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## Accepted Manuscript

Reduced-cost Chlamydia trachomatis-specific multiplex real-time PCR diagnostic assay evaluated for ocular swabs and use by trachoma research programmes

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**REVISED****Reduced-cost *Chlamydia trachomatis*-specific multiplex real-time PCR diagnostic assay evaluated for ocular swabs and use by trachoma research programmes**

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**Running title**

Open-platform qPCR for *Chlamydia trachomatis* in ocular specimens

**Keywords**

*Chlamydia trachomatis*; diagnosis; quantitative PCR; trachoma

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## Abstract

### Introduction

Trachoma, caused by the intracellular bacterium *Chlamydia trachomatis* (*Ct*) is the leading infectious cause of preventable blindness. Many commercial platforms are available that provide highly sensitive and specific detection of *Ct* DNA. However, the majority of these commercial platforms are inaccessible for population-level surveys in resource-limited settings typical to trachoma control programmes. We developed two low-cost quantitative PCR (qPCR) tests for *Ct* using readily available reagents on standard real-time thermocyclers.

### Methods

Each multiplex quantitative PCR test targets one genomic and one plasmid *Ct* target in addition to an endogenous positive control for *Homo sapiens* DNA. The quantitative performance of the qPCR assays in clinical samples was determined by comparison to a previously evaluated droplet digital PCR (ddPCR) test. The diagnostic performance of the qPCR assays were evaluated against a commercial assay (*artus C. trachomatis* Plus RG PCR, Qiagen Ltd) using molecular diagnostics quality control standards and clinical samples. We examined the yield of *Ct* DNA prepared from five different DNA extraction kits and a cold-chain free dry-sample preservation method using swabs 'spiked' with fixed concentrations of human and *Ct* DNA.

### Results

The qPCR assay was highly reproducible (*Ct* plasmid and genomic targets mean total coefficients of variance 41.5% and 48.3%, respectively). The assay detected 8/8 core specimens upon testing of a quality control panel and performed well in comparison to commercially marketed comparator test (sensitivity and specificity >90%). Optimal extraction and sample preservation methods for research applications were identified.

### Conclusion

We describe a pipeline from collection to diagnosis providing the most efficient sample preservation and extraction with significant per test cost savings over a commercial qPCR diagnostic assay. The assay and its evaluation should allow control programs wishing to conduct independent research within the context of trachoma control, access to an affordable test with defined performance characteristics.

ACCEPTED MANUSCRIPT

68 **Introduction**

69

70 *Chlamydia trachomatis* (*Ct*) is the cause of trachoma, which is the leading cause of infection-  
71 related blindness worldwide (1,2). *Ct* is also the most commonly diagnosed bacterial  
72 sexually transmitted infection (3). Diagnosis of trachoma is made by the observation of a  
73 clinical sign which is the appearance of lymphoid follicles and inflammation on the tarsal  
74 conjunctiva (4). This clinical sign is not highly specific (5) and in low-prevalence or post-  
75 treatment settings can correlate poorly with for ocular *Ct* infection (6–8). Control programs  
76 use azithromycin mass drug administration (MDA) in trachoma endemic communities as part  
77 of an overall strategy to control transmission, but the drop in prevalence of infection results  
78 in a decrease in the positive predictive value of clinical signs of disease (8).

79

80 Determination of infection load data offers additional benefits to a qualitative diagnostic  
81 assay because load of infection is associated with disease severity (5). **Reference-free**  
82 **methods for quantitation of nucleic acids using digital droplet PCR technology have also**  
83 **been evaluated (9); these have been useful in demonstrating that infection load may be**  
84 **involved in transmission (10).** In populations that have been treated *en masse* with  
85 azithromycin, the loads of individual infections are usually low (11). Identifying sub-  
86 populations in which there are infections with higher than average loads can identify  
87 communities and subgroup ‘hotspots’ that are reservoirs of infection in otherwise trachoma-  
88 free areas (12). Conversely, infections in low prevalence or post-treatment settings may not  
89 be of high enough load to sustain transmission and the community or burden of infection  
90 may decline; this is referred to as the Allee effect (7,13). A quantitative diagnostic test may  
91 therefore assist in programmatic decisions such as when to continue, cease or target  
92 azithromycin MDA (14).

93

94 Nucleic acid amplification tests (NAATs) have become the gold standard for *Ct*-specific tests  
95 of infection, due to their superior sensitivity and throughput when compared to culture and  
96 antigen detection techniques (15). There are many accredited commercial assays for the  
97 diagnosis of sexually transmitted *Ct* infections but very few are evaluated for testing with  
98 ocular swabs. Diagnostic tests with quantitative capabilities, such as the Abbott RealTime  
99 CT/NG *m2000* (16) platform is widely distributed in many low- and middle-income countries  
100 yet per-specimen testing costs remain beyond trachoma control and research programs.

101

102 NAATs for *Ct* are not currently required by the international guidelines for implementation or  
103 cessation of the “SAFE” (**S**urgery for the correction of in-turned eyelashes, **A**ntibiotics to  
104 treat infection, promotion of **F**acial hygiene and **E**nvironmental improvement to reduce

105 transmission) strategy for trachoma control (17). Diagnosis of current *Ct* infection can be a  
106 valuable component of the monitoring and evaluation of trachoma control programs (18).  
107 Where NAATs have been used, both commercial (19) and non-commercial (20,21) tests  
108 have yielded important results that have shaped the scientific agenda for control program  
109 evaluation, the research activities of government/non-government organisations and the  
110 policy of funding bodies. The cost efficacy of using a commercial NAAT to guide MDA  
111 cessation has been evaluated (22) and it was found that a low-cost commercial NAAT can  
112 be cost effective for the control program as test costs were offset by savings from the  
113 distribution of further unnecessary annual treatment rounds of MDA. In addition to the cost  
114 benefit, avoiding unnecessary rounds of MDA would reduce community antibiotic exposure  
115 and risk of emergence of antibiotic resistance.

116  
117 There are many situations in which an open-platform test with evaluated performance  
118 characteristics may enable valuable data to be gathered and we therefore designed and  
119 evaluated the performance of a NAAT for detection and enumeration of ocular *Ct* infections.  
120 The test was required to be high throughput, low cost, quantitative and comparable in  
121 performance to a commercial alternative. Capacity to multiplex targets was also important to  
122 enable concurrent testing of specimen collection and extraction. qPCR was therefore  
123 selected over other technologies suitable for low-resource settings (such as end-point PCR  
124 or loop-mediated amplification (LAMP) (23)).

125 **Methods**

126

127 *Study ethics*

128

129 Samples were collected from trachoma-endemic communities in Tanzania and Guinea-  
130 Bissau as detailed below. Ethical approval for the collection of these samples was obtained  
131 from the following ethics committees; Comitê Nacional de Ética e Saúde (Guinea Bissau),  
132 London School of Hygiene and Tropical Medicine, UK. Kilimanjaro Christian Medical Centre,  
133 Tanzania and the National Institute for Medical Research, Tanzania. The support of local  
134 leaders in every community was ascertained before sample collection began. All participants  
135 were required to provide written, informed consent prior to study enrollment and parents or  
136 guardians provided consent for children.

137

138 *Oligonucleotides*

139

140 Primer and hydrolysis probe sequences used in this study are shown in Table 1. Primers  
141 targeting highly conserved species-specific regions of plasmid open reading frame 2  
142 (pORF2) and outer membrane protein complex B (*omcB*) of *Ct* were previously described by  
143 Pickett and colleagues (24). pORF2 and *omcB* probe sequences were designed using  
144 Primer Express v3 (Life technologies, Paisley, UK). Oligonucleotides for use on Applied  
145 Biosystems (ABI) real time thermocyclers were synthesized by Life Technologies.  
146 Oligonucleotides for use on the Corbett Rotor-Gene (a.k.a Qiagen Rotor-Q) were  
147 synthesized by Sigma (Sigma-Aldrich, UK). Endogenous control primers and probes specific  
148 to the *Homo sapiens* RNase P/MRP 30-kDa subunit (*RPP30*) gene were previously  
149 described by Luo and colleagues (25). There is no variation in primer or probe binding sites  
150 in published *Ct* genome and plasmid sequences (NCBI Blastn search January 2017). *omcB*  
151 and pORF2 targets are present in a single copy per chlamydial genome and plasmid,  
152 respectively. Studies have estimated copy number in clinical specimens to be between 1  
153 and 18 copies per genome (26).

154

155 *qPCR*

156

157 For ABI thermal cyclers, each 10- $\mu$ L qPCR contained final concentrations of 1 $\times$  TaqMan  
158 Universal Mastermix II, with **Uracil-DNA N glycosylase (UNG, a common method to minimize**  
159 **PCR cross-contamination by enzymatic degradation of previous PCR products with**  
160 **incorporated dUTP**; Life technologies, Paisley, UK), each oligonucleotide at 0.3  $\mu$ M and 2  $\mu$ L



161 template DNA in aqueous solution. In the United Kingdom, the assay was performed on an  
162 ABI 7900HT Fast Real Time PCR machine (Life Technologies, Paisley, UK). In Tanzania,  
163 the assay was performed on an Applied Biosystems ViiA 7 Real Time PCR machine (Life  
164 Technologies, Paisley, UK). Both instruments utilized a 384-well format.

165  
166 For Rotor-Gene thermal cyclers samples were run in a 72-well rotor format on a Corbett  
167 Rotor-Gene 3000. Each 20- $\mu$ L qPCR contained 1 $\times$  qPCRBIO Probe Mix No-Rox (PCR  
168 Biosystems, London, UK), each oligonucleotide at 0.3  $\mu$ M and 8  $\mu$ L template DNA in  
169 aqueous solution.

170  
171 No-template controls and serial dilutions of known-concentration PCR product were  
172 included on each run on all systems. Thermal cycling conditions for all systems were 50°C  
173 for 2 minutes, 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C  
174 for 60 seconds. Samples with quantitation cycle ( $C_q$ ) values <15 cycles were diluted and  
175 retested.

176  
177 *Calibration curve*

178  
179 Calibration standards were prepared using DNA extracted from cultured Human Epithelial  
180 type-2 (HEp-2) cells, which had been infected with *Ct* strain A2497. The Novogen KOD PCR  
181 kit (Novogen, Sydney, Australia) was used to amplify the pORF2, *omcB* and *RPP30* targets  
182 using the primers described in table 1. PCR products were purified and extracted using the  
183 Promega gel clean-up kit and Wizard SV PCR spin columns (Promega, Madison, WI. USA)  
184 according to manufacturer's guidelines. PCR products were then diluted 1:10<sup>7</sup> in 1 mM Tris-  
185 Cl 0.1 mM EDTA (0.1 $\times$  TE) buffer on a background of 2 ng/ $\mu$ L herring sperm DNA (Sigma  
186 Aldrich, St Louis, LA. USA). These standards were ten-fold serially diluted through ten steps  
187 to create a calibration curve. A droplet digital PCR (ddPCR) assay (26,9) was used to  
188 estimate the number of chlamydial and human targets in each standard. The limit of  
189 detection was defined as the lowest analyte concentration at which all ten repeat  
190 measurements of a specific dilution returned a positive result.

191  
192 *Analysis of clinical samples*

193  
194 To assess the performance of the qPCR test in clinical specimens, we compared the results  
195 of testing by *artus C. trachomatis* Plus RG PCR, qPCR and ddPCR. This analysis used 99  
196 randomly selected samples from a collection of clinical ocular-swab derived DNA specimens

197 that originated from a 2014 study of children aged 1-9 years selected from trachoma-  
198 endemic communities on the Bijagos Islands, Guinea-Bissau. Sample collection protocols  
199 and *Ct* ddPCR results were described previously (27). In Tanzania we also tested 523  
200 samples with qPCR referenced against ddPCR. These clinical samples were from a single  
201 cross-sectional time point of a 4-year longitudinal study of a cohort of children aged 5–10  
202 years of age. In each study, samples were collected prior to community treatment with  
203 azithromycin.

204

205 The *artus C. trachomatis* Plus RG PCR Kit (96) CE (4559265) was used to test the samples  
206 from the Guinea-Bissau cohort. Testing was performed as per manufacturer's instructions  
207 using the kit internal control to monitor possible PCR inhibition by adding directly to the  
208 reaction mixture.

209

#### 210 *Quality control molecular diagnostics panel performance*

211

212 Using the assays described above, we used an external quality control molecular  
213 diagnostics panel of samples, (Quality Control in Molecular Diagnostics (QCMD) programme  
214 ([www.qcmd.org](http://www.qcmd.org)) (28)). The panel consisted of positive and negative samples which  
215 participants were expected to detect (termed 'core' samples) and low-load samples termed  
216 'educational' that contained <1 genome equivalent per microlitre of sample. In 'educational'  
217 specimens, the load is so low that only extremely sensitive tests (i.e. those using target  
218 enrichment or transcription-assisted amplification) would be expected to routinely identify  
219 these as positive. The lyophilized samples were rehydrated according to QCMD protocol, in  
220 4 mL of sterile molecular biology-grade water, of which DNA was extracted from 1 mL and  
221 eluted into 100  $\mu$ L. Following DNA extraction, the assays were performed as described.

222

#### 223 *Testing costs of extracted DNA from clinical swabs by artus C. trachomatis Plus RG PCR,* 224 *qPCR and ddPCR*

225

226 The laboratory cost of processing the samples by each assay was calculated for both  
227 personnel time and consumables. Costs of consumables were taken from current UK list  
228 prices at the time of publication and were expressed in **US Dollars (US\$)**. Personnel costs  
229 were based on a salary for a junior laboratory technician at Kilimanjaro Christian Medical  
230 Centre in Moshi, Tanzania. Per-sample costs were calculated based on the time taken to  
231 complete one run on each analysis platform and then expressed as a per sample cost  
232 including overheads. Equipment procurement costs were not included in the analysis, as

233 thermocycler availability and use will vary between country and laboratory. However, prices  
234 for the thermocyclers used in this manuscript are stated in the footnote for Table 6.

235

#### 236 *Preparation of 'spiked' swabs for use in storage assessment experiments*

237

238 A suspension of cultured HEp-2 cells and serovar A *Ct* EBs in phosphate-buffered saline  
239 (PBS) was inoculated onto swabs. HEp-2 cells were spiked into PBS at approximately  
240 400,000 per 1 mL of PBS. Elementary bodies (EBs) were spiked into the same suspension  
241 at a dilution of 2  $\mu$ L of EBs per 1 mL of PBS to achieve a high concentration of *Ct* targets per  
242 PCR reaction. The suspension was homogenized and 50  $\mu$ L was aliquoted onto polyester-  
243 coated swabs (Puritan Medical Products, Guilford, USA). Swabs were allocated to storage in  
244 one of three conditions: dry storage in polystyrene tubes at room temperature (uncontrolled,  
245 typically 22-25°C), dry storage in paper envelopes at room temperature inside a domestic  
246 vacuum-sealed container with silica desiccant, or dry storage in polystyrene tubes at -20°C.  
247 Four swabs were prepared and processed per time point per storage condition. Swabs were  
248 removed from storage at 7, 30, 90 and 180 days and DNA was extracted using the swab  
249 protocol of the QIAamp DNA mini kit (Qiagen, Manchester, UK) according to manufacturer's  
250 instructions. Swabs were tested using ABI qPCR.

251

#### 252 *Comparison of DNA extraction kits and recovery of Chlamydia trachomatis nucleic acids*

253

254 Peripheral blood mononuclear cells (PBMCs) were extracted from the blood of a healthy  
255 volunteer. PBMCs were suspended in PBS and aliquots spiked with high, medium and low  
256 loads of *Ct* A/2497 elementary bodies (EBs). One aliquot did not have any EBs added to act  
257 as negative control. A 50- $\mu$ L aliquot of suspension was pipetted directly onto swabs. A total  
258 of 25 swabs were prepared at each concentration level and refrigerated overnight. Five  
259 swabs were selected at random from each concentration group. Each swab was rehydrated  
260 in 400  $\mu$ L of PBS. They were then vortexed at full speed for 2 minutes and the swab was  
261 removed and discarded, expressing any excess liquid on the side of the tube. DNA was then  
262 prepared following the manufacturer's recommendations for each respective kit. The elution  
263 volume was standardised to 100  $\mu$ L. Five extraction kits were compared: MTB Isolation  
264 (Elisabeth Pharmacon), Blood and Serum DNA Isolation Kit (BioChain), Cador Pathogen  
265 (QIAGEN), QIAamp Mini DNA Extraction (QIAGEN) and Power Soil DNA Isolation Kit  
266 (MoBio), which includes a mechanical lysis step (specimen lysed in PowerBead tube at 6  
267 m/s for 40 seconds). Four 1  $\mu$ L aliquots of eluate were tested per swab, resulting in 20 test  
268 wells per condition.  $C_q$  values from high-, medium- and low-load sample eluates were

269 collated into a single dataset for each extraction kit. QIAmp DNA mini kit was used as our  
270 standard reference as it is used widely in many studies, and our group has used this kit  
271 extensively in trachoma studies (9,29,30).

272

### 273 *Data analysis*

274

275 Data were reported in accordance with Minimum Information for Publication of Quantitative  
276 Real-Time PCR Experiments (MIQE) guidelines (31) (see supplementary information).  
277 Clinical specimens and PCR product dilutions were classified as positive for *Ct* if the test  
278 detected amplification of the plasmid target in any well within 40 cycles for the ABI assay, or  
279 35 cycles for the Rotor-Gene assay. The load of infection was determined by extrapolation  
280 from an eight-step, ten-fold dilution of PCR product standards of known concentration; these  
281 were tested in triplicate on each plate.

282

283 SDS 2.4 software (Life Technologies, Paisley, UK) was used for data analysis. Baseline  
284 fluorescence intensity values were determined by analysis of mean fluorescence between  
285 cycles 3 and 15 on both platforms. The  $C_q$  boundary line was set at 0.2 for all three  
286 fluorescence channels (FAM/VIC/NED) on the ABI instrument, and at 0.1 on the Rotor-Gene  
287 instrument.  $C_q$  data were exported from SDS 2.4 and further analysed using R version 3.2.2  
288 (32). Linear regression was used to determine whether the  $C_q$  decreased significantly with  
289 time under different storage conditions. The gradients of linear models were examined to  
290 determine whether a significant downward trend was identified. To determine if there were  
291 significant differences between *Ct* DNA recovery from extraction kits, homogeneity of  
292 variance within the total dataset was assessed using Fligners test. One-way Analysis of  
293 Variance (ANOVA) with Tukey's Honest Significant Difference (HSD) post-hoc test was used  
294 to determine which of the observed differences were significant.

295

296 **Results**

297

298 *Assay performance*

299

300 The assay characteristics derived from repeat testing of a standard curve are presented in  
301 Table 2. The experimentally determined dynamic range of the assay was between 1 and  $1 \times$   
302  $10^6$  copies of *omcB* and/or pORF2 per reaction. The coefficients of determination for all  
303 three targets in both assays was greater than 0.99. Amplification of all targets was highly  
304 (>95%) efficient. *omcB*, pORF2 and *RPP30* were reproducibly detected at concentrations of  
305 0.9–8.3 copies per test, but not below. No-template controls tested negative on every run.

306

307 The mean coefficient of variance around all data points across the whole dynamic range was  
308 48.3% for the *omcB* target, and 41.5% for the pORF2 target, approximately equivalent to 0.6  
309 and 0.5 PCR cycles, respectively. For the Rotor-Gene assay, the coefficient of variance was  
310 35.3% for *omcB* and 20.3% for pORF2, equivalent to approximately 0.4 and 0.3 cycles,  
311 respectively. The largest contributor to assay variance on both platforms was between-run  
312 variation. The coefficient of variance generally increased at lower concentrations, possibly  
313 reflecting the increased chance of sampling handling error where analytes are rare.  
314 Interestingly, when C<sub>q</sub> values were compared between ABI 7900HT and ABI ViiA7  
315 machines, the assay parameters were mostly similar with the exception that absolute *omcB*  
316 C<sub>q</sub> values were consistently between 0.5 and 1.5 cycles higher when tested in Tanzania than  
317 when tested in the UK.

318

319 *Quality control panel performance*

320

321 All three assays (*artus*, ABI and Rotor-Gene) performed well when used to test external  
322 quality control molecular diagnostics samples, correctly diagnosing 8/8 (100%) of 'core'  
323 samples. Two low-load 'educational' samples, which were below the measured limit of  
324 reproducible detection for the assay were not classified as positive. The results are shown in  
325 Table 3.

326

327 *Clinical specimens*

328

329 According to the validated commercial kit (*artus*), 28/99 Guinea Bissau specimens were  
330 positive for *Ct*. The mean C<sub>q</sub> of those positive specimens was 30.3. On the ABI platform  
331 26/99 of the Guinea Bissau samples were positive. Of the same sample set, 23/99 samples  
332 tested positive by Rotor-Gene. Of the *artus*-positive results, only one was negative by all

333 other methods. The load estimates from samples where both targets were detected is shown  
334 in Figure 1. *omcB* was not detected in two of the ABI positive specimens, which had a  
335 plasmid load of 2 and 3 copies/ $\mu\text{L}$ , respectively. The median *artus*  $C_q$  of *artus+* qPCR- (false  
336 negative) specimens was 34.9 and 32.4 cycles for the ABI and Rotor-Gene assays,  
337 respectively (figure 1). The median load of *artus-* qPCR+ specimens was 29 and 3 copies/ $\mu\text{L}$   
338 for the ABI and Rotor-Gene assays, respectively. For *omcB*, the median load of the ddPCR+  
339 qPCR- samples was 1.2 and 0.7 copies/ $\mu\text{L}$  for the ABI and Rotor-Gene platforms,  
340 respectively (figure 2). There were no ddPCR- qPCR+ samples.

341

342 In Tanzania we tested a further 523 clinical samples by ABI qPCR that had also been tested  
343 by ddPCR at LSHTM. The overall prevalence of infection by ddPCR was 12.4% (65/523). By  
344 qPCR there were 78/523 positive samples leading to a sensitivity and specificity of 100%  
345 (95% CI 94.5 – 100) and 97.2% (95% CI 95.2 – 98.5). The data are shown in Table 4.

346

#### 347 *Comparative efficiency of sample extraction and yield*

348

349 DNA prepared using the PowerSoil DNA kit yielded the most variable estimates of *Ct* burden  
350 overall (Figure 3). Qiagen Cador and Biochain kits recovered the highest amounts of *Ct* DNA  
351 measured by the quantity of *omcB* ( $p = 0.001$  and  $p = 0.0004$ , respectively) and pORF2 ( $p$   
352  $= 0.0004$  and  $p = 0.002$ , respectively) load compared to QIAmp DNA mini extraction. Using  
353 one-way Analysis of Variance (ANOVA) was considered appropriate as there was no  
354 significant heterogeneity in the variance between comparator groups (Fligner's test (*omcB*  $p$   
355  $= 0.31$  and pORF2  $p = 0.66$ )). There were significant differences within the model for both  
356 targets (*omcB*  $p = 0.00005$  and pORF2  $p = 0.00003$ ). Pair-wise analyses using Tukey's  
357 Honest Significant Difference (HSD) *post-hoc* test, found that both Qiagen Cador and  
358 Biochain kits had significantly higher yield when compared to MTB, QIAmp DNA mini and  
359 PowerSoil DNA kits. The results of the pair-wise comparisons were consistent for both *omcB*  
360 and pORF2 targets.

361

#### 362 *Sample preservation and storage*

363

364 *Ct* and human DNA was readily detectable in all samples at all time points, with no  
365 diagnostic failures by 6 months storage at room temperature (Figure 4). All three treatments  
366 showed a significant increase in  $C_q$  required to detect *Ct* over 6 months according to linear  
367 regression models, indicating a decrease in target abundance (Table 5). Based on these  
368 models the estimated rate of reduction in detectable load was 0.01-0.02% of the 7-day

369 specimen  $C_q$  per day. After 6 months, the mean  $C_q$  for detection of plasmid had increased by  
370 18% for the frozen swabs, and by 21% and 14%, respectively, for the desktop and vacuum  
371 contained room temperature swabs. The  $C_q$  for omcB target detection had increased by 9%  
372 for the frozen swabs, and by 17% and 14%, respectively, for the desktop and vacuum  
373 contained room temperature swabs.

374

375 *Testing costs of extracted DNA from clinical swabs by artus C. trachomatis Plus RG PCR,*  
376 *qPCR and ddPCR*

377

378 Overall costs and component parts can be found in Table 6. The commercial test *artus* was  
379 the most expensive at **\$25.04** per sample, and the least expensive was the Rotor-Gene at  
380 **\$9.51** per sample. Calculated costs include trained laboratory technician time for a  
381 Tanzanian Junior Laboratory Technician and therefore all testing runs can be prepared  
382 within a reasonably short period of time. A single ABI qPCR plate of up to 88 samples run  
383 with four technical replicates (plus standards) takes an experienced operator approximately  
384 1.5 hours to prepare and 1.75 hours to run. The operator time is increased for ddPCR to 2  
385 hours preparation time for up to 94 single reaction samples and 2.5 hours run time. Time  
386 savings can be found in the use of the Rotor-Gene which takes approximately 1 hour to  
387 prepare and 1 hour to run 63 single reaction samples. The latter is comparable to the time  
388 required to test 70 samples by *artus*.

389

390

391 **Discussion**

392

393 We evaluated a qPCR assay that detects *Ct* plasmid and genomic targets, whilst assessing  
394 specimen sufficiency with the presence of human DNA. The assay has a linear analyte  
395 response for all three targets that is reproducible across a wide dynamic range (1 - 10<sup>6</sup>  
396 copies/test) of both plasmid and chromosomal targets. The limits of reproducible detection  
397 for both *Ct* targets are below 10 targets per test, which is comparable to other non-  
398 commercial PCR tests (24,33). The absolute sensitivity of the *omcB* and pORF2 tests is  
399 similar, however, due to the relative abundance of the plasmid target in clinical specimens,  
400 the plasmid test detects a lower absolute number of chlamydial equivalents and therefore  
401 has a superior diagnostic performance.

402

403 The total assay variance within-centre was consistently <1 PCR cycle. There was significant  
404 variation within-run (*omcB* mean: 24.7%, pORF2 mean: 19.5%), suggesting that, where  
405 precise quantitation is required, specimens should ideally be run in multiple wells. Where a  
406 qualitative diagnostic result is sufficient, running assays in a single well would only result in  
407 diagnostic failure due to assay variability at very low loads. The between-laboratory variance  
408 is higher, which could be attributed to instrument differences between the 7900HT and the  
409 Viiia 7 (laser excitation versus halogen light source), however the assay was highly linear on  
410 both platforms and the total variance on either target was <1.5 cycles.

411

412 External quality control exercises that included masked testing were used to evaluate these  
413 qPCR assays. During this exercise we successfully identified *Ct* infection in a specimen that  
414 carried a well-characterised plasmid deletion (34) (table 4). An endogenous control target  
415 confirms that the specimen comes from a human and has been stored and processed in a  
416 way that has not compromised the DNA quality therefore differentiating between infection  
417 negative tests and assays that have failed through PCR inhibition or absence of a testable  
418 DNA analyte.

419

420 Diagnostic performance compared to a commercially marketed *Ct* diagnostic kit (*artus C.*  
421 *trachomatis Plus RG*) was good, offering sensitivity and specificity >90%. The median load  
422 of the false negative specimens was much lower than the load of the dual positive  
423 specimens, with the exception of one specimen detected by *artus* with a C<sub>q</sub> of 23 cycles that  
424 was not detected by ddPCR or either qPCR assay. Agreement between assays was not  
425 perfect for any of the tested pairings between ddPCR, qPCR and *artus*.

426



427 Comparative *omcB* load analysis between the qPCR assay and ddPCR assay showed high  
428 concordance, with discordant results occurring at or below the limit of detection of the qPCR  
429 assay. At such low concentrations, sampling volume limitation impacts on the reproducibility  
430 of a positive result. Targets are sporadically detected in samples where the analyte  
431 concentration is below the limit of detection and the likelihood of a positive and negative  
432 result is limited by dilution/concentration and fits a poisson distribution (35).

433  
434 Assuming the level of technical replication described in this paper and UK list prices from  
435 2017, the qPCR assay costs roughly \$11.51 per sample, inclusive of DNA extraction (\$3.75).  
436 Whereas testing using the commercial kit *artus*, costs more than twice as much at \$25.05  
437 per sample. Reducing the number of technical replicates, reducing the volume of the assay  
438 to 5  $\mu$ L, or omitting primer–probe sets for nondiagnostic targets (*omcB* or *RPP30*) could  
439 reduce the overall cost of the assay further, whilst the use of a larger DNA aliquot could  
440 offset potential loss of sensitivity. Use of the Rotor-Gene and ddPCR platform allows a larger  
441 sample volume to be assayed in a single reaction and is competitively costed against *artus* at  
442 respectively \$9.51 and \$15.64 per sample. Proprietary fluorophores may also be  
443 interchanged for non-proprietary equivalents to reduce cost or enable the assay to run on  
444 real-time thermocyclers from other manufacturers.

445  
446 Along with other NAAT methods, qPCR is a useful research tool. In this study we utilize  
447 qPCR for three key purposes: (1) to determine the loss of material during extraction under  
448 differing conditions, (2) to determine the rate of degradation of DNA under different long-  
449 term storage conditions and (3) to analyse the concentration of diagnostic discrepant results.  
450 The BioChain extraction kit performed best in this study. For sample storage room  
451 temperature preservation rather than frozen, regardless of of dessication method, did not  
452 increase the rate of loss of detectable *Ct* DNA suggesting that control programs without  
453 access to a freezer may be able to store swabs at room temperature without loss of  
454 diagnostic performance. This has previously been described for *Ct* stored for long periods in  
455 transport media, and in short-term dry storage at room temperature (36,37).

456  
457 Together, these findings describe an optimal pipeline of sample handling and processing in  
458 a budget-conscious research setting. By demonstrating variability at each step of the  
459 pipeline, this study illustrates the flexible nature of qPCR that allows parameters to be  
460 modified according to user requirement (38). The qPCR method described may offer an  
461 effective and affordable solution for quantitative estimates of *Ct* loads in trachoma studies.

462

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464

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473

474 **Contributions**

475

476 Conceived the study: JH, RB, AR, TD, ChR, MJH

477 Collected specimens: ARL, PAM, MJB

478 Performed experiments: JH, RB, AR, BH, TD

479 Analysed data: JH, RB, AR, TD, ChR, MJH

480 Wrote manuscript: JH, RB, ChR, MJH

481 Reviewed and approved manuscript: JH, RB, ARL, AR, BH, TD, PAM, MJB, ChR, MJH

482

483

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- 602
- 603

604 Table 1. Oligonucleotides used in this study.

605

Target	Oligo	Sequence (5'-3')	Amplicon size (bp)
<i>C. trachomatis</i> <i>omcB</i>	Primer (F) <sup>†</sup>	GAC ACC AAA GCG AAA GAC AAC AC	106
	Primer (R) <sup>†</sup>	ACT CAT GAA CCG GAG CAA CCT	
	ABI – Probe <sup>§</sup>	[FAM]-CCA CAG CAA AGA GAC TCC CGT AGA CCG- [QSY]	
	Rotor-Gene - Probe <sup>§</sup>	[FAM]-CCA CAG CAA AGA GAC TCC CGT AGA CCG- [BHQ]	
<i>C. trachomatis</i> <i>pORF2</i>	Primer (F) <sup>†</sup>	CAG CTT GTA GTC CTG CTT GAG AGA	109
	Primer (R) <sup>†</sup>	CAA GAG TAC ATC GGT CAA CGA AGA	
	ABI Probe <sup>§</sup>	[NED]-CGG GCG ATT TGC CTT-[MGBNFQ]	
	Rotor-Gene - Probe <sup>§</sup>	[JOE]-CCC CAC CAT TTT TCC GGA GCG A-[BHQ1]	
<i>H. sapiens</i> <i>RPP30</i>	Primer (F) <sup>†</sup>	AGA TTT GGA CCT GCG AGC G	65
	Primer (R) <sup>†</sup>	GAG CGG CTG TCT CCA CAA GT	
	ABI - Probe <sup>§</sup>	[VIC]-TTC TGA CCT GAA GGC TCT GCG CG-[QSY]	
	Rotor-Gene - Probe <sup>§</sup>	[Cy5]-TTC TGA CCT GAA GGC TCT GCG CG-[BHQ2]	

Bp: base pairs; F: forward; *omcB*: outer membrane complex B; *pORF2*: plasmid open reading frame 2; R: reverse; *RPP30*: RNase P/MRP 30-kDa subunit;

<sup>†</sup> Primers purified by desalting;

<sup>§</sup> Probes purified by high-performance liquid chromatography.

606

607

608 **Table 2:** Assay characteristics derived from repeat-tested standard curve.

Assay	Target	CoV	Gradient	CoD	Efficiency	LoD	C <sub>q</sub> range at LoD
ABI 7900HT	pORF2	41.5	-3.4	0.990	96.7	8.3	34.9–37.0
	omcB	48.3	-3.3	0.998	100.1	4.5	37.4–39.7
Rotor-Gene 3000	pORF2	20.3	-3.3	0.999	100.2	0.9	30.0–31.1
	omcB	35.3	-3.3	0.999	100.2	1.4	31.5–32.6

C<sub>q</sub>: quantitation cycle; CoD: coefficient of determination; CoV: coefficient of variance; LoD: limit of detection in copies/reaction; *omcB*: outer membrane protein complex B; pORF2: plasmid open reading frame 2

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612 Table 3. External Quality Assessment panel performance of qPCR assays.

Sample ID	Sample	Sample type	QCMD concentration (copies/mL)	artus Qualitative result	ABI-qPCR Qualitative result	Rotor-Gene Qualitative result
CTA13-01	<i>Ct</i> <sup>+</sup> urine	Core	Positive (250)	Positive	Positive	Positive
CTA13-02	<i>Ct</i> <sup>+</sup> urine	Educational	Positive 63	NOT DETERMINED	Negative	Positive
CTA13-03	nv <i>Ct</i> <sup>+</sup> urine	Core	Positive 10,000	Positive	Positive	Positive
CTA13-04	<i>Ct</i> <sup>+</sup> urine	Core	Positive 1000	Positive	Positive	Positive
CTA13-05	<i>Ct</i> urine	Core	Negative 0	Negative	Negative	Negative
CTA13-06	nv <i>Ct</i> <sup>+</sup> urine	Core	Positive 10,000	Positive	Positive	Positive
CTA13-07	<i>Ct</i> <sup>+</sup> swab	Educational	Positive 13	Positive	Negative	Positive
CTA13-08	<i>Ct</i> swab	Core	Negative 0	Negative	Negative	Negative
CTA13-09	<i>Ct</i> <sup>+</sup> swab	Core	Positive 250	Positive	Positive	Positive
CTA13-10	<i>Ct</i> <sup>+</sup> swab	Core	Positive 63	Positive	Positive	Positive
Core performance				8/8	8/8	8/8
Educational performance				2/2	0/2	2/2

*Ct*: *Chlamydia trachomatis*

613

614 **Table 4.** Diagnostic comparison of noncommercial qPCR assays to commercially marketed  
 615 comparator.

616

	<i>artus C. trachomatis</i> Plus RG PCR		
	versus		
	qPCR (ABI 7900HT)	qPCR (Rotor-Gene 3000)	ddPCR (Bio-Rad QX100)
<b>Sensitivity</b>	90 (73.5 – 97.9)	90.6 (75 – 98)	90.6 (75 – 98)
<b>Specificity</b>	97.3 (96.0 – 99.7)	94.6 (86.7 – 98.5)	94.6 (86.7 – 98.5)
<b>PPV</b>	93.1 (77.4 – 98.6)	87.9 (73.5 – 95)	87.9 (73.5 – 95)
<b>NPV</b>	96 (89.2 – 98.6)	95.9 (88.8 – 98.6)	95.9 (88.8 – 98.6)
<b>Cohens Kappa</b>	0.82	0.78	0.83

NPV: Negative predictive value; PPV: positive predictive value

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623 **Table 5:** Coefficients from linear regression models examining the relationship between cycle threshold and time  
624 in days  
625

Treatment	Ct pORF2		Ct omcB	
	Gradient	p-value	Gradient	p-value
Dry, frozen	0.019	< 0.0001	0.011	< 0.0001
Dry, desktop, room temperature	0.018	< 0.0001	0.018	< 0.0001
Dry, vacuum box, room temperature	0.015	< 0.0001	0.016	< 0.0001

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631 **Table 6:** qPCR assay costs

632

Costing Category	Assay Platform Cost (US\$)			
	<i>artus</i>	ABI	ddPCR	Rotor-Gene
DNA Extraction (kit & consumables)	4.70	4.70	4.70	4.70
PCR Reaction mix (inclusive of oligos)	17.18	1.70	2.84	1.10
Lab Consumables	0.41	0.12	1.84	0.35
Personnel monthly salary Multiplied by 1.2 for overheads and divided by 160 for hourly rate (based on a trained laboratory technician from Moshi, Tanzania)	3.37	5.06	6.74	3.37
Total	\$25.04	\$11.51	\$15.64	\$9.51

633

634 NOTE: Purchase equipment costs for an ABI 7900HT, Rotor-Gene Q 5plex (equivalent to the 3000 which is no  
635 longer available for purchase) and ddPCR working platform are \$62,445, \$38,142 and \$115,154  
636 respectively.

637

638

639 **Figure 1.** Comparison of plasmid load estimate from qPCR compared to *artus* cycle  
640 threshold. **(A)**. ABI 7900HT. **(B)**. Rotor-Gene 3000. The main plots show concordant results  
641 (ABI n = 24, Rotor-Gene n = 20), side panels show discordant results.

642 ND: Not detected.

643

644 **Figure 2.** Agreement between load estimates from ddPCR and qPCR. **(A)** ABI 7900HT. **(B)**  
645 Rotor-Gene 3000. Main panels show concordant results, side bars show discrepant results.

646 ND: Not detected.

647

648

649 **Figure 3.** Recovery of **(A)** *omcB* and **(B)** pORF2 from *Ct*-spiked swabs by five different  
650 extraction kits. Boxes represent median, inter-quartile range and range of all swabs for each  
651 treatment. Circles represent swabs spiked with high-load elementary bodies, triangles  
652 represent medium-load spiking, and crosses represent low-load spiking. There is variation in  
653 the number of targets recovered by different extraction kits. Biochain and Qiagen cador kits  
654 appear to yield more *Ct* DNA than comparators.

655

656 **Figure 4:** Change in recovered load of **(A)** *Ct* plasmid and **(B)** genomic targets following long-term storage frozen  
657 and at room temperature. Points represent mean of four swabs per time point per condition. Dashed lines  
658 represent linear regression model between load and time.

659