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Development of an accurate CRISPR/Cas13 diagnostic system for *Wuchereria bancrofti*

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ABSTRACT

Disease surveillance is critical for WHO's Global Programme to Eliminate Lymphatic Filariasis (LF), but current field-ready diagnostics exhibit insufficient sensitivity and/or specificity in low-endemic settings. We aim to develop an ultra-sensitive and field-friendly CRISPR/Cas13-based SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) tool for point-of-care detection of *Wuchereria bancrofti* infection. The SHERLOCK assay, combining recombinase polymerase amplification (RPA) with CRISPR/Cas13a detection, was performed in two-step and one-pot formats, with sensitivity compared to qPCR (the emerging gold standard). The *W. bancrofti* SHERLOCK assays achieved a detection limit of 0.1 fg/µL gBlock DNA and showed no cross-reaction with five other filarial species. Assays were validated using 149 pooled mosquito and 185 human blood samples from Samoa, which were previously tested for LF antigen using WHO-recommended Filariasis Test Strips (FTS) and microscopically examined for microfilariae (Mf). For both mosquito and human samples, the two-step SHERLOCK assay reached 100% sensitivity and specificity, while one-pot SHERLOCK showed 94.0–96.4% sensitivity and 100% specificity. To improve the field deployability of the assay, we also used lyophilized one-pot SHERLOCK master mix and assessed its thermostability after storage at 25, 4, and −20°C. The lyophilized one-pot SHERLOCK mix maintained a detection limit of 10 fg/µL *W. bancrofti* gBlock after 10 days at 25°C, indicating reduced reliance on cold-chain for reagent storage and transport. However, when testing human blood samples with lyophilized SHERLOCK, reduced sensitivity (59.7%) was observed. This CRISPR-based platform offers a promising field-ready next-generation approach to LF diagnostics, potentially addressing current limitations, enhancing surveillance, and elimination efforts.

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KEYWORDS Mosquito; *Wuchereria bancrofti*; microfilaria; CRISPR/Cas13 diagnostic tool; one-pot reaction; qPCR diagnosis

Introduction

Lymphatic filariasis (LF) is a mosquito-borne parasitic disease afflicting more than 120 million people in 72 countries globally [1]. LF is caused primarily by infection with *Wuchereria bancrofti* (responsible for approximately 90% of cases worldwide) [2], with the remaining caused by species of the genus *Brugia*. WHO's Global Programme to Eliminate LF is one of the largest public health programmes in the world, aiming to eliminate the disease as a public health problem through multiple rounds of annual mass drug administration (MDA), morbidity management, and disability prevention in affected persons. Significant progress has been achieved since 2000, with more than 10 billion drug treatments administered to over 943.4 million people through MDA programmes [1]. As transmission decreases after MDA, and elimination targets draw closer, more sensitive and precise diagnostic tools are urgently needed to reliably identify remaining infections and transmission hotspots.

According to WHO monitoring and evaluation guidelines [3], programmatic decisions (for starting and stopping MDA) are based on surveys undertaken using LF diagnostic tools. Currently, three conventional tools are available, including:

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(1) Immunological detection in human blood samples. Identification of circulating filarial antigen (CFA) using the Filariasis Test Strip (FTS, Abbott Diagnostics, Scarborough, USA) [4] or Q Filariasis Antigen Test (QFAT, SD Bioline, Korea) [5] has been widely used in *W. bancrofti*-endemic areas, while rapid antibody-based diagnostics, such as the Brugia Rapid Test, are employed for the detection of *Brugia* spp [6]. These diagnostic tools are field-friendly and relatively sensitive in resource-limited settings. However, immunological detection is limited by antigen/antibody persistence after parasite clearance, inability to distinguish active from past infection, reduced sensitivity in low-transmission settings, and potential cross-reactivity with other helminths, leading to false positives and underestimation of the true impact of MDA on LF transmission [7,8].

(2) Detection of microfilariae (Mf) by microscopy in human blood. This method is labour-intensive, relatively insensitive, and requires the collection and microscopic examination of blood samples. In most endemic settings, blood must be collected at night to coincide with peak microfilarial densities in peripheral blood. Although daytime sampling is feasible in parts of the Pacific, such as Samoa, where *W. bancrofti* is diurnally subperiodic, Mf detection remains operationally challenging [9]. This may result in underestimation of infection prevalence, allowing residual infections to remain undetected and potentially sustaining ongoing LF transmission [10].

(3) Nucleic acid detection methods for detecting parasite DNA in samples. qPCR is highly sensitive and specific for detecting parasite DNA in human and mosquito samples [11], but its use is limited by specialized laboratory infrastructure and the need for trained personnel. Molecular xenomonitoring (MX) detects filarial DNA in mosquitoes (commonly using qPCR) as a non-invasive indicator of ongoing transmission [12], but it cannot identify infected individuals and requires extensive mosquito collection and costly equipment /expertise, also limiting its feasibility in resource-limited settings.

In the past 5 years, CRISPR-based diagnostics have enabled a new generation of nucleic acid diagnostics with high sensitivity, user-friendliness, and potential for low-cost and point-of-care (POC) use in resource-limited settings [13]. This platform leverages programmable CRISPR-associated (Cas) enzymes (Cas12 and Cas13) to detect target DNA or RNA targets and has been applied to several pathogens, including protozoan parasites (malaria *Plasmodium* spp., *Cryptosporidium parvum*, *Enterocytozoon hepatopenaei*, *Leishmania* spp., and *Trypanosoma* spp.) [14]. The CRISPR/Cas13-based SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) platform was a key component of diagnostic assays developed at QIMR Berghofer Medical Research Institute that enabled highly sensitive and specific detection of two human schistosome species and represents one of the first applications of CRISPR-based diagnostics for helminths [15]. SHERLOCK combines recombinase polymerase amplification (RPA) with CRISPR/Cas13a detection, measured via fluorescent or colorimetric readouts. It operates with minimal equipment (37°C heat block and simple readout device) while achieving qPCR-comparable sensitivity. Compared with PCR, CRISPR-based assays offer programmable, single-base specificity with reduced false positives and are well suited for decentralized, field-based diagnostics.

In this study, we aim to develop accurate, field-friendly CRISPR/Cas13-based tools (two-step and one-pot SHERLOCK) to detect *W. bancrofti* infection and measure their sensitivity and specificity against qPCR, antigen (detected by FTS), and Mf. SHERLOCK assays were evaluated using large banks of mosquito and human blood samples collected in Samoa, where lymphatic filariasis remains endemic despite multiple rounds of MDA over recent decades.

Materials and methods

Biosafety and ethics

Ethics approvals were granted by the Samoan Ministry of Health and the University of Queensland Human Research Ethics Committee (HREC) (protocol 2021/HE000895). The Australian National University HREC and Federation University HREC provided recognition of approval by another HREC (Federation University HREC approval 2023/154). Informed consent, including consent for minors, was obtained in accordance with a written informed consent protocol. The field survey for sample collection was conducted in close collaboration with the Samoa Ministry of Health, the WHO country office in Samoa, and the Samoa Red Cross. All the SHERLOCK/qPCR tests performed in this study were approved by the QIMR Berghofer Medical Research Institute Animal and Human Ethics Committee (P3705) and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th ed., 2004).

Sample collection, FTS and Mf testing

Mosquitoes and human blood samples were collected in Samoa as part of the Surveillance and Monitoring for the Elimination of Lymphatic Filariasis (SaMELFS) program's 2023 survey [16]. Mosquitoes were trapped on the main island of Upolu as described [17]. Males were discarded and females were sorted to species level and pooled with a maximum of 25 mosquitoes

Table 1. Sequences of primers, crRNA/probes, and synthetic gBlocks used in RPA/SHERLOCK and qPCR assays.

	Sequences (5′–3′)		
Assays	Forward Primer	Reverse Primer	crRNA/Probe
SHERLOCK	GAAATTAATACGACTCACTATAGGGTTGCTGAAA AACATTCGCTTTTGAATGATCTGAT	CTTGGGTAATTAAACCGGTGATCCTACCTGCACCA	ACUACCCCAAAAACGAAGGGGACUAAA ACUCCCAUAUAGUUGUUGUGCAUUC AACAU
qPCR	TTGCTGAAAAACATTCGCTTTTGAA	CTTGGGTAATTAAACCGGTGATCC	/56FAM/ACAACAACT/ZEN/ATATGGGAA TGGTGCAGGT/3IABKFQ/
gBlock	TTTGTTTGATCCGTTTGCTGAAAAACATTCGCTTTTGAATGATCTGATGAATGTTGAATGCACAACAACTATATGGGAATGGTGCAGGTAGG ATCACCGGTTTAATTACCCAAGTTTGAGGTAAT		

per pool. Pools were oven-dried for 3 h at 60°C. A total of 149 pools of mosquitoes (60 qPCR-positive and 89 qPCR-negative) were used for this study.

During household visits (14:00–20:00), consenting participants provided finger-prick blood samples (≤500 µL) in heparin microtainers, which were stored at 4°C (≤48 h), and tested for LF antigen using FTS [5]. Ag-positive samples were used to prepare up to three thick blood smears per WHO guidelines [18]; slides were dehaemoglobinized, Giemsa-stained following standard procedures [5], and microscopically examined by two experienced technicians. In total, 461 samples, comprising 203 Ag-positive samples (including 81 Mf-positive samples) and 258 Ag-negative samples, were analysed at QIMR Berghofer using qPCR and SHERLOCK.

DNA extraction

Genomic DNA was extracted from pooled mosquitoes using the same DNA extraction approach as previously described [19], and from heparinized human blood using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. This work was performed at Federation University (Australia).

RPA primer and crRNA design

Specific RPA primers and crRNAs were designed [15], targeting the high-copy *W. bancrofti* Tandem Repeat #1 (*WbTR1*) [20], a highly conserved and sequence-stable genomic region with minimal homology with other filarial nematodes (including *Brugia malayi*, *Onchocerca volvulus*, *Loa loa*, and *Mansonella perstans*) [21]. Furthermore, a BLAST analysis of the targeted *WbTR1* sequence was performed in WormBase to assess its sequence specificity. A T7 promoter sequence (5′-GAAATTAATACGACTCACTA-TAGGG-3′) was incorporated into the 5′ end of the RPA forward primer for SHERLOCK assays [22]. A synthetic gBlock gene fragment containing the target *WbTR1* sequence was designed and synthesized by Integrated DNA Technologies (IDT, Singapore) and used as a positive control in both SHERLOCK and qPCR assays. The list of oligonucleotides (including the RPA primers, crRNA, qPCR primers and probe, gBlock) used in this study is provided in Table 1. All oligonucleotides were synthesized by IDT.

SHERLOCK assays

Two-step SHERLOCK

RPA pre-amplification reactions were performed using the TwistAmp Basic kit (TwistDx, Maidenhead, UK) according to the manufacturer's instructions with RPA pellets resuspended in TwistDx Rehydration Buffer, primers and ddH_2O, with modifications as previously described [15]. Reactions were incubated at 37°C for 30 min. Fluorescence-based SHERLOCK detection was performed [15] in triplicate 20 µL reactions comprising 45 nM LwCas13a (GenScript, Piscataway, USA), 1 mM rNTP mix (New England Biolabs; NEB, Ipswich, USA), 20 mM HEPES pH 6.8, 9 mM MgCl2, 0.6 µL Murine RNase inhibitor (ScienCell, Carlsbad, USA), 0.5 µL T7 RNA Polymerase (NEB), 10 ng crRNA (IDT, Singapore), 125 nM fluorescent Poly-U reporter (IDT), and 1 µL RPA product.

One-pot SHERLOCK

Standard (liquid format) one-pot fluorescence-based SHERLOCK detection was performed in duplicate or triplicate 20 µL reactions comprising 5.76 µL of reconstituted RPA reaction mix, 50 nM LwCas13a, 1 mM rNTP mix, 5 mM $MgCl_2$, 1 U Murine RNase inhibitor, 1 U T7 RNA Polymerase, 25 nM crRNA, 125 nM fluorescent Poly-U reporter, 14 mM magnesium acetate, and 1 µL sample.

Lyophilized one-pot SHERLOCK

The first version (v.1) of the assay used the same reagent composition as the standard (liquid format) one-pot SHERLOCK assay (except magnesium acetate was initially omitted), with the master mix flash frozen and lyophilized using the Heto LyoLab 3000 Freeze Dryer (Thermo Fisher Scientific, Waltham, USA) overnight. The second assay version (v.2) was formulated by supplementing the v.1 master mix with 5% (w/v) sucrose and 150 mM mannitol.

The stability of the lyophilized one-pot master mix was assessed by storage at room temperature (RT), 4 and −20°C, and testing at 0, 5, 10, 15, and 20 days post-lyophilization, using serial dilutions of

W. bancrofti gBlock DNA (1 pg/µL–0.01 fg/µL) as the template. Each pellet was rehydrated in 45 µL H_2O and 2.5 µL 280 mM magnesium acetate, with 2.5 µL DNA sample added. Fluorescence-based SHERLOCK detection was performed in duplicate or triplicate 20 µL reactions.

All the SHERLOCK reactions were incubated at 37°C for 1–3 h on a BioTek Synergy Neo2 Hybrid Multi-mode plate reader (Agilent, Santa Clara, USA) with excitation at 480 nm and emission at 525 nm. Each run included a positive control (gBlock, 1 pg/µL), a no-template "Blank" (H_2O instead of RPA product) and a RPA no-template control (NTC). To calculate background-subtracted fluorescence (BSF), the absolute fluorescence values of no-template "Blank" wells were subtracted from sample well. A test result was classified as positive when the fluorescence signal exceeded the mean fluorescence of the negative controls for the corresponding sample type by more than 5 standard deviations (SD). A sample was considered positive overall if ≥60% of replicates tested positive.

qPCR with specific probe

qPCR was performed using TaqPath ProAmp Master Mix (Thermo Fisher Scientific, USA) in 2–3 technical replicates, with 125 nM of each primer, 250 nM probe (IDT), and 1 µL of DNA template. Previously validated *WbTR1* assay primers and TaqMan probe (FAM/ZEN/IABkFQ) were used [21], as shown in Table 1. The qPCR reactions were performed in 2- using the Mic qPCR cycler (Bio Molecular Systems, Upper Coomera, Australia), and the cycling conditions consisted of 50°C incubation for 2 min, denaturation at 95°C (10 min), with 40 cycles of denaturation at 95°C (15 s) and annealing at 60°C (1 min). Each run contained a NTC (ddH_2O as input) and gBlock DNA (1 pg/µL) served as a positive control. Data were analysed with Mic qPCR software (Bio Molecular Systems) based on cycle threshold (*Ct*) values [23]. A positive qPCR result was defined as successful amplification (positive *Ct* value) in ≥66.6% technical replicates.

Comparison between SHERLOCK assays and qPCR using serially diluted W. bancrofti *gBlock DNA*

To assess the sensitivity of two-step, one-pot SHERLOCK and qPCR (with specific probe) assays targeting *WbTR1*, assays were conducted using 10-fold serial dilutions of *W. bancrofti* gBlock DNA (Table 1) as the template (1 pg/µL–0.01 fg/µL) and H_2O as a NTC.

Analytical specificity of W. bancrofti *SHERLOCK targeting WbTR1*

To assess the specificity of *WbTR1*, the targeted 100-bp sequence was BLAST-searched against the WormBase database to identify potential matches with other helminth species.

The specificity of the SHERLOCK assays was evaluated using DNA (1 µL of 1 ng/µL) extracted from five different species of nematode parasites, including *Dirofilaria asiatica*, coinfection of *Stephanofilaria* spp. and *Onchocerca gibsoni*, *O.lupi*, *D. repens*, and *D. immitis*.

Statistical analysis

Statistical analyses were conducted using Prism Graph-Pad (Version 9, GraphPad Software, La Jolla, CA, USA). Each experiment was performed at least in duplicate, and results are presented as mean ± SE of technical replicates. Differences between groups were assessed using one-way ANOVA with Dunnett's correction for multiple comparisons, as described [15].

Using human and mosquito samples from Samoa, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of SHERLOCK were calculated. Corresponding 95% confidence intervals for each proportion were calculated using the exact binomial (Clopper–Pearson) method. Using qPCR as the reference, a range of comparative metrics were calculated in R version 4.2.2 [24] for each of the SHERLOCK assay variants (two-step, one-pot, and lyophilized one-pot). Overall metrics were calculated for all samples combined, as well as stratified by Ag and Mf status. Concordance was calculated as the proportion of samples with identical results between each SHERLOCK assay and the reference test (qPCR) (defined as true positive + true negative / number of samples), with 95% confidence intervals estimated using exact binomial methods. Cohen's kappa statistic was also calculated. All metrics were calculated for both the human and mosquito samples.

Results

We describe the development and validation of SHERLOCK diagnostic platforms (two-step and one-pot formats) for the detection of *W. bancrofti* using field-relevant mosquitoes and human blood specimens collected from Samoa. The SHERLOCK assay employs RPA for isothermal pre-amplification of *WbTR1* at 37°C, and CRISPR/Cas13a-mediated detection (Figure 1). Upon recognition of the *WbTR1* target by the specific programmed crRNA, the Cas13a/crRNA complex is activated, triggering collateral cleavage of a FAM-labelled single-stranded RNA reporter molecule, resulting in an increase in FAM fluorescence signal. RPA amplification and CRISPR/Cas13 detection are performed separately in two-step SHERLOCK assays, or can be combined in one-pot SHERLOCK

assays to simplify workflow and reduce contamination risk. One-pot assays can also be lyophilized to further enhance ease-of-use and reaction stability.

Detection limitations of W. bancrofti *SHERLOCK (two-step and one-pot) assay and cross-reactivity with other filarial species*

We found the qPCR (with specific probe), two-step and one-pot SHERLOCK assays exhibited the same limit of detection (LoD) of 0.05 fg/μL of *W. bancrofti* gBlock DNA (Figure 2(a)–(c) and Supplementary Table 1). The corresponding inter- and intra-assay CVs are detailed in Supplementary Tables 2 and 3. This LoD (0.05 fg/μL) was detectable in 2/3 of replicates for qPCR and one-pot SHERLOCK, and 3/3 of replicates for two-step SHERLOCK. The specificity of the *W. bancrofti* one-pot SHERLOCK assay was confirmed by testing DNA extracted from various species of filarial nematodes (Figure 3). No detectable cross-reactivity was observed under the conditions tested for any of these filarial nematode species, including *D. asiatica*, mixed infections of *Stephanofilaria* spp. and *O. gibsoni*, *O. lupi*, *D. repens*, and *D. immitis*. Furthermore, the high specificity of *WbTR1* is also supported by our BLAST analysis in WormBase. It shows *B. pahangi*, *B. malayi*, and *B. timori* share the highest sequence similarity with *W. bancrofti* (Supplementary Table 4). However, the *WbTR1* forward RPA primer of the SHERLOCK assay spans all nucleotide variations (23 nt) that distinguish *W. bancrofti* from these three closely related *Brugia* species (Supplementary Table 4). Therefore, the SHERLOCK targeting *WbTR1* is highly specific for *W. bancrofti*, as successful amplification requires the presence of the forward primer-binding region, containing *W. bancrofti*-specific sequences that are absent in the *Brugia* homologues.

Evaluation of the W. bancrofti *SHERLOCK (two-step and one-pot) assay against qPCR using pooled mosquito samples*

A total of 149 pooled mosquito DNA samples were tested by qPCR and two-step SHERLOCK, and 144 of these samples were also tested using the one-pot SHERLOCK assay. A detailed summary of results is presented in Supplementary Table 5. It was noted that of the 60 qPCR-positive samples, five were excluded from the one-pot assay due to insufficient DNA volume. Two had weak-positive qPCR results (*Ct* 34.86–37.29), while three had stronger positive results (*Ct* 30.82–33.32), suggesting that the exclusion was not specifically biased towards weak-positive samples (Supplementary Table 5). The two-step SHERLOCK assay successfully detected *W. bancrofti* DNA in all 60 qPCR-positive mosquito samples, showing 100% sensitivity [95% confidence interval (CI) (94.0%–100%)], and was negative for all 89 qPCR-negative samples, presenting 100% specificity [95% CI (95.9%–100%)]. Meanwhile, the one-pot SHERLOCK assay was also able to detect *W. bancrofti* in mosquito samples with 96.4% sensitivity (53/55) [95% CI (87.5%–99.6%)], and 100% specificity (89/89) [95% CI (95.9%–100%)] relative to qPCR (with probe).

Comparison of two-step and one-pot SHERLOCK fluorescence-based assay results (Figure 4(a), Table 2) demonstrated that both assays had high concordance with qPCR [two-step: 1.00, 95% CI (0.98–1.00), one-pot: 0.99, 95% CI (0.95–1.00)]. Both assays showed a bimodal distribution of average BSF values, with qPCR-negative and qPCR-positive samples clustering in distinct groups (Figure 4(b) and (c)), indicating clear separation between negative and positive samples. Results of two-step and one-pot SHERLOCK (including individual replicates) for all 60 qPCR-positive samples and 10 randomly selected qPCR-negative samples are shown in Supplementary Figure 1.

Evaluation of the W. bancrofti *SHERLOCK (two-step and one-pot) assay against qPCR using human blood samples with known Ag and Mf status*

In a total of 461 human blood samples from the SaMELFS study, 203 (44.0%) were Ag-positive by FTS (of which 81 were also Mf-positive), and 258 (56.0%) were Ag-negative (assumed to also be Mf-negative) (Figure 5(a) and Supplementary Table 6). Of the 81 Ag-positive/Mf-positive samples, 65 (80.2%) were qPCR-positive. The 114 Ag-positive/Mf-negative samples were all qPCR-negative (Figure 5(a) and Supplementary Table 6). All 258 Ag-negative/Mf-negative samples were also qPCR-negative.

A subset of 185 human blood samples (Figure 5(b)) was used to validate the SHERLOCK assays, including all 67 qPCR-positives and 118 qPCR-negatives randomly selected from the 394 qPCR-negative samples. A summary of the two-step and one-pot SHERLOCK assay results (stratified by Ag and Mf status) is presented in Figure 5(b), with detailed results shown in Supplementary Table 7 and Supplementary Figure 2. The two-step SHERLOCK assay successfully detected *W. bancrofti* in 100% (67/67) of qPCR-positive and 0% (0/118) of qPCR-negative blood samples (Supplementary Figure 2 and Supplementary Table 7). It showed 100% sensitivity [95% CI (94.6%–100%)] and 100% specificity [95% CI (96.9%–100%)] relative to qPCR. Meanwhile, the one-pot SHERLOCK assay showed 94.0% sensitivity (63/67 qPCR-positives detected) [95% CI (85.4%–98.3%)] and 100% specificity [95% CI (96.9%−100%)] compared to qPCR for

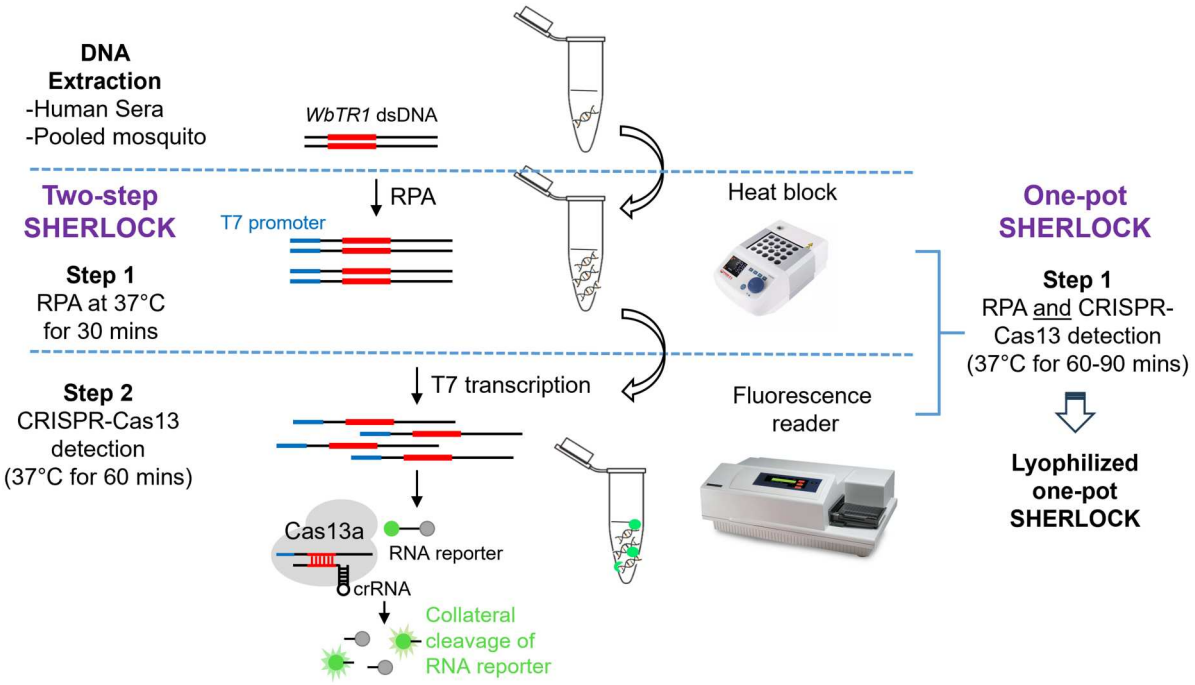


Figure 1. Workflow of SHERLOCK assay for the detection of *Wuchereria bancrofti*. DNA is extracted from pooled mosquito and human blood samples collected from Samoa. For the two-step SHERLOCK assay, in step 1 the target sequence is first amplified by a recombinase polymerase amplification (RPA) reaction undertaken at a single temperature (37°C) for 30 min. In step 2, the resulting double-stranded DNA (dsDNA) amplicons are transcribed into single-stranded RNA (ssRNA) by T7 RNA polymerase, then sequence-specific recognition of the target ssRNA by the Cas13a–crRNA complex activates the collateral RNase activity of Cas13a, resulting in non-specific cleavage of the quenched fluorescent ssRNA reporters. Reporter cleavage is detected using a fluorescence plate reader after incubation at 37°C for 60 min. For one-pot SHERLOCK, steps 1 and 2 are combined into a single reaction performed at 37°C for 60–90 min. One-pot reactions can also be lyophilized to enhance thermostability and ease-of-use, enhancing field applicability.

the detection of *W. bancrofti* DNA in the human blood. Comparison of two-step and one-pot SHERLOCK fluorescence-based assays against qPCR (Figure 6(a), Table 2) showed excellent agreement for both formats, with concordance values [two-step: 1.00, 95% CI (0.98–1.00), one-pot: 0.98, 95% CI (0.95–0.99)]. Both assays showed a clear bimodal distribution of average BSF values, enabling robust separation of positive and negative samples (Figure 6(b) and (c)). However, the one-pot assay exhibited greater variability than the two-step format, suggesting reduced signal consistency.

When compared with Mf detection results, the two-step SHERLOCK reached 94.2% sensitivity (65/69 Mf-

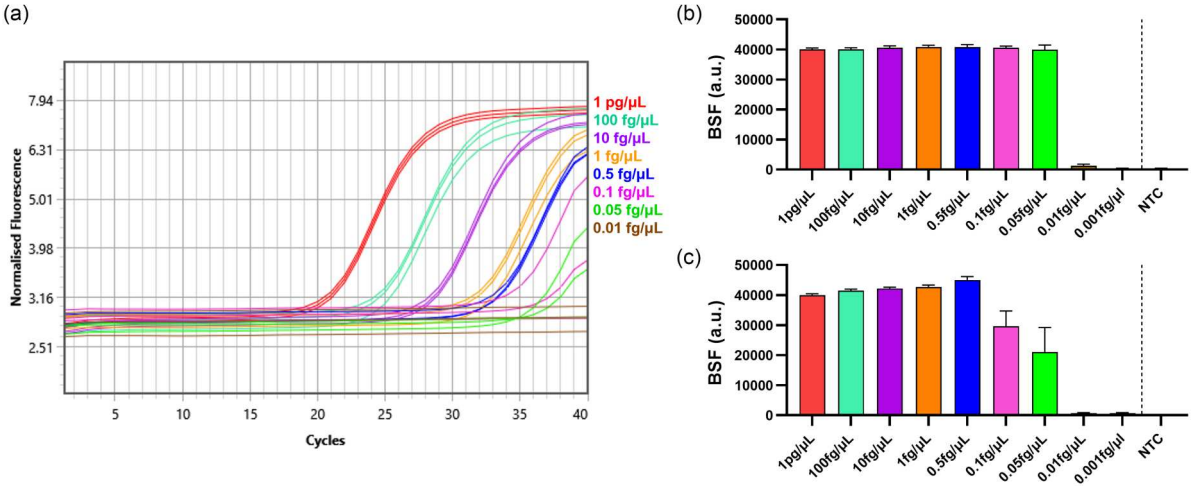


Figure 2. Diagnostic sensitivity of qPCR and SHERLOCK assays targeting *WbTR1* for the detection of *W. bancrofti*. Serially diluted gBlock DNA served as positive *W. bancrofti* DNA (1 pg/µL–0.01 fg/µL) and H_2O was used as a no-template control (NTC). (a) qPCR amplification plots; (b) fluorescence-based two-step SHERLOCK assay at 60 min; and (c) one-pot SHERLOCK assay with signal detected at 60 min.

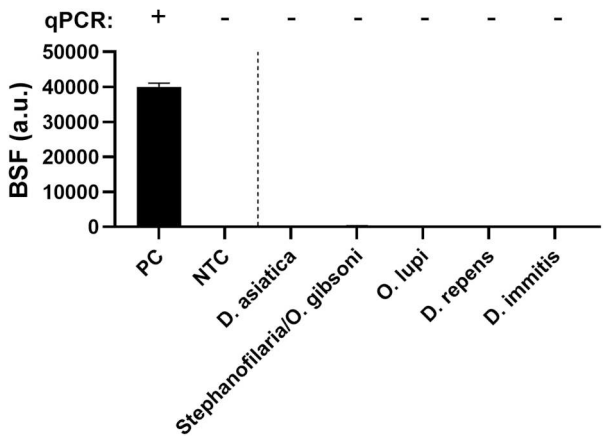


Figure 3. Specificity of one-pot SHERLOCK assay targeting *WbTR1* for the detection of *W. bancrofti*. Tested DNA samples include *Dirofilaria asiatica*, coinfection of *Stephanofilaria* spp. and *Onchocerca gibsoni*, *O. lupi*, *D.repens,* and *D. immitis*. Signal detected at 60 min (mean ± SEM of two biological replicates).

positives detected) [95% CI (86.8%−98.1%)] and 100% specificity [95% CI (96.8%−100%)], while one-pot format showed 89.9% sensitivity (62/69 Mf-positives detected) [95% CI (80.2%−95.8%)] and 100% specificity [95% CI (96.8%−100%)]. SHERLOCK demonstrated high concordance with Mf detection [two-step:0.98 (95% CI, 0.95–0.99), one-pot: 0.96 (95% CI, 0.92–0.99)] (Supplementary Figure 3). Both assays displayed a bimodal distribution of average BSF values, while the one-pot format exhibited a broader signal distribution, consistent with its relatively lower concordance and increased variability.

Freeze-dried one-pot W. bancrofti *SHERLOCK assay development and testing*

To facilitate cold-chain independence for reagent storage, we developed two versions (v.1 and v.2) of lyophilized one-pot master mix for the detection of *W. bancrofti* gBlock DNA. A comparison of the stability of v.1 and v.2 lyophilized one-pot SHERLOCK assays was shown in Figure 7 and Supplementary Table 8. The v.1 assay could detect *W. bancrofti* gBlock DNA at 100 fg/µL after 10 days of storage at −20 or 4°C but not at day 20, and detected 10 fg/µL DNA at day 10 when stored at −20 but not at 4°C (Figure 7(a) and (b)). Meanwhile, the v.2 formulation (containing 5% sucrose, 150 mM mannitol [25]) was able to detect DNA at both 100 and 10 fg/µL throughout the 20 days when stored at −20 or 4°C (Figure 7(c) and (d)). When assays were stored at RT, the v.2 assay detected both 100 and 10 fg/µL DNA up to day 10, while the v.1 assay could not detect either concentration, even on day 5.

To validate the diagnostic capabilities of the v.2 lyophilized one-pot SHERLOCK assay, human samples ($n = 97$) collected from Samoa, including 67 qPCR-positives and 30 qPCR-negatives, were tested using the lyophilized one-pot SHERLOCK master mix after 5 days storage at −20°C (Figure 8). The v.2 lyophilized pellet was able to detect *W. bancrofti* DNA in 59.7% (40/67) of qPCR-positive samples and 0% (30/30) of qPCR-negative samples, corresponding to a sensitivity of 59.7% [95% CI (47.0%−71.5%)] and specificity of 100% [95% CI (88.4%−100%)] relative to qPCR. This is a reduction compared to the 94.0% sensitivity relative to qPCR achieved by the standard (liquid format) one-pot SHERLOCK *W. bancrofti* assay.

Statistical analysis for SHERLOCK assays compared to qPCR using human and mosquito samples

For both human and mosquito samples, two-step SHERLOCK showed perfect agreement with qPCR, with sensitivity, specificity, concordance, and kappa equal to 1.00 across all Ag, Mf, and mosquito categories (Table 2). One-pot SHERLOCK had lower sensitivity than two-step SHERLOCK overall for all human samples [0.94, 95% CI (0.85–0.98) vs 1.00, 95% CI (0.95–1.00)], as well as for the mosquito samples [0.96, 95% CI (0.87–1.00) vs 1.00, 95% CI (0.94–1.00)]. The lyophilized one-pot SHERLOCK assay (human samples only) had lower kappa values than the other two assays as a result of lower sensitivity (Table 2). All three SHERLOCK assays were negative for all Mf-negative samples. Additional metrics [including true positive (TP), false positive (FP), true negative (TN), false negative (FN), and positive predictive value (PPV) and negative predictive value (NPV)] are presented in Supplementary Table 9.

Discussion

The CRISPR/Cas13-based *W. bancrofti* diagnostic assays developed in this study show strong potential as next-generation field diagnostics, providing sensitive and field-deployable detection in a user-friendly format. Their availability could improve the detection of residual infections and facilitate the identification of LF transmission hotspots. Implementation of these assays in endemic settings could strengthen surveillance programmes by enabling more reliable detection of low-level infections, particularly as transmission declines and elimination efforts progress into the post-MDA phase. Their suitability for use in remote and resource-limited areas may further support efforts to achieve and sustain the global LF elimination.

To address the important practical gap in LF diagnosis, the SHERLOCK assays combine ease of use with sensitivity comparable to that of qPCR (the emerging standard). The estimated cost per sample, including DNA extraction for both SHERLOCK and qPCR,

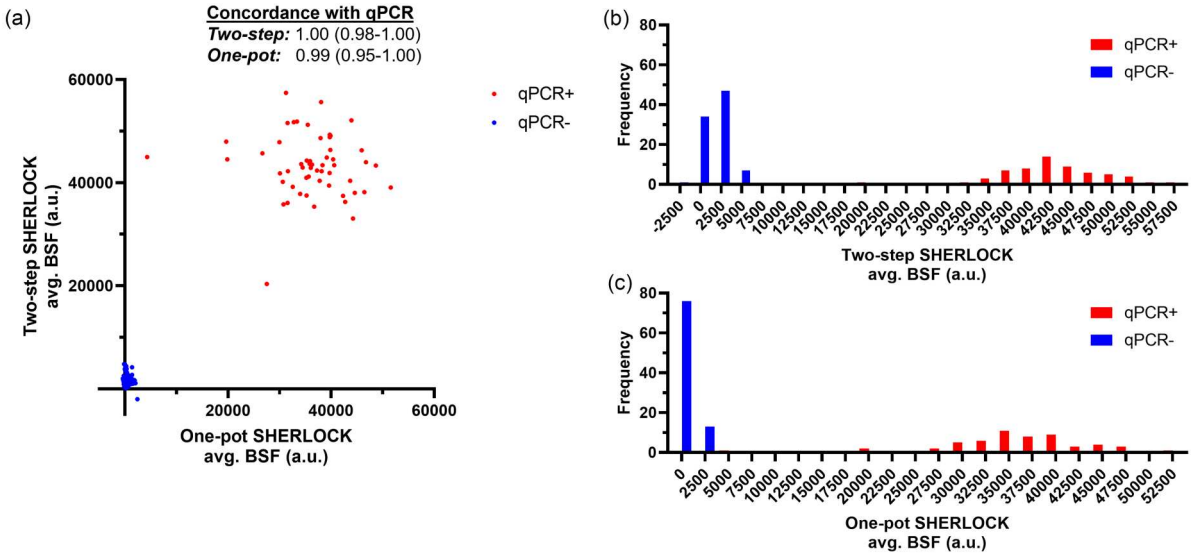


Figure 4. Fluorescence-based two-step and one-pot SHERLOCK detection of *W. bancrofti* (targeting *WbTR1*) in DNA extracted from pooled mosquito samples collected from Samoa, where *W. bancrofti* is endemic. (a) Comparison of average sample BSF values for two-step (*Y*-axis) and one-pot (*X*-axis) SHERLOCK in qPCR-positive ($n = 55$) and qPCR-negative samples ($n = 89$); Frequency distribution plot of average sample BSF values for (b) two-step SHERLOCK ($n = 149$), and (c) one-pot SHERLOCK ($n = 144$). Signal was detected at 60 min for two-step SHERLOCK and 90 min for one-pot.

was AU$9.10 for the two-step SHERLOCK assay, AU $11.30 for the one-pot SHERLOCK assay, AU$7.10 for qPCR, and AU$10.00 for FTS lateral-flow detection. Although qPCR had the lowest reagent cost, its implementation requires a relatively expensive real-time PCR thermocycler and supporting laboratory infrastructure. In contrast, SHERLOCK can be performed using only a 37°C heat block and offers flexible readout options. A fluorescent plate reader can facilitate high-throughput screening, while more field-

Table 2. Performance (sensitivity, specificity, and concordance) of two-step, one-pot, and lyophilized one-pot (one-pot Ly) SHERLOCK assays compared to qPCR for human and mosquito samples from Samoa.

Sample type	Subset	SHERLOCK assay type (n)	qPCR positive n (%)	SHERLOCK positive n (%)	Sensitivity (95% CI)	Specificity (95% CI)	Concordance (95% CI)	Kappa
Human	All	Two-step (185)	67 (36.22%)	67 (36.22%)	1.00 (0.95–1.00)	1.00 (0.97–1.00)	1.00 (0.98–1.00)	1.00
		One-pot (185)	67 (36.22%)	63 (34.05%)	0.94 (0.85–0.98)	1.00 (0.97–1.00)	0.98 (0.95–0.99)	0.95
		One-pot Ly (97)	67 (69.07%)	40 (41.24%)	0.60 (0.47–0.72)	1.00 (0.88–1.00)	0.72 (0.62–0.81)	0.48
	Ag-positive	Two-step (122)	67 (54.92%)	67 (54.92%)	1.00 (0.95–1.00)	1.00 (0.94–1.00)	1.00 (0.97–1.00)	1.00
		One-pot (122)	67 (54.92%)	63 (51.64%)	0.94 (0.85–0.98)	1.00 (0.94–1.00)	0.97 (0.92–0.99)	0.93
		One-pot Ly (91)	67 (73.63%)	40 (43.96%)	0.60 (0.47–0.72)	1.00 (0.86–1.00)	0.70 (0.6–0.79)	0.44
	Ag-negative	Two-step (63)	0 (0.00%)	0 (0.00%)	N/A	1.00 (0.94–1.00)	1.00 (0.94–1.00)	NA
		One-pot (63)	0 (0.00%)	0 (0.00%)	N/A	1.00 (0.94–1.00)	1.00 (0.94–1.00)	NA
		One-pot Ly (6)	0 (0.00%)	0 (0.00%)	N/A	1.00 (0.54–1.00)	1.00 (0.54–1.00)	NA
	Mf-Positive	Two-step (69)	65 (94.20%)	65 (94.20%)	1.00 (0.94–1)	1.00 (0.4–1.00)	1.00 (0.95–1.00)	1.00
		One-pot (69)	65 (94.20%)	61 (88.41%)	0.94 (0.85–0.98)	1.00 (0.4–1.00)	0.94 (0.86–0.98)	0.64
		One-pot Ly (67)	65 (97.01%)	40 (59.70%)	0.62 (0.49–0.73)	1.00 (0.16–1.00)	0.63 (0.5–0.74)	0.09
	Mf-Negative	Two-step (113)	0 (0.00%)	0 (0.00%)	N/A	1.00 (0.97–1.00)	1.00 (0.97–1.00)	NA
		One-pot (113)	0 (0.00%)	0 (0.00%)	N/A	1.00 (0.97–1.00)	1.00 (0.97–1.00)	NA
		One-pot Ly (28)	0 (0.00%)	0 (0.00%)	N/A	1.00 (0.88–1.00)	1.00 (0.88–1.00)	NA
Mosquito	All	Two-step (149)	60 (40.27%)	60 (40.27%)	1.00 (0.94–1.00)	1.00 (0.96–1.00)	1.00 (0.98–1.00)	1.00
		One-pot (144)	55 (38.19%)	53 (36.81%)	0.96 (0.87–1.00)	1.00 (0.96–1.00)	0.99 (0.95–1.00)	0.97

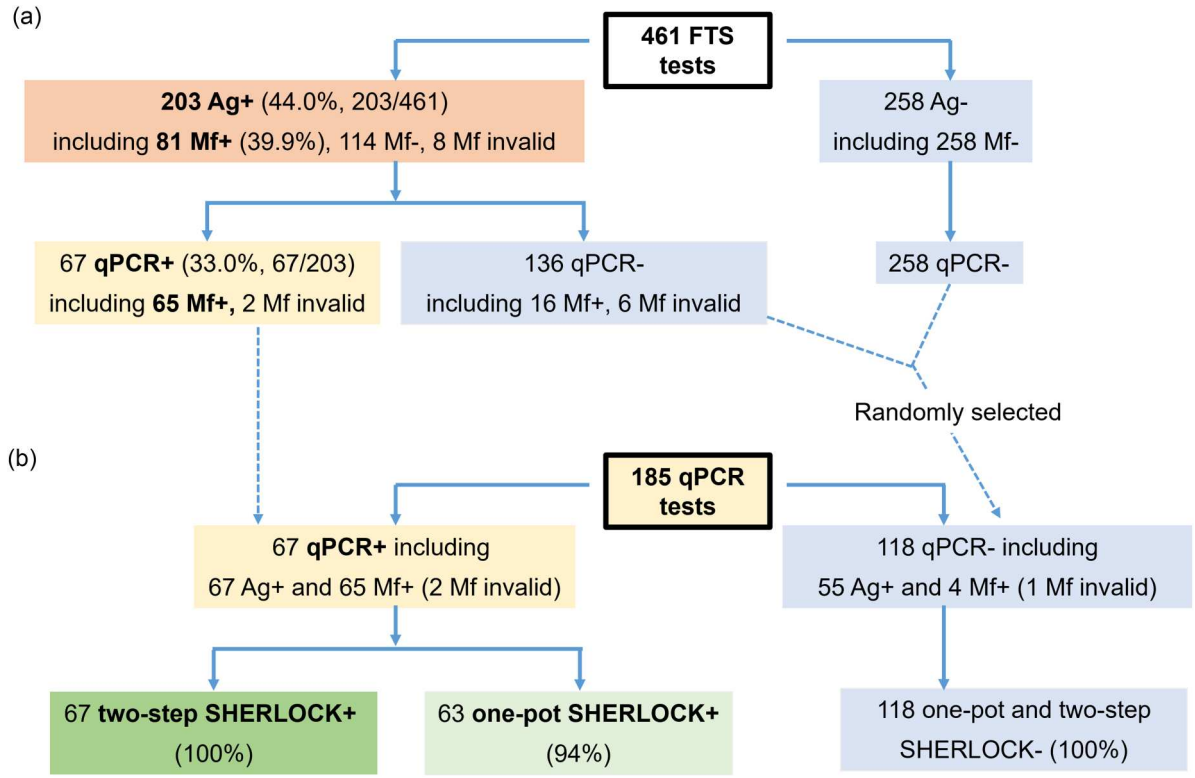


Figure 5. Process diagram outlining the testing of 461 human blood samples collected from human subjects living in Samoa, a *W. bancrofti*-endemic region. Different methods of LF detection, including FTS, Mf, qPCR, two-step and one-pot SHERLOCK were used. (a) A total of 461 human blood samples were tested by qPCR, FTS, and Mf tests conducted as part of the SaMELFS 2023 survey [41]. Among 203 Ag-positive samples, 81 were Mf-positive (39.9%) and 67 were qPCR-positive (33.0%). (b) A total of 185 samples were tested by SHERLOCK (67 qPCR-positive and 118 randomly selected qPCR-negative). All qPCR-positive samples were detected by two-step SHERLOCK (100%) and 63 (94.0%) by one-pot SHERLOCK, while all qPCR-negative samples were negative in both assays. "Mf invalid" refers to samples where the Mf test result was not obtained or could not be determined.

deployable formats include in-tube fluorescence detection visualized with a handheld UV torch, as previously demonstrated [15], and colorimetric detection using lateral-flow strips [15]. Although FTS also provides a simple and field-compatible readout, it detects circulating filarial antigen and therefore may not reliably distinguish active infection status (Figure 5). Overall, SHERLOCK provides a practical balance between cost, analytical performance, equipment requirements, and field applicability, effectively bridging the gap between laboratory-based qPCR and simple lateral-flow FTS testing.

Although LF one-pot SHERLOCK showed slightly lower sensitivity than two-step SHERLOCK and qPCR when evaluated using 149 pooled mosquito samples and 185 human blood samples from Samoa, it retains important field-friendly advantages, including a simplified reaction setup, reduced contamination risk, and the potential to minimize cold-chain requirements for reagent storage. The reduced sensitivity of the one-pot assay is plausibly due to suboptimal compatibility among the RPA, T7 transcription, and Cas13 detection reactions when they occur simultaneously, consistent with previous studies [26]. In the two-step format, RPA amplification is performed separately before the amplified product is transferred to the Cas13 detection reaction, allowing each stage to operate under more favourable conditions. In contrast, the one-pot SHERLOCK requires all enzymes to function under shared conditions, which may reduce the efficiency of target amplification, transcription, or detection, particularly in borderline-positive samples with low target concentrations. Previous studies indicate that optimizing buffer composition, Mg^{2+} concentration, primer concentration, and other reaction parameters can improve one-pot SHERLOCK performance [27]. Thus, the slightly reduced sensitivity of the one-pot assay relative to the two-step assay when evaluated against qPCR and Mf detection likely reflects the challenge of balancing multiple enzymatic reactions in a single mixture rather than an intrinsic limitation of Cas13 detection.

Lyophilization can further enhance the field applicability of one-pot SHERLOCK by decreasing reliance on ultra-low temperature freezers for reagent storage and transport, increasing shelf life [25]. Encouragingly, we found that with the addition of sucrose/mannitol (v.2 lyophilized one-pot SHERLOCK), the activity of the assay was maintained even after 10 days storage at RT (Figure 7). However, when testing human blood DNA samples using the v.2 lyophilized one-pot SHERLOCK mix stored at −20°C, only

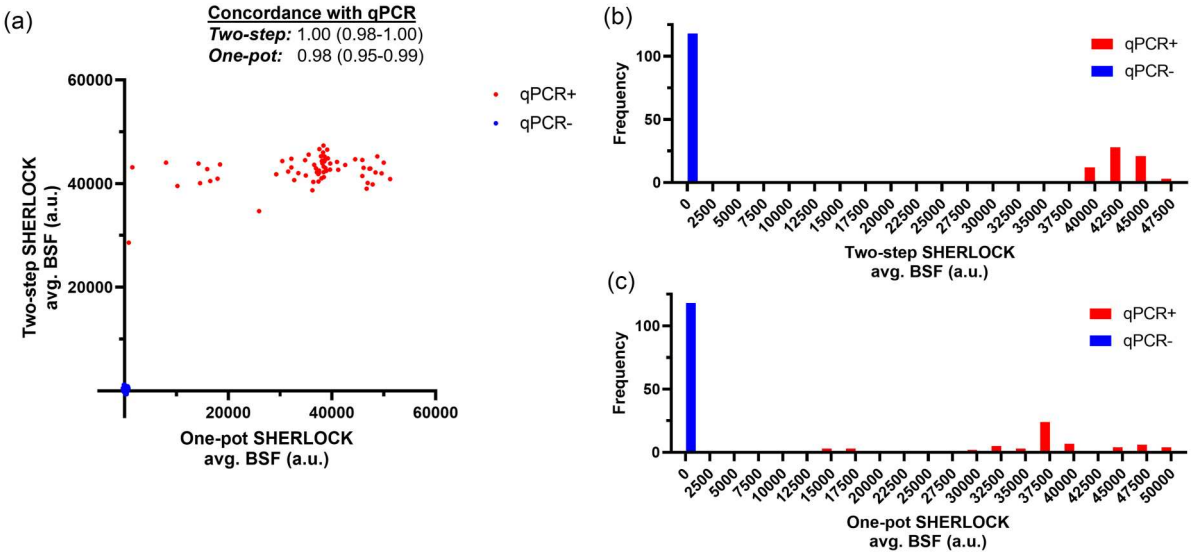


Figure 6. Comparison of fluorescence-based two-step and one-pot SHERLOCK assays with qPCR for detection of *W. bancrofti* (targeting *WbTR1*) in DNA samples extracted from human blood collected from Samoa, a *W. bancrofti*-endemic region. (a) Comparison of average sample BSF values for two-step (*Y*-axis) and one-pot (*X*-axis) SHERLOCK in qPCR-positive ($n = 67$) and qPCR-negative samples ($n = 118$); Frequency distribution plot of average sample BSF values for (b) two-step SHERLOCK, and (c) one-pot SHERLOCK ($n = 185$). Signal was detected at 60 min for two-step SHERLOCK and 90 min for one-pot.

59.7% sensitivity was observed compared to qPCR assays (Figure 8), suggesting that further improvement is needed to enhance the stability and activity of the lyophilized one-pot SHERLOCK assay. Two strategies to improve thermostability and usability are worth exploring in future work: (1) Optimization of lyophilization using chemical stabilizers. These include glycine/arginine, which have been found to improve protein recovery and activity retention after freeze-drying [28]; 2′-O-Methyl modified crRNAs that

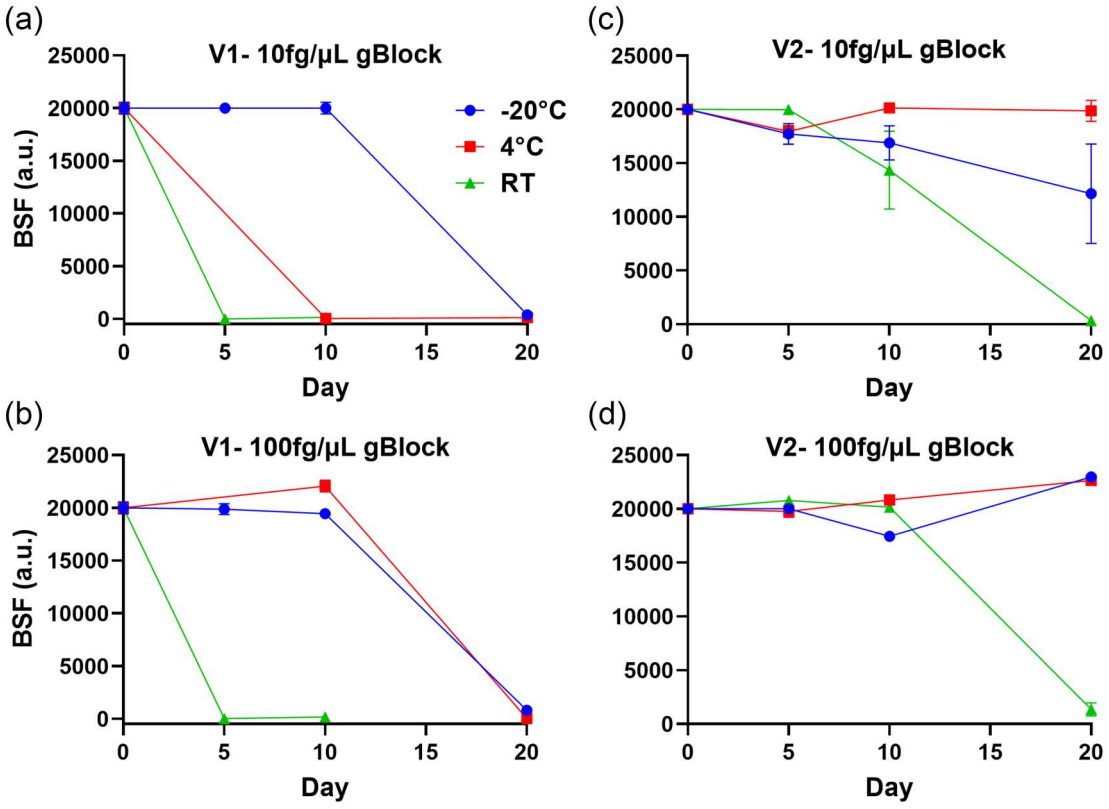


Figure 7. The stability of lyophilized v.1 and v.2 one-pot SHERLOCK pellets after storage at RT, 4°C, and −20°C for 0, 5, 10, 15, and 20 days, using 10 and 100 fg/µL of *W. bancrofti* gBlock DNA as the template. Lyophilized one-pot SHERLOCK v.1 tested using (a) 10 fg/µL and (b) 100 fg/µL *W. bancrofti* gBlock DNA; Lyophilized one-pot SHERLOCK v.2 tested using (c) 10 fg/µL and (d) 100 fg/µL *W. bancrofti* gBlock DNA.

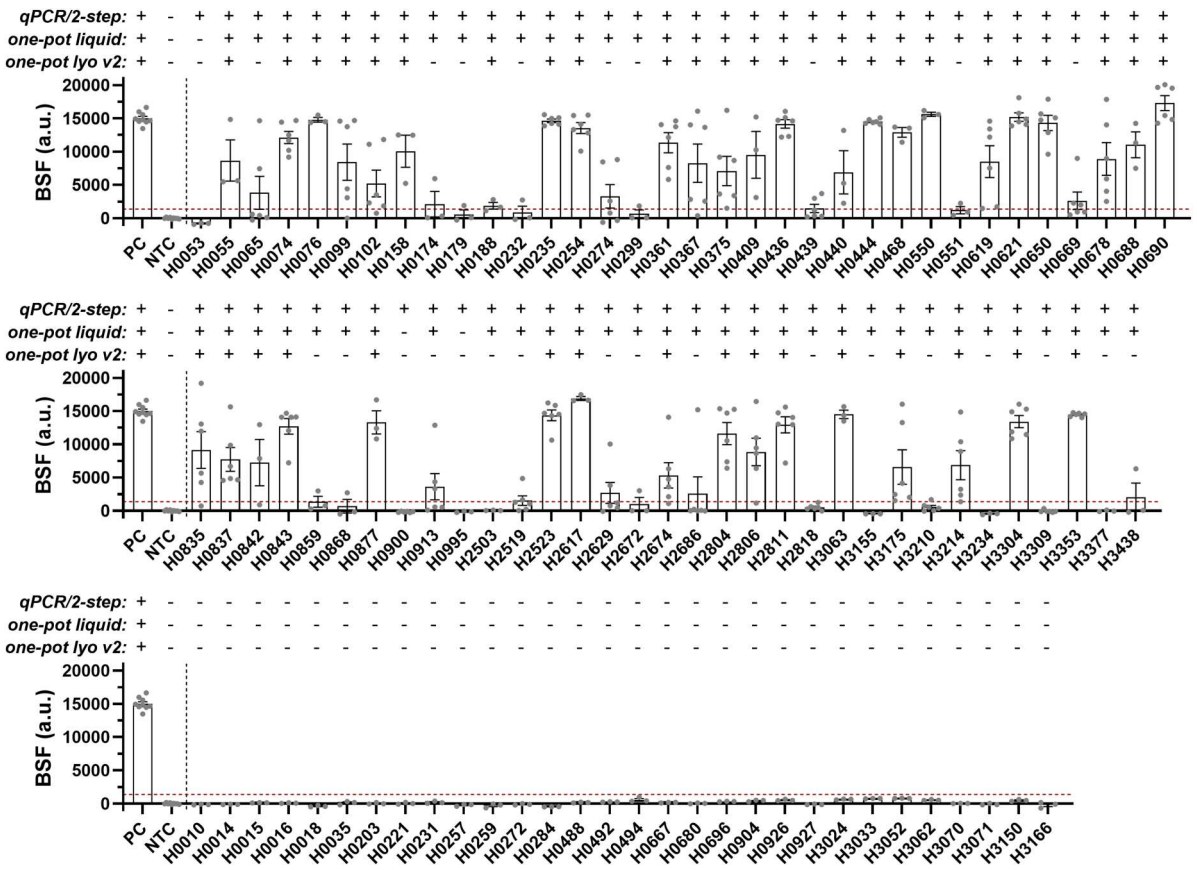


Figure 8. Fluorescence-based v.2 lyophilized one-pot SHERLOCK detection of *W. bancrofti* (targeting *WbTR1*) in DNA samples extracted from human blood (including 67 qPCR-positives and 30 qPCR-negatives) collected from Samoa. v.2 lyophilized one-pot SHERLOCK pellets were tested after 5 days storage at −20°C. BSF values are given as the mean ± SEM after 2 h incubation. Results of qPCR/two-step SHERLOCK, standard one-pot SHERLOCK and v.2 lyophilized one-pot SHERLOCK assays are shown at the top of each sample (+, positive; −, negative). Red line = cut off.

enhance nuclease resistance and maintain CRISPR activity after storage [29]; and locked nucleic acids that increase thermal stability through higher melting temperatures [30] and may further improve the structural and thermal stability of lyophilized CRISPR diagnostic reagents. (2) Replacing LwCas13 with thermostable Cas enzymes such as TccCas13a from *Thermoclostridium caenicola* [31] which exhibits strong trans-cleavage activity across 37–70°C, supporting enhanced thermal tolerance, and BrCas12b from *Brevibacillus* sp., engineered for improved thermostability and efficient activity at 37–50°C, facilitating cold-chain-free diagnostic applications [32].

To further enhance field applicability, CRISPR-based diagnostics should be integrated with rapid, field-friendly DNA extraction methods such as magnetic bead-based or filter paper/cotton approaches [33,34]. Isothermal amplification methods are generally more tolerant to inhibitors than PCR [35], allowing them to work effectively with simplified, extraction-free workflows using minimal lysis steps, enabling detection from blood, saliva, and urine with limited processing [13]. The field is rapidly evolving towards multiplexed, quantitative, amplification-free assays, and integrated platforms using nanotechnology and microfluidics to further simplify workflows [36].

In this study, the *W. bancrofti* SHERLOCK assay was also compared with the antigen-based FTS, which is currently the most widely used field tool for TAS, mapping and monitoring LF incidence in infected regions [37]. Despite its widespread use, several factors can lead to inaccurate FTS results:

(1) Persistent antigenemia after worm clearance. CFA can remain detectable in the bloodstream for months to years after adult worms have died following treatment, causing false-positive FTS results [38]. This is also supported by our finding that only 33% of FTS-positive samples were positive by two-step SHERLOCK or qPCR (Figure 5(a)). The remaining >60% may represent early or resolved infections, as FTS cannot distinguish active from past infection. Molecular diagnostics may detect DNA from dead parasites for several days post-treatment [39], but this persistence is much shorter than that of CFA. In addition, Mf detection showed 0.96–0.98 concordance relative to SHERLOCK (Supplementary Figure 3), suggesting Mf remains a reliable

indicator of active infection, in contrast to FTS, although the limitations of microscopy in detecting low-density infections are well recognized.

(2) Cross-reactivity with other filarial species, leading to false positives [7]. Our *W. bancrofti* SHERLOCK assays demonstrated no cross-reactivity with *D. repens* or *O. lupi*, which are known to cause false-positive FTS results due to shared antigen epitopes [40]. The high cross-reactivity is also recognized as a major obstacle to LF elimination in loiasis-endemic areas, where LF-FTS often detects *L. loa* antigens [7].

Overall, CRISPR-based diagnostics offer a powerful approach to field diagnostics, and their versatility, ultra-sensitivity, and potential for low-cost portable deployment position SHERLOCK as a game-changing platform for detecting a broad range of neglected tropical diseases (NTDs). Although further optimization is required for field use, the first *W. bancrofti* SHERLOCK assays developed in this study, together with our previously developed assays for the detection of schistosomiasis, offer a practical approach to address WHO-identified gaps, including rapid helminth mapping, monitoring and evaluation of control programmes towards elimination targets.

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Author contributions

CRediT: **Skye R. MacGregor:** Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing; **Richard S. Bradbury:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing; **Malcolm K. Jones:** Conceptualization, Investigation, Writing – review & editing; **Asrhyella Seeler:** Data curation, Validation; **Helen J. Mayfield:** Data curation, Formal analysis, Resources, Software, Validation; **Patricia M. Graves:** Formal analysis, Resources, Validation, Writing – review & editing; **Lisa Rigby:** Investigation, Methodology, Validation, Writing – review & editing; **Shannon M. Hedtke:** Validation; **Vito Colella:** Investigation, Resources; **Beatris Mario Martine:** Investigation, Validation; **Yuhai Bi:** Methodology, Writing – review & editing; **Robert Thomsen:** Data curation, Methodology, Resources, Validation; **Satupaitea Viali:** Data curation, Investigation, Resources, Validation; **Colleen L. Lau:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Resources, Supervision, Validation, Writing – review & editing; **Hong You:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Use of generative artificial intelligence

A generative artificial intelligence (AI) tool, ChatGPT (OpenAI; GPT-5), was used solely to improve the grammar and readability of the manuscript text. It was not used for data analysis, interpretation of results, figure generation or manipulation, creation of original data, or formulation of scientific conclusions. All outputs were reviewed, edited, and verified by the authors, who take full responsibility for the content's accuracy and integrity.

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