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A simple and rapid DNA extraction method for *Chlamydia trachomatis* detection from urogenital swabs

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Abstract

A highly sensitive and specific *Chlamydia trachomatis* (CT) diagnostic test was developed by combining filtration isolation of nucleic acid (FINA) extraction with quantitative polymerase chain reaction including an internal control to identify test inhibition. A pilot study of 40 clinical specimens yielded 100% sensitivity and specificity.

Keywords

Chlamydia trachomatis; diagnosis; sample prep; quantitative PCR; sexually transmitted disease

Filtration isolation of nucleic acids (FINA) is a simple, rapid DNA extraction process (<2 minutes) developed for proviral HIV-1 detection from blood (1, 2). Genomic DNA is trapped in a glass-fiber disk, washed to remove inhibitors, and qPCR amplified from filter. A sample-to-answer point-of-care (POC) qPCR system is under development where the disk is inserted into a disposable assay card, and the battery-operated analyzer rehydrates lyophilized PCR reagents and performs qPCR (3).

Chlamydia trachomatis (CT), an obligate intracellular parasite, causes a common sexually transmitted infection (STI) (4). In women, infections are frequently asymptomatic, and may result in reproductive complications and loss of fertility (5, 6). A POC test that offers immediate diagnosis allowing prompt treatment could reduce STI prevalence and transmission in high risk populations (7). CT screening in women who are at higher infection risk will reduce reproductive health morbidities (5).

Here we apply FINA testing to CT DNA detection thus broadening the potential of our FINA POC analyzer. The assay targets *parA* located on the multi-copy cryptic plasmid found in all CT serovars including the Swedish nvCT serovar (8, 9) plus the hydroxypyruvate reductase gene of *Cucurbita pepo* (pumpkin), which is unlikely to cross-

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react with urogenital pathogens as a non-competitive internal control (IC) (2, 10). McCoy cells infected by CT [ATCC®VR-346™ Serovar F; (American Type Culture Collection; Manassas, VA)] were used as the model system for infected urogenital epithelial cells to demonstrate that plasmid DNA can be co-isolated with gDNA. Accurate identification of 40 CT clinical specimens confirmed this observation.

DNA was extracted from CT-infected McCoy cells or swabs by FINA method (1, 2, 11) with minor modifications. A DNA capture membrane (D=5.1-mm; Fusion 5, GE Healthcare, Pittsburgh, PA) was sandwiched between 707 blotter pad (25X25-mm; VWR International, West Chester, PA) and PARAFILM (Bemis Flexible Packaging; Neenah, WI) with a 4.3-mm-diameter hole exposing the center of DNA capture membrane (11) (Figure 1A). Cells were lysed by Triton-X100 (1% final concentration; Sigma Chemicals; St. Louis MO), added to the DNA capture membrane, and washed with 400µl of 10mM NaOH. The membrane was placed into a 200µl PCR tube prepared with double-sided adhesive (3M Double-Coated Polyester Diagnostic tape, #9965; D=4.3-mm; 3M Medical Specialties, St. Paul, MN) which holds the membrane out of the light path during amplification (Figure 1A).

PCR master mix consisted of: 0.2mg/ml bovine serum albumin (Life Technologies Corporation; Grand Island, NY), 0.2mg/ml Surfact-Amps®20 (Thermo Scientific; Rockford, IL), 150mM trehalose (Sigma), 10% glycerol (Affymetrix, Incorporated.; Santa Clara, CA), 62.5mM bicine pH8 (Affymetrix), 135mM CH₃CO₂K (Affymetrix), 2.0mM MgCl₂ (Sigma), 0.325mM dNTPs (Life Technologies), 32mM Tris, pH8.0 (Life Technologies), 3.75U Z05 DNA polymerase (Roche Molecular Systems, Inc., Branchburg, NJ), and sequence-specific oligonucleotides. MIQE guidelines (12) allow publication of reference sequence, anchor nucleotide (defined as a nucleotide located in probe sequence), and amplicon length in lieu of DNA sequences; CT and IC amplicons are: CP015307.1, 6300, 91bp and U01067.1, 330, 103bp, respectively. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The CT amplicon was detected via 5'-fluorescein-conjugated double-quenched ZEN™ probe (green), and the IC amplicon was detected via 5'-conjugated double-quenched TAO™ Cy5 probe (red). PCR conditions of CT single copy gene, *omcB*, were the same as above with forward primer 5'-GACACCAAAGCGAAAGACAA-3', reverse primer 5'-GCAAGAATCCTGTTTAGGTCC-3', and probe 5'-FAM-AGCAAAAAAGCAAGAAAAAACCACAGC-BHQ1-3'. Amplification was performed in Stratagene Mx3005p cyclor: 95°C for 2:00; 50 cycles of 95 °C 0:15 and 60 °C 0:45. Data were acquired in green and red channels and analyzed by Stratagene software. CT genomic copies in infected McCoy cells were determined by extracting total DNA with the BD GeneOhm Lysis Kit™ (Franklin Lakes, NJ), and quantifying CT genomic copies with an *omcB* standard curve with gDNA copy standards.

To verify true negative results, 500 copies of IC plasmid (2) was included in each reaction. Negative CT samples with no IC amplification are considered invalid. Standard curves of 100µl CT-infected McCoy cells were tested with and without IC amplification, and assay performance was unaffected (Figure 1B). C_q values were plotted *versus* log₁₀ genomic copies/reaction. Slopes were estimated by linear regression (XLStat) and used to calculate qPCR efficiencies (13): +IC = 92.7% (slope -3.51; 95% CI -3.71,-3.32), and -IC = 95.7%

(slope -3.43 ; 95% CI $-3.53, -3.34$), indicating very similar assay performance. Additionally, the limit of detection of the FINA assay was estimated at 32 CT genomic copies by testing 7 replicates of CT- infected McCoy cells containing 32, 16, 8, and 4 genomic copies/reaction and determining the percent positives at each concentration: 100%, 71%, 57% and 43%, respectively.

CT oligonucleotide sequences were designed to not cross-react to human, viral, or bacterial genomes in the NCBI database. Additionally, assay specificity was assessed by testing DNA isolated from a specificity panel (SP) of either closely related species or species frequently found in the urogenital tract: *Neisseria gonorrhoeae* ATCC19424, *Neisseria lactamica* ATCC23970, *Trichomonas vaginalis* (patient isolate), *Staphylococcus epidermidis* ATCC14990, *Acinetobacter lwoffii* ATCC15309, *Candida albicans* ATCC96268, *Chlamydia muridarum* ATCC VR-123, and *Escherichia coli* OP50. 500,000 genomic copies of each panel member were spiked into qPCR reactions, and no amplification was observed. Amplification interference was tested by assaying 500,000 copies of each SP member plus 5,000 copies of CT DNA in triplicate. The average Cq without SP DNA was 24.6 ± 0.03 (N=3), and with SP DNA was 24.4 ± 0.07 (N=24) demonstrating no interference.

FINA was also used successfully with urogenital swabs. Forty (20 positive/20 negative) de-identified dried swabs from the Center for Point-of-Care Tests for Sexually Transmitted Diseases (IRB approved study NA 00023037; second swab collected after standard of care specimen) were tested by resuspending swab in 3ml of 10mM Tris, pH8.0 and 0.01% Surfact-Amps®20, mixing vigorously, and testing 25µl (0.8%) of specimen. All swabs were correctly identified versus Aptima® *Chlamydia trachomatis* Assay yielding test sensitivity and specificity of 100% (95% CI: 80.0–100%). Positive Cqs ranged from 21.4–35.5 (Table 1), and no amplification was observed for negative specimens. The average IC Cq was 31.1 ± 0.3 and average negative control Cq was 31.6 ± 0.2 (N=4) indicating no qPCR inhibition. FINA extraction of urogenital swabs was combined with qPCR amplification for sensitive CT detection.

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Abbreviations

FINA	filtration isolation of nucleic acids
CT	<i>Chlamydia trachomatis</i>
HPR	hydroxypyruvate reductase
IC	internal control
qPCR	quantitative polymerase chain reaction

IFU	inclusion forming unit
Cq	quantitation cycle
CI	95% confidence interval
SP	specificity panel

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Highlights

- Highly sensitive and specific *Chlamydia trachomatis* assay is reported
- Utilizes filtration isolation of nucleic acids (FINA) sample prep method
- Assay performance was validated with 40 clinical swab specimens

Figure 1.

A) FINA sample preparation module qPCR tube with double sided tape. Sample preparation steps: 1. Lysed sample is added to DNA capture disk; 2. Disk is washed with 10 mM NaOH; 3. Disk is attached to side of qPCR tube by double sided tape; 4. DNA is amplified off capture disk. B) Amplification plots obtained from 100 μ l of 4 replicates of UTM spiked with 3,204,000, 320,400, 32,040, 3204, 320 and 32 CT genomic copies/pcr reaction with and without 500 copies/reaction of the internal control; gray lines=+IC; black lines=-IC. Y-axis is fluorescence units and X-axis is qPCR cycle number. Inset plot contains standard curves of Cq values calculated from amplification plot versus log genomic copies/reaction; Gray * represent samples with IC, $y = -3.51x + 41.66$ [95% CI slope: $-3.71, -3.31$]; $R^2 =$

0.986; black open circles represent samples without IC, $y = -3.43x + 41.51$ [95% CI slope: $-3.53, -3.34$]; $R^2 = 0.996$.

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Cqs of the CT and IC qPCR assays as compared to the negative/positive result of the Aptima® *Chlamydia trachomatis* Assay provided by the Johns Hopkins Center for STD Point-of-Care Tests.

Table 1

Swab	Aptima	CT	IC	Swab	Aptima	CT	IC
1	Neg	No Cq	31.2	3	Pos	30.1	30.9
2	Neg	No Cq	31.4	6	Pos	22.3	31.0
4	Neg	No Cq	31.2	7	Pos	23.8	31.1
5	Neg	No Cq	31.3	11	Pos	33.2	31.3
8	Neg	No Cq	31.3	13	Pos	22.8	31.0
9	Neg	No Cq	30.9	14	Pos	31.3	31.1
10	Neg	No Cq	31.4	16	Pos	22.1	30.8
12	Neg	No Cq	31.1	18	Pos	23.5	30.7
15	Neg	No Cq	31.3	19	Pos	21.7	30.7
17	Neg	No Cq	31.3	21	Pos	23.2	30.5
20	Neg	No Cq	31.6	22	Pos	35.5	31.3
24	Neg	No Cq	31.4	23	Pos	22.9	30.7
25	Neg	No Cq	31.3	26	Pos	21.4	30.6
29	Neg	No Cq	31.3	27	Pos	22.5	31.3
30	Neg	No Cq	30.9	28	Pos	23.4	31.3
31	Neg	No Cq	31.5	34	Pos	25.0	30.5
32	Neg	No Cq	31.7	35	Pos	21.4	30.8
33	Neg	No Cq	31.5	36	Pos	21.7	31.3
38	Neg	No Cq	30.9	37	Pos	22.0	31.7
39	Neg	No Cq	31.6	40	Pos	30.4	31.2