

Multiplex Mediator Displacement Loop-Mediated Isothermal Amplification for Detection of *Treponema pallidum* and *Haemophilus ducreyi*

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Yaws, a neglected tropical disease caused by the bacterium *Treponema pallidum* subspecies *pertenue*, manifests as ulcerative skin lesions. Nucleic acid amplification tests, like loop-mediated isothermal amplification (LAMP), are versatile tools to distinguish yaws from infections that cause similar skin lesions, primarily *Haemophilus ducreyi*. We developed a novel molecular test to simultaneously detect *T. pallidum* and *H. ducreyi* based on mediator displacement LAMP. We validated the *T. pallidum* and *H. ducreyi* LAMP (TPHD-LAMP) by testing 293 clinical samples from patients with yaws-like lesions. Compared with quantitative PCR, the TPHD-LAMP demonstrated high sensitivity and specificity for *T. pallidum* (84.7% sensitivity, 95.7% specificity) and *H. ducreyi* (91.6% sensitivity, 84.8% specificity). This novel assay provided rapid molecular confirmation of *T. pallidum* and *H. ducreyi* DNA and might be suitable for use at the point of care. TPHD-LAMP could support yaws eradication by improving access to molecular diagnostic tests at the district hospital level.

Yaws, a neglected tropical disease caused by the bacterium *Treponema pallidum* subspecies *pertenue*, predominantly affects children living in low-income, rural communities of warm and humid regions (1). Clinical manifestations include lesions of the skin, bone, and cartilage, progressing to severe destructive lesions if left untreated (2). Manifestations of primary yaws include papillomas or ulcerative lesions; manifestations of secondary yaws include a wide range of rashes, often accompanied by bone and joint involvement (2). Currently, 15 countries in West and Central Africa, Southeast Asia, and the Pacific region are known to be yaws-endemic. The World Health Organization (WHO) released a yaws eradication strategy (the Morges strategy) in 2012 (3). The mainstay of the strategy is mass drug administration (MDA) with single-dose azithromycin in yaws-endemic communities, followed by routine surveillance and retreatment for 3–6 months until no cases remain (3).

Serologic tests, including the *T. pallidum* particle agglutination and rapid plasma reagin tests, remain the primary diagnostic tools for yaws (2). Newer point-of-care serologic tests have replaced traditional laboratory-based serologic assays in many settings (4–7). Despite their central role in yaws diagnosis, serologic assays have several limitations. First, treponemal serologic assays usually remain positive over a patient's lifetime, and these tests cannot distinguish previous from current infection. Second, studies in Africa and in countries in the Pacific region have demonstrated that *Haemophilus ducreyi* causes cutaneous lesions similar to those observed in yaws (8–11). Persons with clinically suspicious lesions caused by

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H. ducreyi can have a reactive serologic test for yaws because of latent *T. pallidum* infection. Nucleic acid amplification tests (NAATs) can distinguish active yaws, involving a lesion with detectable *T. pallidum* DNA, from latent yaws, in which patients will have reactive serology without detectable *T. pallidum* DNA from lesions. In addition, before seroconversion, a small proportion of patients with early active yaws will have a positive NAAT but negative serologic results.

NAATs could play a central role in yaws eradication efforts, particularly for diagnosis and surveillance after MDA in yaws-endemic areas (12). PCR has been standard for molecular diagnosis and has a high specificity and sensitivity for *T. pallidum* and *H. ducreyi*, but the process is time-consuming and requires expensive laboratory equipment. Most yaws-endemic countries have limited access to PCR to aid national yaws eradication programs. A point-of-care NAAT could provide reliable post-MDA molecular surveillance, as well as help in monitoring for azithromycin resistance. Loop-mediated isothermal amplification (LAMP) is an alternative for molecular diagnosis that might be more suitable than PCR as a point-of-care NAAT in resource-limited environments. LAMP has fast processing times and high specificity and can be performed on less expensive devices than those needed for PCR.

Multiplex technologies, such as mediator displacement (MD) LAMP (13), have extended the usability of LAMP for simultaneous detection of >1 target and could be an efficient and cost-effective solution. MD detection uses an MD probe composed of a generic mediator attached to a generic overhang of a DNA target-specific sequence and a universal reporter molecule with a fluorophore and quencher for detection. We developed and validated a biplex MD LAMP assay to simultaneously identify *T. pallidum* and *H. ducreyi*.

Methods

Participants

We obtained samples from larger trials conducted on Lihir Island (n = 57) and Karkar Island (n = 184), Papua New Guinea; and in Ghana (n = 52). Details of the studies in which the samples were collected are provided elsewhere (14,15). In brief, samples were collected as part of a randomized control trial comparing azithromycin doses of 30 mg/kg against doses of 20 mg/kg to treat patients in a pilot study for yaws elimination (14,15). Swabs were collected from persons with yaws-like ulcers and placed in AssayAssure Multilock (Sierra Molecular, <https://sierramolecular.com>) transport medium, then frozen

at -20°C until transported to Mast Diagnostica GmbH laboratory in Reinfeld, Germany. DNA was extracted from the samples by using innuPREP MP Basic Kit A (Analytik Jena, <https://www.analytik-jena.com>) according to manufacturer's instructions. Isolated DNA was kept frozen at -20°C until it was used for biplex *T. pallidum* and *H. ducreyi* LAMP (TPHD-LAMP), singleplex *T. pallidum* and *H. ducreyi* LAMP assays, and quantitative PCR (qPCR) testing.

Ethics Approval

Participants, or parents or guardians of persons <18 years of age, provided written consent for inclusion in clinical surveys and etiologic studies. Children also provided assent when appropriate. The studies were approved by the National Medical Research Advisory Committee of the Papua New Guinea Ministry of Health (MRAC nos. 12.36 and 14.31), the Ghana Health Service (approval no. GHS 13/11/14), the London School of Hygiene & Tropical Medicine (approval no. 8832), and WHO (approval no. RPC720).

TPHD-LAMP Assay

We devised the TPHD-LAMP assay on the basis of 2 previously published assays: a singleplex LAMP assay (16), which we modified by adding an MD probe; and a biplex LAMP assay of *T. pallidum* and *H. ducreyi* (13). TPHD-LAMP primers target the polymerase I (polA) gene of *T. pallidum* and the 16S ribosomal RNA (16S rRNA) of *H. ducreyi*. We further optimized the assays for improved functionality by redesigning primers and probes and modifying reagent concentrations (Appendix Tables 1–3, <https://wwwnc.cdc.gov/EID/article/26/2/19-0505-App1.pdf>).

We performed a 2-step validation of the TPHD-LAMP assay. In the first step, we assessed the analytical sensitivity and specificity of the assay. In the second step, we used clinical samples collected in Ghana and Papua New Guinea to compare the performance of TPHD-LAMP against qPCR for individual targets. In a secondary analysis, we compared the performance of singleplex LAMP assays for each individual target against qPCR assays.

Assessment of Analytical Performance

We determined the analytical limit of detection (LOD) for the TPHD-LAMP assay by using target sequences cloned into plasmids. We determined the LOD of each of the 2 components separately, as well as the LOD of the biplex TPHD-LAMP assay (Appendix). We varied the plasmid DNA concentrations between 3×10^1 copies/reaction and 3×10^5 copies/reaction in 8 replicates to reproduce the *Treponema*

bacterial load in skin infections, which ranges from 10^2 – 10^4 copies/reaction (17). In addition, we tested the TPHD-LAMP in the presence of a high number of copies, 3×10^5 copies/reaction, of *H. ducreyi* or *T. pallidum* in the presence of a low number of copies of the second target to optimize each component and to simulate clinical samples that might contain both targets. We conducted primer titration experiments to minimize the preferential amplification of *H. ducreyi* DNA targets in persons with both infections. We estimated the LOD by counting the fraction of positive amplifications and performed probit regression analysis by using SPSS Statistics 25 (IBM, <https://www.ibm.com>).

We assessed the analytical specificity of the primer sets in silico by using ortholog target gene sequences from GenBank (Appendix Table 4) and found all primer sets were highly specific for *T. pallidum* and *H. ducreyi*. Based on these results, we tested the specificity of TPHD-LAMP in vitro against endemic pathogens associated with cutaneous ulcerative syndromes by using a panel of 13 organisms: *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Salmonella enterica* (Paratyphi and Typhi), *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Corynebacterium diphtheria*, *Corynebacterium ulcerans*, *Proteus mirabilis*, and *Enterococcus faecalis* (Appendix). We calculated interassay and intraassay variability of the TPHD-LAMP assay by using 3 batches of the TPHD-LAMP mix, prepared individually on 3 separate days and processed in different runs of 3 replicates per batch (Appendix).

Clinical Performance of the TPHD-LAMP

We performed clinical validation by comparing the performance of the TPHD-LAMP and qPCR assays to identify *T. pallidum* and *H. ducreyi* in patient samples collected in Ghana and Papua New Guinea. TPHD-LAMP reactions (10 μ L per assay) were composed of 1 \times RM MPM buffer (MAST Diagnostica GmbH, <https://mast-group.com>), 8 U Bst 2.0 WarmStart DNA Polymerase (New England Biolabs, <https://www.neb.com>), 0.05 μ mol/L universal reporter, and MD primer mix (Appendix). We incubated primer mixes for 5 m at 70°C before LAMP to prevent non-specific amplification initiated by primer dimerization. We performed real-time TPHD-LAMP reactions at 64°C in a Rotor-Gene Q (QIAGEN, <https://www.qiagen.com>) and acquired fluorescence signals every minute by using the Cy5-readout gain for *T. pallidum* and the FAM-readout gain for *H. ducreyi*. The single-

plex LAMP reactions (10 μ L per assay) using intercalating dye were composed of 1 \times RM MPM buffer, 8 U Bst 2.0 WarmStart DNA Polymerase, and 1 μ L of 10 \times SYBR Green staining reagent, DNA free (AppliChem, <https://www.applichem.com>) and primer mix (Appendix Table 1). We also performed singleplex LAMP reactions in a Rotor-Gene Q at 63°C with the FAM-readout gain. We used a cutoff of 60 m for biplex TPHD-LAMP and singleplex LAMP assays and considered samples with amplification beyond 60 m negative.

For performance analyses, we compared the TPHD-LAMP assay against TaqMan qPCR assays targeting *polA* of *T. pallidum* (18) and an optimized TaqMan qPCR assay targeting the 16S rRNA gene of *H. ducreyi* on the same DNA extract (Appendix Table 4, Figure 1). The 16S rRNA gene has been previously used in qPCR assays to detect *H. ducreyi* (19). We ran all tests in duplicate and included positive controls and DNA-free negative controls in each run. We used an identical sample volume, 2.5 μ L/reaction, for TPHD-LAMP, singleplex LAMP, and qPCR. For samples that tested negative by qPCR but positive by TPHD-LAMP, we repeated qPCR in a single reaction with higher sample volumes (3 μ L) to identify true negative test results.

Statistical Analysis

For clinical validation, we compared the sensitivity and specificity of the TPHD-LAMP assay against TaqMan qPCR assays. In a secondary analysis, we compared the performance of singleplex LAMP assays to qPCR. We performed all analysis by using R version 3.4.3 (<https://www.R-project.org>).

Results

Analytical Sensitivity and Specificity

The LOD for the TPHD-LAMP assay was 357 copies/reaction (95% CI 265–535 copies/reaction) for *T. pallidum* and 293 copies/reaction (95% CI 199–490 copies/reaction) for *H. ducreyi*. When we added the second target at the higher concentration of 3×10^5 copies/reaction to simulate clinical samples from persons infected with both bacteria, the LOD increased to 808 copies/reaction (95% CI 550–2,128 copies/reaction) for *T. pallidum* and 622 copies/reaction (95% CI 415–1,687 copies/reaction) for *H. ducreyi* (Appendix Figure 2). The TPHD-LAMP assay was negative for all other pathogens tested within 60 m, demonstrating high analytical specificity (Appendix Figure 3). We observed a minimal interassay or intraassay variation (Appendix Figure 4).

Validation of TPHD-LAMP in Clinical Samples

For clinical validation, we used a sample set consisting of 293 lesion swabs collected from patients with suspected *T. pallidum* infection. Samples were collected in Lihir Island (n = 57; 19.5%) and Karkar Island (n = 184; 62.8%), Papua New Guinea; and in Ghana (n = 52; 17.7%). A total of 184 (62.8%) cases were in male patients and 109 (37.2%) in female patients; the median age of case-patients was 10 years (interquartile range [IQR] 8–12 years).

Using qPCR, we detected *T. pallidum* in 59 (20.1%) samples, *H. ducreyi* in 155 (52.9%) samples, and *T. pallidum* and *H. ducreyi* co-infection in 19 (6.5%) samples. When tested by TPHD-LAMP, we detected *T. pallidum* in 60 (20.5%) samples and *H. ducreyi* in 163 (55.6%) samples. We detected both targets in 12 (4.1%) samples. Taking qPCR as the reference standard, the diagnostic sensitivity of the TPHD-LAMP assay for *T. pallidum* was 84.7% and the specificity was 95.7%. For *H. ducreyi*, the sensitivity of the TPHD-LAMP assay was 91.6% and the specificity was 84.8% (Table 1). Kappa coefficients (κ), ranging from 0.7 to 0.9 for the detection of *T. pallidum* and from 0.7 to 0.8 for *H. ducreyi*, show substantial to excellent agreement between qPCR and TPHD-LAMP. Moderate agreement between qPCR and TPHD-LAMP ($\kappa = 0.5$) also was demonstrated for the simultaneous detection of both targets. The median time to amplification of *T. pallidum* was 11 min (IQR 9–15 min) and the median time to amplification of *H. ducreyi* was 10 min (IQR 8–24 min).

For samples in which only 1 organism was detected by qPCR, the sensitivity of the TPHD-LAMP assay was higher for both *T. pallidum* (92.5%) and *H. ducreyi* (94.1%) than for samples with both organisms confirmed by qPCR. For samples confirmed to contain both bacteria by qPCR, sensitivity for *T. pallidum* was 68.4% ($p = 0.048$) and sensitivity for *H. ducreyi* was 73.7% ($p = 0.01$) (Table 1).

Using qPCR as the reference standard, the singleplex *T. pallidum* LAMP assay had a sensitivity of 78.0% and specificity of 97.9%; for the singleplex *H. ducreyi* LAMP assay the sensitivity was 91.0% and specificity was 75.3% (Table 2). We did not see a noticeable variation in the performance of the biplex TPHD-LAMP and singleplex LAMP assays between locations from which samples were collected (Tables 1 and 2).

Discussion

We provide data demonstrating a high analytical performance of a multiplex LAMP assay for *T. pallidum* and *H. ducreyi* and a high sensitivity and specificity comparable to qPCR. The TPHD-LAMP assay also performed better than singleplex LAMP assays, likely reflecting better performance of the MD technology used in the biplex LAMP compared with standard intercalating dyes used in singleplex LAMP assays.

The LOD of the TPHD-LAMP assay was 300 copies/reaction for both targets, which is comparable to qPCR, which has standard reproducibility in a range

Table 1. Comparison of clinical performance of biplex loop-mediated isothermal amplification for detection of *Treponema pallidum* and *Haemophilus ducreyi* (TPHD-LAMP) against singleplex TaqMan quantitative PCR*

Characteristics	Sample size	<i>Treponema pallidum</i>	<i>Haemophilus ducreyi</i>
Total samples, no.	293		
No. positive		60	163
Sensitivity, % (95% CI)		84.7 (72.5–92.4)	91.6 (85.8–95.3)
Specificity, % (95% CI)		95.7 (92.0–97.8)	84.8 (77.4–90.1)
Lesions containing a single pathogen†	195		
No. positive		48	151
Sensitivity, % (95% CI)		92.5 (78.5–98.0)	94.1 (88.4–97.2)
Specificity, % (95% CI)		95.7 (92.0–97.8)	84.8 (77.4–90.1)
Lesions containing both pathogen†	19		
No. positive		12	12
Sensitivity, % (95% CI)		68.4 (43.5–86.4)	73.7 (48.6–89.9)
Specificity, % (95% CI)		NA	NA
Samples from Lihir Island, no.	57		
No. positive		21	13
Sensitivity, % (95% CI)		90.5 (68.2–98.3)	76.5 (50.0–92.2)
Specificity, % (95% CI)		94.4 (80.0–99.0)	100.0 (89.1–100)
Samples from Karkar Island, no.	184		
No. positive		33	119
Sensitivity, % (95% CI)		78.1 (59.6–90.1)	94.2 (87.5–97.7)
Specificity, % (95% CI)		94.7 (89.5–97.5)	74.7 (63.4–83.5)
Samples from Ghana, no.	52		
No. positive		6	31
Sensitivity, % (95% CI)		100.0 (51.7–100)	90.9 (75.5–97.6)
Specificity, % (95% CI)		100.0 (90.4–100)	94.7 (71.9–99.7)

*NA, not applicable.

†Determined by quantitative PCR.

Table 2. Comparison of clinical performance of singleplex loop-mediated isothermal amplification for detection of *Treponema pallidum* and *Haemophilus ducreyi* against singleplex TaqMan quantitative PCR*

Characteristics	Sample size	<i>Treponema pallidum</i>	<i>Haemophilus ducreyi</i>
Total samples, no.	293		
No. positive		51	175
Sensitivity, % (95% CI)		78.0 (64.9–87.3)	91.0 (85.0–94.8)
Specificity, % (95% CI)		97.9 (94.8–99.2)	75.3 (67.2–82.1)
Lesions containing a single pathogen†	195		
No. positive		34	158
Sensitivity, % (95% CI)		82.5 (66.6–92.1)	92.6 (86.5–96.2)
Specificity, % (95% CI)		97.9 (94.8–99.2)	75.4 (67.2–82.1)
Lesions containing both pathogens†	19		
No. positive samples		17	17
Sensitivity, % (95% CI)		68.4 (43.5–86.4)	78.9 (53.9–93.0)
Specificity, % (95% CI)		NA	NA

*NA, not applicable.

†Determined by quantitative PCR.

of 10^1 – 10^6 copies/reaction. The LOD increased to ≈ 600 copies/reaction in samples that contained both targets, which is consistent with our clinical validation of the TPHD-LAMP; sensitivity for both bacteria was slightly higher when samples contained only a single target. Kappa coefficients confirmed substantial agreement ($\kappa > 0.7$) for the individual targets and moderate agreement ($\kappa = 0.5$) for simultaneous detection of both targets in a sample.

Detection of *T. pallidum* is the programmatic priority, but detection of *H. ducreyi* is beneficial for clinical management of patients with suspected yaws. The median time to amplification was < 15 m for both *T. pallidum* and *H. ducreyi*, indicating the TPHD-LAMP assay could provide rapid, molecular confirmation of the presence of *T. pallidum* or *H. ducreyi*. Further optimization of the assay to enhance the performance of the *T. pallidum* component, particularly in the context of co-infection, will be required to ensure cases of yaws are not missed.

Implementing qPCR at the point of care is operationally challenging because it requires relatively expensive equipment, in particular thermocyclers, which can cost up to 10 times as much as a tubescanner capable of performing the TPHD-LAMP assay. Because qPCR is available only in a limited number of national and international reference laboratories, TPHD-LAMP might be an alternative molecular test to support expansion of yaws eradication activities. We did not conduct a cost-effectiveness analysis of the TPHD-LAMP assay, but such an assessment should consider equipment costs, cost per assay, and the relative performance of each assay to assess the cost per case diagnosed. However, our data suggest that the TPHD-LAMP assay might be a cost-saving alternative to qPCR, especially at the point of care.

Our study had some limitations. We tested samples from only 2 geographic regions for clinical

validation of the TPHD-LAMP. Primer binding site mutations have affected the performance of other diagnostic assays for *T. pallidum* strains. Although we selected conserved genomic regions when designing the TPHD-LAMP primers, further experimental validation of the TPHD-LAMP assay with samples from a broader range of settings is needed. We conducted clinical validation of the assay in a controlled laboratory setting, but conditions at the point of care, including temperature, humidity, and a range of other environmental factors, might affect reagents in storage and in performing assays. Further optimization, including freeze-dried reagents in combination with dried oligonucleotides, might improve robustness and facilitate rollout of the assay in yaws-endemic countries.

In yaws-endemic countries, clinical manifestations combined with serologic tests are still the standard tool for the clinical management of yaws, but serologic tests have limitations and molecular assays are needed to support WHO yaws eradication efforts (12). Molecular assays also can detect mutations in the 23S RNA gene associated with azithromycin resistance (15,20,21), which is essential to monitor for drug resistance as yaws eradication efforts expand. qPCR is the most common NAAT currently available but remains restricted to a small number of laboratories in yaws-endemic countries. MD LAMP could facilitate surveillance for resistance and we plan further studies to evaluate a modified TPHD-LAMP assay for this purpose. Further, multicountry evaluations are warranted to assess performance of the assay when deployed in yaws-endemic countries and to assess the role the test could play in support of national yaws eradication programs. Nonetheless, the performance characteristics of the TPHD-LAMP suggest it has the potential to increase access to molecular diagnosis of yaws, especially at the point of care.

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Author contributions: L.B. conducted laboratory work, analyzed the data, and wrote the first draft of the manuscript. M.M. and O.M. designed the field studies and analyzed the data. S.K., S.L., S.F., and N.B. contributed to laboratory work or analysis. S.B., Y.A.-S., and K.A. led the field studies. M.B. contributed to laboratory work and analyzed the data. All authors revised the manuscript.

Potential conflicts of interest: S.F. and M.B. are employees of Mast Diagnostica GmbH, which produces and sells LAMP kits and products. A patent covering the technique described in the paper has been applied for by the University of Freiburg, Freiburg, Germany, and Hahn-Schickard, Villingen-Schwenningen, Germany.

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Multiplex Mediator Displacement Loop-Mediated Isothermal Amplification for Detection of *Treponema pallidum* and *Haemophilus ducreyi*

Appendix

Assay Optimization

Oligonucleotide and Primer Design

For the *Treponema pallidum* and *Haemophilus ducreyi* loop-mediated isothermal amplification (TPHD-LAMP) assay, we redesigned and further optimized oligonucleotides previously described by Knauf et al. (1) for detecting *T. pallidum* and by Becherer et al. (2) for detecting *H. ducreyi* to improve assay performance. In brief, we designed primers from GenBank sequences of the 16S ribosomal RNA gene of *H. ducreyi* and the *polA* gene of *T. pallidum* using PrimerExplorer V5 (Fujitsu, <http://primerexplorer.jp>) software.

Mediator displacement (MD) LAMP (2) for the simultaneous detection of multiple targets requires MD probes and fluorogenic universal reporter (UR) molecules for signal generation. An MD probe is comprised of a universal mediator (Med) combined with a modified Loop F (LF) or Loop B (LB) primer. The modified primer (LB/LF_Medc) contains target-specific primer sequences (LF or LB) at the 3'-end and a universal sequence at the 5'-end (Medc) that is complementary to the mediator. We designed MD probes in silico with Visual OMP version 7.8.42.0 (DNA Software, <https://www.dnasoftware.com>) software, as described previously (2), and used URs described by Lehnert et al. (4) and Becherer et al. (2). Oligonucleotides were synthesized and cartridge purified by Biomers (<https://www.biomers.net>) (Appendix Table 1).

LAMP Primer Sets

The MD LAMP included 6 primers per target, as described by Nagamine et al. (5), and an MD probe comprised of LB_Medc or LF_Medc, a Med, and a UR (2). We list oligonucleotides and UR sequences in Appendix Table 1. Standard concentration (1×) of primers, Med probes, and UR for TPHD-LAMP is 1.6 μmol/L of each forward inner primer (FIP) and backward inner primer (BIP), 0.6 μmol/L LF and 0.2 μmol/L LF_Medc, or 0.6 μmol/L LB and 0.2 μmol/L LB_Medc, 0.2 μmol/L of each F3 and B3, 0.1 μmol/L Med, and 0.05 μmol of UR. We used the same 6-primer set for singleplex LAMP assays, which contained 1.6 μmol/L of each FIP and BIP, 0.8 μmol/L of each LF and LB, and 0.2 μmol/L of each F3 and B3. We performed the singleplex LAMP assays using the intercalating dye SYBR green.

Primer Titration for TPHD-LAMP

To simultaneously amplify 2 targets in 1 reaction vessel successfully, both primer sets must perform equally. In a biplex LAMP reaction with an unbalanced assay, the more efficient assay will inhibit the amplification of the second target partially by binding the polymerase to the amplicon (3). Experiments using the standard 1× concentration of primers, mediator, and UR showed that the amplification of *H. ducreyi* is faster than the amplification of *T. pallidum* (data not shown). Consequently, the assay efficiency for *H. ducreyi* amplification is higher and might influence the amplification of *T. pallidum*. We also performed target titration and found that low *T. pallidum* concentrations, 3×10^3 copies/reaction, combined with high *H. ducreyi* concentrations, 3×10^5 copies/reaction, led to false-negative signals for *T. pallidum* (Appendix Table 2).

To solve this problem, we optimized the primer, Med, and UR concentrations used in TPHD-LAMP to focus on sensitivity and time to positive (t_p) and increase the support of the amplification of *T. pallidum*. We increased the concentration of primers, Med, and UR of the *T. pallidum* component to 1.25× and 1.5× and left the concentrations for the *H. ducreyi* component at the standard 1× concentration per reaction. Both concentrations for *T. pallidum* component showed better results; the concentration of 1.25× demonstrated best results and t_p . When we combined 3×10^3 copies/reaction of *T. pallidum* with 3×10^5 copies/reaction of *H. ducreyi*, we could still detect positive signals for both targets (data not shown). We fixed 1.25× concentration for *T. pallidum* component and 1× concentration for *H. ducreyi* component of the TPHD LAMP and used these for the subsequent testing of clinical samples (Appendix Table 3).

Plasmid Design

To determine analytical performance parameters of the assay and primer optimization, we obtained plasmid targets from Eurofins Scientific (<https://www.eurofins.com>) to use as quantified standard. Plasmids contained 300 bp of a defined conserved region of the 16S gene for *H. ducreyi* and the *polA* gene for *T. pallidum*. We diluted plasmid DNA in 10 mM Tris (pH 8) to adjust concentrations.

Clinical Performance of the TPHD-LAMP

Reference Assays

We performed a TaqMan real-time PCR targeting a 67-bp fragment of the *T. pallidum* polymerase I (*polA*) gene using previously described primers and probes (7). We used a plasmid containing the amplified fragment of the *polA* gene as a quantification standard covering the range 10^1 – 10^6 gene copies, but modified the reaction mix. In brief, the reaction encompassed 10 μ L TaqMan Universal Master Mix II without Uracil-N glycosylase (Applied Biosystems, <https://www.thermofisher.com>) and 1.8 μ L each of 10 μ mol/L primer and the hydrolysis probe. We completed the reaction with 1 μ L of the genomic DNA sample, independent of the DNA concentration. We used molecular-grade water to adjust the reaction volume to 20 μ L and used the following cycling conditions: 50°C for 2 m, 95°C for 10 m, then 40 cycles each at 95°C for 15 s and 60°C for 60 s.

We retrieved ortholog sequence data of the *Haemophilus* 16S rRNA gene from GenBank (Appendix Figure 1, Appendix Table 4) and aligned the genes by using Geneious R11 (<https://www.geneious.com>). We used V-Xtractor (<http://www.cmde.science.ubc.ca/mohn/software.html>), a Perl-based high-throughput software tool, to locate the hypervariable regions of the 16S rRNA sequences using the Hidden Markov Models option. In silico, we searched for regions that discriminate *H. ducreyi* from ortholog 16S rRNA gene sequences. We found suitable target sequences in the V8 region of the 16S rRNA gene and designed primers to target that region. Prior to use in the qPCR, we ran a PCR using the newly designed sense primer 5'-TAT ACA GAG GGC GGC AAA CC and the antisense primer 5'-CCA ATC CGG ACT TAG ACG TAC. Sanger sequencing of the 66-bp product confirmed the amplification of the targeted sequence of the 16S rRNA gene of *H. ducreyi*. We cloned the

product and used it to generate a plasmid quantification standard for the qPCR covering a 10^1 – 10^6 gene copies. Subsequently, we designed a hydrolysis probe FAM-5' CAA AGG GGA GCG AAT CTC AC-TAMRA and used it to perform the TaqMan qPCR using the same reaction mix and cycling conditions as described for the *polA* qPCR.

LAMP and qPCR assays used the same DNA extracts and included appropriate negative controls. Because of sample restrictions, we analyzed samples as duplicates. All reactions of the *polA* and *H. ducreyi* qPCR were run on a StepOnePlus Real-Time PCR System (Applied Biosystems). We analyzed raw data by using the StepOne Software version 2.3 (Life Technologies, <https://www.thermofisher.com>). We considered positive reactions to be those with exponential increase of delta-Rn, a value that corresponds to the intensity of fluorescence. We excluded samples that increased in fluorescence above the threshold but failed exponential increase.

Validation of TPHD-LAMP in Clinical Samples

To validate the TPHD-LAMP, we used clinical samples to determine the sensitivity and specificity for *T. pallidum* and *H. ducreyi*, which we calculated by using the following formulas:

$$\% \text{ Sensitivity} = \frac{TP}{TP+FN} \times 100$$

$$\% \text{ Specificity} = \frac{TN}{TN+FP} \times 100$$

$$\text{Positive predictive value (\%)} = \frac{TP}{TP+FP} \times 100$$

$$\text{Negative predictive value (\%)} = \frac{TN}{TN+FN} \times 100$$

where TP (true positive) means positive results were confirmed with PCR; TN (true negative) means negative results were confirmed with PCR; FP (false positive) means PCR results were negative; and FN (false negative) means PCR results were positive. We calculated positive predictive values and negative predictive values for TP, TN, FP, and FN of TPHD-LAMP of clinical samples (Appendix Table 5) and for singleplex LAMP assays of clinical samples (Appendix Table 6).

Assessment of Analytical Performance

Analytical Sensitivity and Specificity

We described how we calculated values for the limit of detection (LOD) of the TPHD-LAMP in the main article (Appendix Figure 2). We determined the linearity of the biplex assays, containing both targets, was $R^2(H. ducreyi) = 0.97$ and $R^2(T. pallidum) = 0.95$.

We generated analytical specificity data by in silico analysis. Then we tested a panel including *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Salmonella enterica* (Paratyphi and Typhi), *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Corynebacterium diphtheria*, *Corynebacterium ulcerans*, *Proteus mirabilis*, and *Enterococcus faecalis*. We tested the specificity of primer sets for the single *T. pallidum* and *H. ducreyi* components of the TPHD-LAMP, and for TPHD-LAMP assay. In all cases, assays were negative within 60 m for single components (data not shown) and for TPHD-LAMP (Appendix Figure 3), demonstrating high analytical specificity.

Interassay and Intraassay Variability

Interassay variability describes reproducibility and intraassay variability describes repeatability of assays. We calculated interassay and intraassay variability of the TPHD-LAMP assay by using 3 batches of biplex LAMP mix, individually prepared on 3 separate days, processed in different runs, and ran each batch in 3 replicates. First, we evaluated TPHD-LAMP assays that contained 3×10^4 copies/reaction of a single target; then we tested them for simultaneous detection of both targets in the sample. We determined t_p as the time of the maximum increase of fluorescence, calculated by the first derivative of the fluorescence intensity, as previously described (2,6). We calculated the SD for t_p by the scattering of measurement values between triplicates.

For assays containing a single target, the TPHD-LAMP interassay coefficients of variation (CVs) were 0.9 % for *H. ducreyi* and 2.6 % for *T. pallidum*. The intraassay CVs were 0 for *H. ducreyi* and 3.5 % for *T. pallidum*. For assays containing both pathogens, the interassay CVs were 0.2 % for *H. ducreyi* and 2.8 % for *T. pallidum* and the intraassay CVs were 0.5 % for *H. ducreyi* and 2.0 % for *T. pallidum*. The t_p for *H. ducreyi* were almost constant around 7 m

independent of the presence of *T. pallidum* (Appendix Figure 4). In contrast, t_p for *T. pallidum* increased from 11.4 m \pm 0.4 m in assays without *H. ducreyi* to 30.4 m \pm 0.8 m for TPHD-LAMP in assays containing both targets.

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Appendix Table 1. Sequences of primers, mediator displacement probes, and universal reporters for loop-mediated isothermal amplification for *Treponema pallidum* and *Haemophilus ducreyi**

Target, description	Sequence, 5'→3'	Reference
<i>Haemophilus ducreyi</i>		
F3	ATGTTGGGTTAAGTCCCGC	This work
B3	TCCAATCCGGACTTAGACGT	This work
FIP	CATCCCCACCTTCTCCAGTTTTATCCTTTGTTGCCAGCATG	This work
BIP	CATGGCCCTTACGAGTAGGGCCCCCTTGCAGGTTTGCC	This work
LF	GCAGTCTCCTTTGAGTTCCCA	This work
LB	TACACACGTGCTACAATGGCg	This work
LB_Medc ₁	<u>GGTCGTAGAGCCCAGAACGAGATGAGTGGTACACACGTgcTACAATGGCg</u>	This work
Med ₁	CCACTCATCTCGTTCTGGGCTCTACGACC	This work
UR ₁	BMN-Q-535-ATTGCGGGAGATGAGACCCGCAA-dTFAM- TGTTGGTCTAGAGCCCAGAACGA-C3	(2,6)
<i>Treponema pallidum</i>		
F3	GATTGGTCCTAAGACGGC	This work
B3	GGAATACAACAGGAATCTTCGA	This work
FIP	CAGCGCTTCTTTAAGGAATAGGTATGCACATCTTCTCCACTG	This work
BIP	TGTGGGAAGAAAAGATGCATTTTTTAAAAAACACATGGTACATCGT	This work
LF	CGATAAATACCATCAAGTGTGCCA	This work
LB	CGTTCACTCATTGAGTTGCGTG	This work
LF_Medc ₂	<u>CACTGACCGAACTGAGCTCCTGAGGCATGGTTTTCGATAAATACCATCAAGTGTGCCA</u>	This work
Med ₂	CCATGCCTCAGGAGCTCAGTTCGGTCAGTG	(2)
UR ₂	BMN-Q-535-CACCGGCCAAGACGCGCCGG-dT-Atto-647N- GTGTTCACTGACCGAACTGGAGCA-C3	(2,6)

*Underlined sequences illustrate complementary regions between LF/LB_Medc and mediator (Med). Bold text indicates nucleotides in complementary regions of mediator and universal reporters. Indices 1 and 2 indicate complementary sequences in Med and Medc or UR. BIP, backward inner primer; FIP, forward inner primer; LB, Loop B; LF, Loop F; UR, universal reporter.

Appendix Table 2. Positive results for *Treponema pallidum* and *Haemophilus ducreyi* in 3 TPHD-LAMP assays before primer titration*

<i>T. pallidum</i> , concentration	<i>H. ducreyi</i> , concentration	No. <i>H. ducreyi</i> -positive	No. <i>T. pallidum</i> -positive
3×10 ³ copies/reaction	3×10 ⁵ copies/reaction	3	0
3×10 ⁵ copies/reaction	3×10 ⁵ copies/reaction	3	3
3×10 ⁵ copies/reaction	3×10 ³ copies/reaction	3	3
3×10 ³ copies/reaction	3×10 ³ copies/reaction	3	3
3×10 ⁴ copies/reaction	3×10 ⁴ copies/reaction	3	3
0	0	0	0

*Positive results reflect number of positive results in 3 reactions at each combined concentration of *Treponema pallidum* and *Haemophilus ducreyi* loop-mediated isothermal amplification (LAMP).

Appendix Table 3. Primer composition for the biplex loop-mediated isothermal amplification of *Treponema pallidum* and *Haemophilus ducreyi* for the clinical validation.

Target	Oligonucleotide	Concentration, μmol/L
<i>H. ducreyi</i>	F3	0.20
	B3	0.20
	FIP	1.60
	BIP	1.60
	LF	0.80
	LB	0.60
	LB_Medc ₁	0.20
<i>T. pallidum</i>	Med ₁	0.10
	F3	0.25
	B3	0.25
	FIP	2.00
	BIP	2.00
	LF	0.75
	LB	1.00
LF_Medc ₂	0.25	
Med ₂	0.13	

Appendix Table 4. Ortholog sequence data of the *Haemophilus* 16S rRNA gene retrieved from GenBank and used for the *H. ducreyi* TaqMan qPCR design

GenBank accession no.	Pathogen	Strain no.
DQ851143_1	<i>Haemophilus simiae</i>	ROG53
AF224307_1	<i>Haemophilus quentini</i>	NA
CP008740_1	<i>Haemophilus influenzae</i>	2019
CP008740_1_2	<i>Haemophilus influenzae</i>	2019
AY613457_1	<i>Haemophilus influenzae</i>	M8943
AY613451_1	<i>Haemophilus influenzae</i>	M9741
CP007470_1_6	<i>Haemophilus influenzae</i>	477
CP007470_1_5	<i>Haemophilus influenzae</i>	477
CP007470_1_4	<i>Haemophilus influenzae</i>	477
CP007470_1_3	<i>Haemophilus influenzae</i>	477
CP007470_1	<i>Haemophilus influenzae</i>	477
CP007470_1_2	<i>Haemophilus influenzae</i>	477
EF399173_1	<i>Unclutred bacterium</i>	SJTU_F_12_59
AB597550_1	<i>Unclutred gamma proteobacterium</i>	NA
AB597543_1	<i>Unclutred gamma proteobacterium</i>	NA
AB597538_1	<i>Unclutred gamma proteobacterium</i>	NA
FQ312002_1_6	<i>Haemophilus parainfluenza</i>	T3T1
FQ312002_1_2	<i>Haemophilus parainfluenza</i>	T3T1
FQ312002_1	<i>Haemophilus parainfluenza</i>	T3T1
FQ312002_1_5	<i>Haemophilus parainfluenza</i>	T3T1
CP006956_1	<i>Bibersteinia trehalosi</i>	USDA-ARS-USMARC-190
CP006956_1_2	<i>Bibersteinia trehalosi</i>	USDA-ARS-USMARC-190
CP006956_1_6	<i>Bibersteinia trehalosi</i>	USDA-ARS-USMARC-190
CP006955_1_4	<i>Bibersteinia trehalosi</i>	USDA-ARS-USMARC-189
CP003745_1_3	<i>Bibersteinia trehalosi</i>	USDA-ARS-USMARC-192
CP006955_1_2	<i>Bibersteinia trehalosi</i>	USDA-ARS-USMARC-189
CP006955_1	<i>Bibersteinia trehalosi</i>	USDA-ARS-USMARC-189
CP003745_1	<i>Bibersteinia trehalosi</i>	USDA-ARS-USMARC-192
CP006944_1	<i>Mannheimia varigena</i>	USDA-ARS-USMARC-1312
CP006944_1_5	<i>Mannheimia varigena</i>	USDA-ARS-USMARC-1312
CP006953_1_3	<i>Mannheimia varigena</i>	USDA-ARS-USMARC-1388
CP006953_1	<i>Mannheimia varigena</i>	USDA-ARS-USMARC-1388
CP006943_1_2	<i>Mannheimia varigena</i>	USDA-ARS-USMARC-1296
CP006943_1_5	<i>Mannheimia varigena</i>	USDA-ARS-USMARC-1296
CP006943_1_6	<i>Mannheimia varigena</i>	USDA-ARS-USMARC-1296
CP006943_1_3	<i>Mannheimia varigena</i>	USDA-ARS-USMARC-1296
LN795822_1	<i>Mannheimia sp.</i>	MG13
AF053895_1	<i>Mannheimia sp.</i>	HPA102
AF053890_1	<i>Mannheimia glucosida</i>	UT18
KU051693.1	<i>Mannheimia haemolytica</i>	A2
CP011099_1_6	<i>Mannheimia haemolytica</i>	89010807N
CP004753_2_6	<i>Mannheimia haemolytica</i>	USDA-ARS-USMARC-185
CP005972_1_6	<i>Mannheimia haemolytica</i>	D153
CP023044_1_4	<i>Mannheimia haemolytica</i>	191
CP006574_1_4	<i>Mannheimia haemolytica</i>	D174
CP023043_1_2	<i>Mannheimia haemolytica</i>	193
CP005972_1	<i>Mannheimia haemolytica</i>	D153
DQ301920_1	<i>Mannheimia haemolytica</i>	PHL213
CP005383_1_6	<i>Mannheimia haemolytica</i>	M42548
CP006957_2_6	<i>Mannheimia haemolytica</i>	USDA-ARS-USMARC-184
CP006957_2_4	<i>Mannheimia haemolytica</i>	USDA-ARS-USMARC-184
CP006957_2_2	<i>Mannheimia haemolytica</i>	USDA-ARS-USMARC-184
GQ358868_1	<i>Uncultured bacterium</i>	clone 8837-D0-O-7D
M75079_1	<i>Haemophilus ducreyi</i>	35000
M75084_1	<i>Haemophilus ducreyi</i>	KC57
M75078_1	<i>Haemophilus ducreyi</i>	CPI 542
CP015434_1_5	<i>Haemophilus ducreyi</i>	GHA9
CP015434_1	<i>Haemophilus ducreyi</i>	GHA9
AE017143_1_2	<i>Haemophilus ducreyi</i>	35000
ST16SrRNA_3_1490596	<i>Haemophilus ducreyi</i>	1490596
AF525028_1	<i>Haemophilus ducreyi</i>	isolate Amsterdam
CP015432_1	<i>Haemophilus ducreyi</i>	GHA5
CP015426_1_2	<i>Haemophilus ducreyi</i>	VAN3
NR_044741_1	<i>Haemophilus ducreyi</i>	CIP 54.2
CP015425_1_2	<i>Haemophilus ducreyi</i>	VAN2
NZ_CP015429	<i>Haemophilus ducreyi</i>	GHA1
CP015430_1_5	<i>Haemophilus ducreyi</i>	GHA2

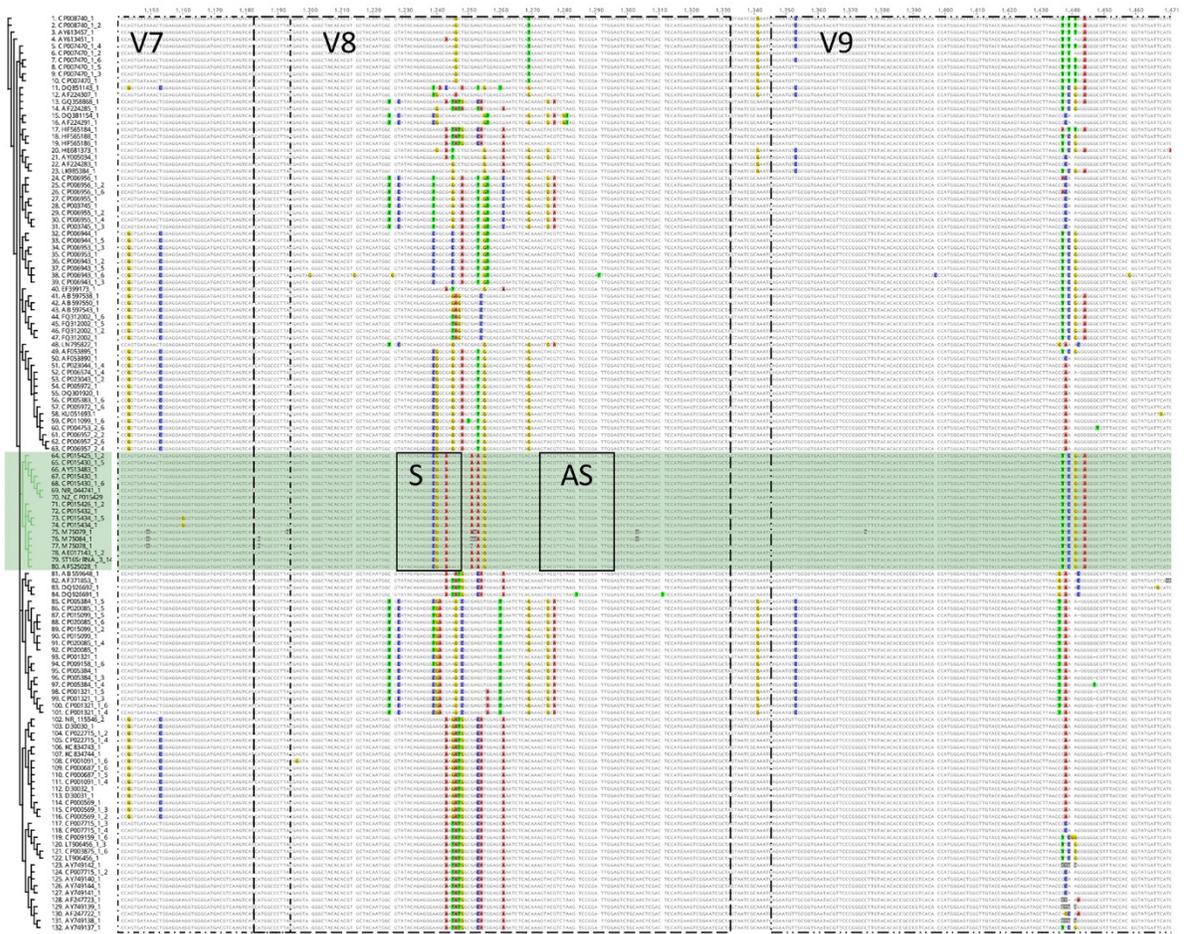
GenBank accession no.	Pathogen	Strain no.
CP015430_1_6	<i>Haemophilus ducreyi</i>	GHA2
CP015430_1	<i>Haemophilus ducreyi</i>	GHA2
AY513483_1	<i>Haemophilus ducreyi</i>	ATCC 33921
HE681373_1	Uncultured bacterium	clone 7q_13
AY005034_1	<i>Haemophilus</i> sp.	clone BJ021
AF224283_1	<i>Actinobacillus pleuropneumoniae</i>	MCCM 00189
LK985384_1	<i>Haemophilus parahaemolyticus</i>	isolate G321
AF224285_1	<i>Actinobacillus capsulatus</i>	CCUG 37035
CP009158_1_6	<i>Haemophilus parasuis</i>	SH03
CP005384_1	<i>Haemophilus parasuis</i>	ZJ0906
CP001321_1	<i>Haemophilus parasuis</i>	SH0165
CP001321_1_6	<i>Haemophilus parasuis</i>	SH0165
CP001321_1_4	<i>Haemophilus parasuis</i>	SH0165
CP001321_1_5	<i>Haemophilus parasuis</i>	SH0165
CP001321_1_3	<i>Haemophilus parasuis</i>	SH0165
CP005384_1_3	<i>Haemophilus parasuis</i>	ZJ0906
CP005384_1_4	<i>Haemophilus parasuis</i>	ZJ0906
CP005384_1_5	<i>Haemophilus parasuis</i>	ZJ0906
CP020085_1_5	<i>Haemophilus parasuis</i>	CL120103
CP015099_1_5	<i>Haemophilus parasuis</i>	SC1401
CP020085_1_6	<i>Haemophilus parasuis</i>	CL120103
CP015099_1_2	<i>Haemophilus parasuis</i>	SC1401
CP015099_1	<i>Haemophilus parasuis</i>	SC1401
CP020085_1_4	<i>Haemophilus parasuis</i>	CL120103
CP020085_1	<i>Haemophilus parasuis</i>	CL120103
AB559648_1	Uncultured bacterium	clone c_GA_H2
AF371853_1	Uncultured bacterium	clone bp-2130-s959-2
DQ926692_1	<i>Actinobacillus porciconsillarum</i>	73706
DQ926691_1	<i>Actinobacillus porciconsillarum</i>	71123
DQ381154_1	<i>Pasteurella caballi</i>	NSVL 84679
AF224291_1	<i>Pasteurella caballi</i>	MCCM 00841
HF565184_1	<i>Actinobacillus</i> sp.	MK-2012
HF565188_1	<i>Actinobacillus</i> sp.	MK-2012
HF565186_1	<i>Actinobacillus</i> sp.	MK-2012
KC834743_1	<i>Actinobacillus pleuropneumoniae</i>	TJ12
KC834744_1	<i>Actinobacillus pleuropneumoniae</i>	HB13
NR_115546_2	<i>Actinobacillus pleuropneumoniae</i>	Shope 4074
D30030_1	<i>Actinobacillus pleuropneumoniae</i>	NA
CP022715_1_2	<i>Actinobacillus pleuropneumoniae</i>	KL 16
CP022715_1_4	<i>Actinobacillus pleuropneumoniae</i>	KL 16
CP000569_1_3	<i>Actinobacillus pleuropneumoniae</i>	L20 serotype 5b
CP000569_1_2	<i>Actinobacillus pleuropneumoniae</i>	L20 serotype 5b
CP000569_1	<i>Actinobacillus pleuropneumoniae</i>	L20 serotype 5b
CP001091_1_6	<i>Actinobacillus pleuropneumoniae</i>	serotype 7, str. AP76
CP000687_1_6	<i>Actinobacillus pleuropneumoniae</i>	serotype 3, str. JL03
CP000687_1_5	<i>Actinobacillus pleuropneumoniae</i>	serotype 3, str. JL03
CP001091_1_4	<i>Actinobacillus pleuropneumoniae</i>	serotype 7, str. AP76
D30032_1	<i>Actinobacillus pleuropneumoniae</i>	NA
D30031_1	<i>Actinobacillus pleuropneumoniae</i>	NA
AY749139_1	<i>Actinobacillus genomu</i> sp.	52418-03
AY749138_1	<i>Actinobacillus genomu</i> sp.	52418-03
AF247722_1	<i>Actinobacillus lignieresii</i>	F 127
AY749137_1	<i>Actinobacillus genomu</i> sp.	24593-01
AF247723_1	<i>Actinobacillus lignieresii</i>	F 264
CP003875_1_6	<i>Actinobacillus suis</i>	H91-0380
LT906456_1	<i>Actinobacillus suis</i>	NCTC12996
LT906456_1_3	<i>Actinobacillus suis</i>	NCTC12996
CP009159_1_6	<i>Actinobacillus suis</i>	ATCC 33415
CP007715_1_4	<i>Actinobacillus equuli</i> subsp. <i>equuli</i>	19392
CP007715_1_3	<i>Actinobacillus equuli</i> subsp. <i>equuli</i>	19392
AY749144_1	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>	27368-01
AY749142_1	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>	23611-01
AY749141_1	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>	23596-01
CP007715_1_2	<i>Actinobacillus equuli</i> subsp. <i>equuli</i>	19392
AY749140_1	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>	23337-01

Appendix Table 5. Sensitivity and specificity of bplex loop-mediated isothermal amplification of *Treponema pallidum* and *Haemophilus ducreyi* in samples from 293 patients with suspected *T. pallidum* infection

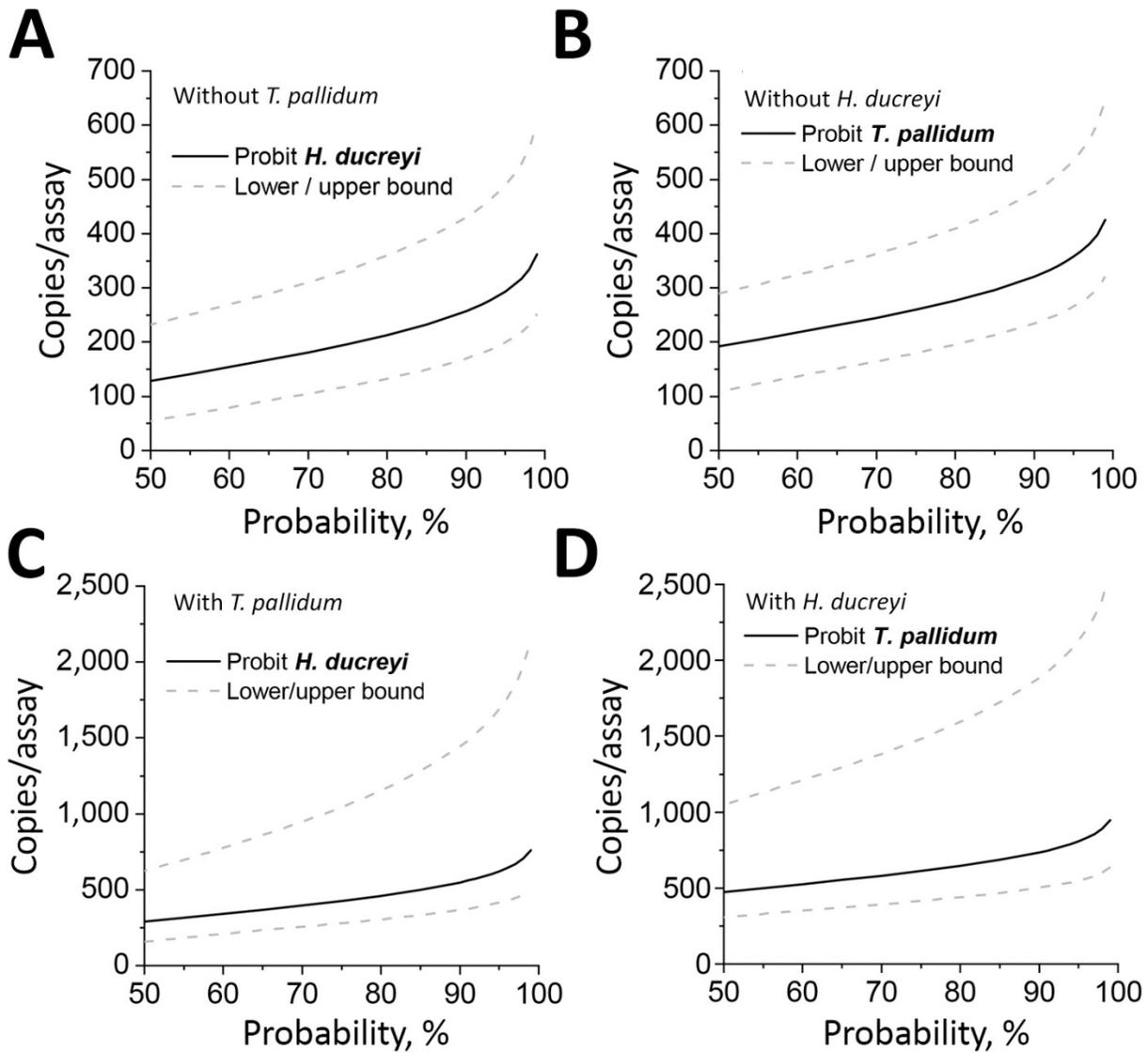
Characteristics	Sample size, no.	<i>Treponema pallidum</i> , no.	<i>Haemophilus ducreyi</i> , no.
All samples	293		
True positive		50	142
True negative		224	116
False positive		10	21
False negative		9	13
Positive predictive value, %		83.3	87.1
Negative predictive value, %		96.1	89.9
Lihir Island	57		
True positive		19	13
True negative		34	39
False positive		2	0
False negative		2	4
Positive predictive value, %		90.5	100
Negative predictive value, %		94.4	90.7
Karkar Island	184		
True positive		25	99
True negative		144	59
False positive		8	20
False negative		7	6
Positive predictive value, %		75.8	83.2
Negative predictive value, %		95.4	90.8
Ghana	52		
True positive		6	30
True negative		46	18
False positive		0	1
False negative		0	3
Positive predictive value, %		100	96.8
Negative predictive value, %		100	85.7

Appendix Table 6. Sensitivity and specificity of singleplex loop-mediated isothermal amplification for *Treponema pallidum* and *Haemophilus ducreyi* in samples from 293 patients with suspected *T. pallidum* infection

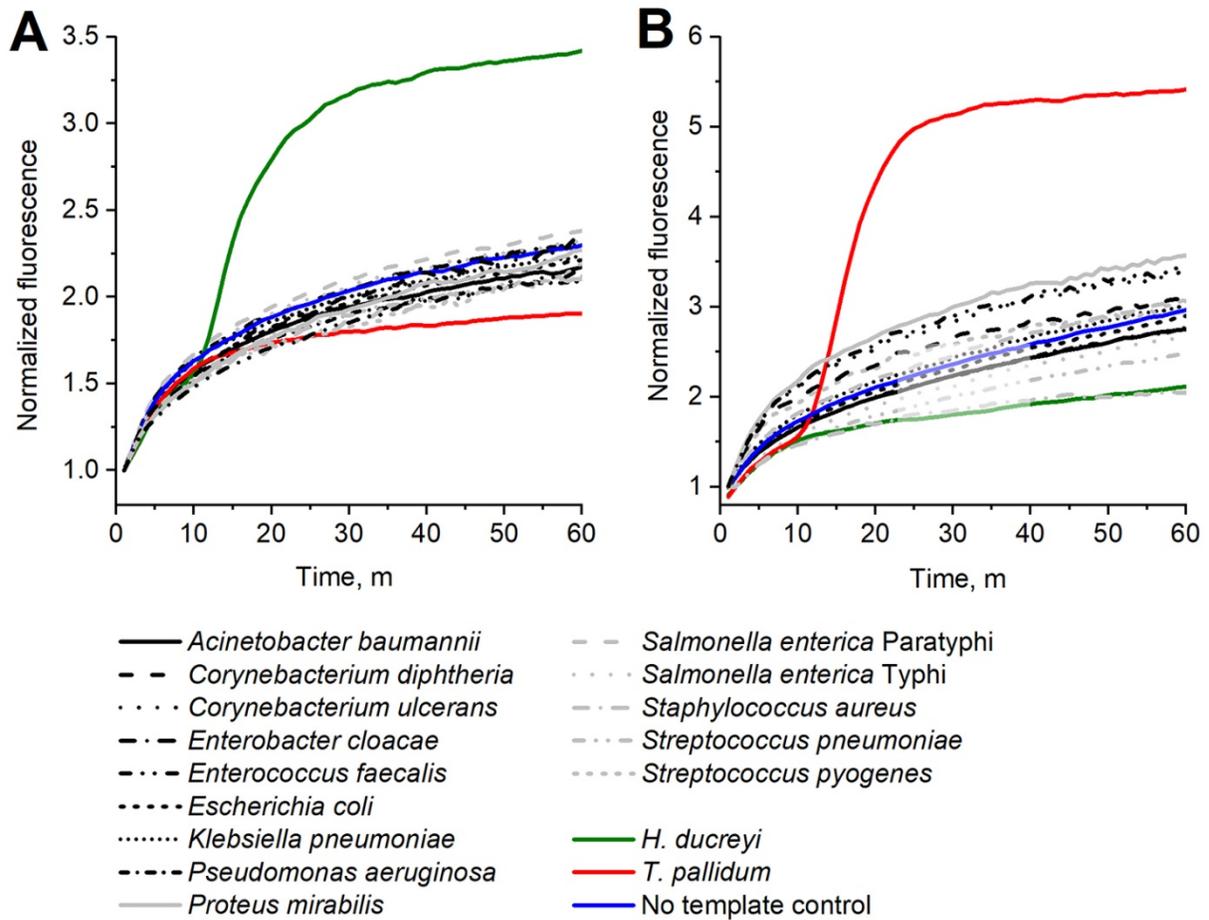
Results	<i>Treponema pallidum</i> , no.	<i>Haemophilus ducreyi</i> , no.
True positive	46	141
True negative	229	103
False positive	5	34
False negative	13	14
Positive predictive value, %	90.2	80.6
Negative predictive value, %	94.6	88.0



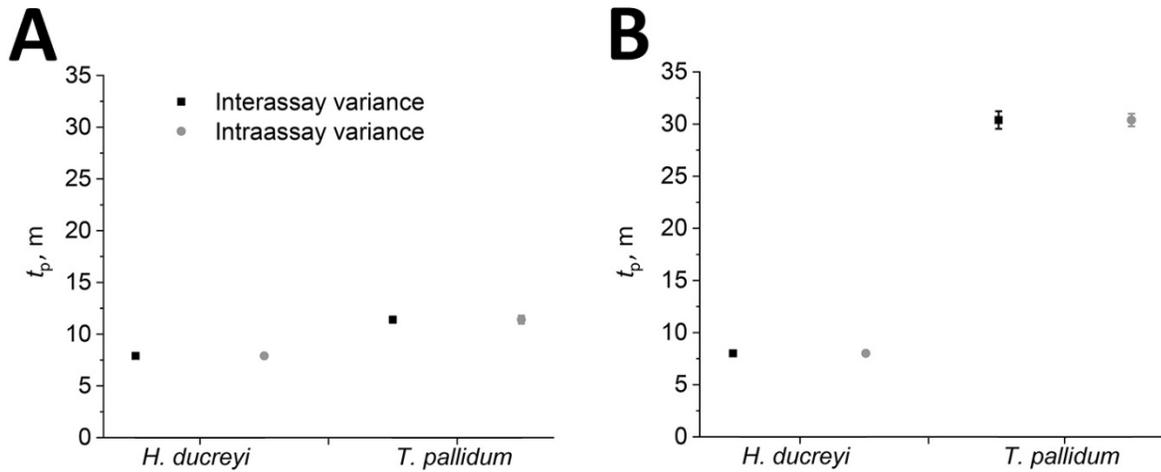
Appendix Figure 1. Nucleotide sequence identity chart for variable regions 7–9 and dendrogram for variable regions 1–9 of orthologue sequence data of the 16S rRNA gene of *Haemophilus ducreyi*. Sequence differences are highlighted. The region inside the dotted line V7 represents variable region 7, the region inside dotted line V8 represents variable region 8, and the region inside dotted line V9 represents the variable region 9 of the 16S rRNA gene. Sequences in green band denote *H. ducreyi* specific sequences. The S-box indicates the binding region of sense primer and the AS-box indicates the binding region of the antisense primer for the optimized qPCR for *H. ducreyi*. Sequences were aligned using Geneious (<https://www.geneious.com>). GenBank accession numbers correspond to the data shown in Appendix Table 4.



Appendix Figure 2. Limit of detection (LOD) for *Treponema pallidum* and *Haemophilus ducreyi* loop-mediated isothermal amplification (TPHD-LAMP) assay. The probability of successful amplification of a given copy number was predicted using Probit analysis. Lower and upper bounds, illustrated as dashed lines, represent 95% CI. A) LOD for *H. ducreyi* in samples without *T. pallidum*. B) LOD for *T. pallidum* in samples without *H. ducreyi*. C) LOD for *H. ducreyi* in the presence of 3×10^5 copies *T. pallidum* plasmid DNA. D) LOD for *T. pallidum* in the presence of 3×10^5 copies *H. ducreyi* plasmid DNA.



Appendix Figure 3. Analytical specificity of *Treponema pallidum* and *Haemophilus ducreyi* loop-mediated isothermal amplification (TPHD-LAMP) assay against a selected panel of pathogens. A) FAM-readout gain for *H. ducreyi*; B) Cy5-readout gain for *T. pallidum*.



Appendix Figure 4. Interassay and intraassay variance of *Treponema pallidum* and *Haemophilus ducreyi* loop-mediated isothermal amplification (TPHD-LAMP) assay illustrated for the amplification of 1 target and of both targets in the sample. The mean value of time to positive (t_p) is plotted against target and type of variability. The variability is illustrated by error bars, which reflect SD values. A) Variance in the presence of 3×10^4 copies/reaction of 1 target, either *T. pallidum* or *H. ducreyi* in the sample. B) Variance in the presence of 3×10^4 copies/reaction of *T. pallidum* and 3×10^4 copies/reaction *H. ducreyi* in the sample.