCTGAGATG	0	0	4
		Reverse Primer	TATTGGAAAGAAGCSCCTAAAGT	0	0	≥6
		Probe	TACAGCATCCTTTTTGACCGT	0	≥6	≥6
		Forward Primer	GACATATGCAGGATGCTGAGATG	0	0	4
		Reverse Primer	TATTGGAAAGAAGCSCCTAAAGT	0	0	≥6
		Probe	ACAAGCTTACTATCTGAAACTGTAG	≥6	0	≥6
		Forward Primer	CCACTTGGTGTGACGCTATCAGCAT	0	0	0
		Reverse Primer	GCATCTTGCATGTGTTTGCCATAAGC	5	5	0
		Probe	TCGTTTGATAAGCCCGCTGTATCGC	≥6	≥6	0
Verweji *et al.*, 2010	*pmpH*	Forward Primer	TCGCTGACGATTCCTTTTGTT	0	0	0
		Reverse Primer	CGCTTCATTGTAACCCCGTTA	0	0	0
		Probe	CTTCTAGTAGTTCTAGTAGTGCT	≥6	0	≥6
Zigangirova *et al.*, 2013	Cryptic plasmid	Forward Primer	GGCGATATTTGGGCATCCGAGTAACG	0	0	0
		Reverse Primer	TCAAATCCAGCGGGTATTAACCGCCT	1	0	1
		Probe	TGGCGGCCAATCTCTCAATCCGCCTAGA	0	0	0
	16S rRNA	Forward Primer	GGGATTCCTGTAACAACAAGTCAGG	0	0	0
		Reverse Primer	CCTCTTCCCCAGAACAATAAGAACAC	0	0	0
		Probe	CTCCCAGAGTACTTCGTGCAAGCGCTTTGA	0	0	1

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