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TrypOne: Fluorescence-Based One-Pot CRISPR-LAMP Assay for Point-of-Need Diagnosis of Trypanosomosis

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Abstract

Human and animal trypanosomosis caused by members of *Trypanosoma brucei* (*T. brucei*) remains a major global One Health challenge. While effective disease control relies on the accurate detection of active infections, existing point-of-need (PON) tests fall short of the performance of gold-standard laboratory polymerase chain reaction (PCR) assays. Here, we developed *TrypOne*, a sensitive, specific, and field-deployable CRISPR-LAMP-based assay for the pan-specific detection of active *T. brucei* infections. *TrypOne* integrates CRISPR-*AapCas12b* cleavage with loop-mediated isothermal amplification (LAMP) targeting the conserved, high-copy RIME retrotransposon in a single one-pot reaction. Under optimized conditions, it detected ≤ 1 trypanosome equivalent per reaction within 60 min at 55 °C, with a naked-eye (NE) fluorescence-based readout. In a murine infection model, it reliably tracked ongoing parasitaemia and treatment response, achieving 100% specificity and sensitivity and showing concordant performance with the gold-standard TBR PCR. *TrypOne* enables rapid molecular confirmation of active infections under field-compatible conditions, facilitating diagnosis and monitoring of treatment response. It represents a promising One Health tool for coordinated surveillance and control of human and animal trypanosomosis.

Introduction

Human and animal trypanosomosis caused by *Trypanosoma brucei* (*T. brucei* – *T. b.*) subspecies comprise a group of neglected parasitic diseases with major medical, veterinary and economic consequences. In humans, *T. b. gambiense* causes the chronic form of human African trypanosomiasis (HAT) in western and central Africa, whereas *T. b. rhodesiense* causes an acute zoonotic form in eastern and southern Africa [1]. In domestic and wild animals, *T. b. brucei*, *T. b. evansi* and *T. b. equiperdum* cause distinct forms of animal trypanosomosis (AT), including nagana, surra and dourine [2-4]. The broader geographical spread of some forms of *T. brucei* trypanosomosis

reflects their transmission beyond the classical tsetse (*Glossina* spp.) cycle, including mechanical transmission by haematophagous insects and, in specific epidemiological settings, mammalian vectors [5, 6]. This transmission flexibility increases overlap among animal reservoirs, vectors and human populations, while climate-driven changes in vector distribution and expanding livestock grazing may further intensify these interfaces, reinforcing the need to consider *T. brucei* trypanosomosis within a broader One Health framework [7, 8].

Control of *T. brucei* trypanosomosis relies on sustained surveillance, case detection, treatment and vector control. Although these interventions have reduced reported HAT cases to historically low levels, effective control across HAT and AT still requires practical tools to guide treatment decisions and support integrated disease management [1, 9]. In the absence of an effective vaccine, control largely depends on accurate diagnosis followed by appropriate intervention. To this end, diagnosis should be based on the detection of ongoing infection, avoiding unnecessary treatment and overestimation of disease incidence [10]. However, current diagnostic approaches do not fully meet this requirement. Microscopy remains one of the most widely used direct diagnostic methods; however, low or intermittent parasitaemia during early or chronic infection can limit its diagnostic performance [11]. Serological antibody (Ab)-detection tests, including the card agglutination test for trypanosomiasis (CATT) and lateral-flow (LF) rapid diagnostic tests, are useful for large-scale screening but cannot reliably distinguish active infection from previous exposure [12]. In addition, reported limitations in the specificity of serological assays become particularly consequential in low-prevalence settings. This is especially relevant in HAT foci with prevalence far below 0.1%, where serological screening can yield approximately 99 false-positive results for every true-positive (TP) case [13]. Under these conditions, some individuals suspected of being affected by *T. brucei* trypanosomosis remain unconfirmed. Accordingly, WHO has defined 99% clinical specificity and sensitivity as the desired target for individual diagnostic tests [10].

Molecular confirmation by polymerase chain reaction (PCR) can address these performance requirements through direct detection of parasite DNA, supporting confirmation of ongoing infection. However, its reliance on specialized laboratory infrastructure, stable power supply, cold-chain reagent storage and controlled multi-step workflows restricts implementation outside reference laboratories, limiting availability at the point of need (PON) [14, 15]. Consequently, unresolved cases often require sample referral for centralized testing, delaying intervention and increasing the risk of disease progression, continued transmission or unnecessary treatment [1]. These constraints highlight the need for molecular diagnostic assays that combine confirmatory performance with operational simplicity. Isothermal DNA amplification assays replace programmed temperature cycling with single-temperature incubation, enabling amplification using portable heat sources such as water baths or compact heaters. Among these assays, loop-mediated isothermal amplification (LAMP) has been widely explored as a simplified alternative to polymerase chain reaction (PCR)-based molecular diagnosis [16]. For pan-*T. brucei* detection, LAMP development has focused on two targets: the single-copy paraflagellar rod protein A (PfrA) gene [17] and the multicopy repetitive insertion mobile elements (RIMEs) [18]. Among these, RIME-LAMP has been further evaluated in clinical and field samples and implemented with multiple readout strategies, including endpoint colorimetric and turbidity-based detection, supporting PON testing [19-21]. However, recurrent non-specific amplification remains a major limitation of LAMP assays, undermining their practical applicability outside controlled laboratory settings [16, 22, 23].

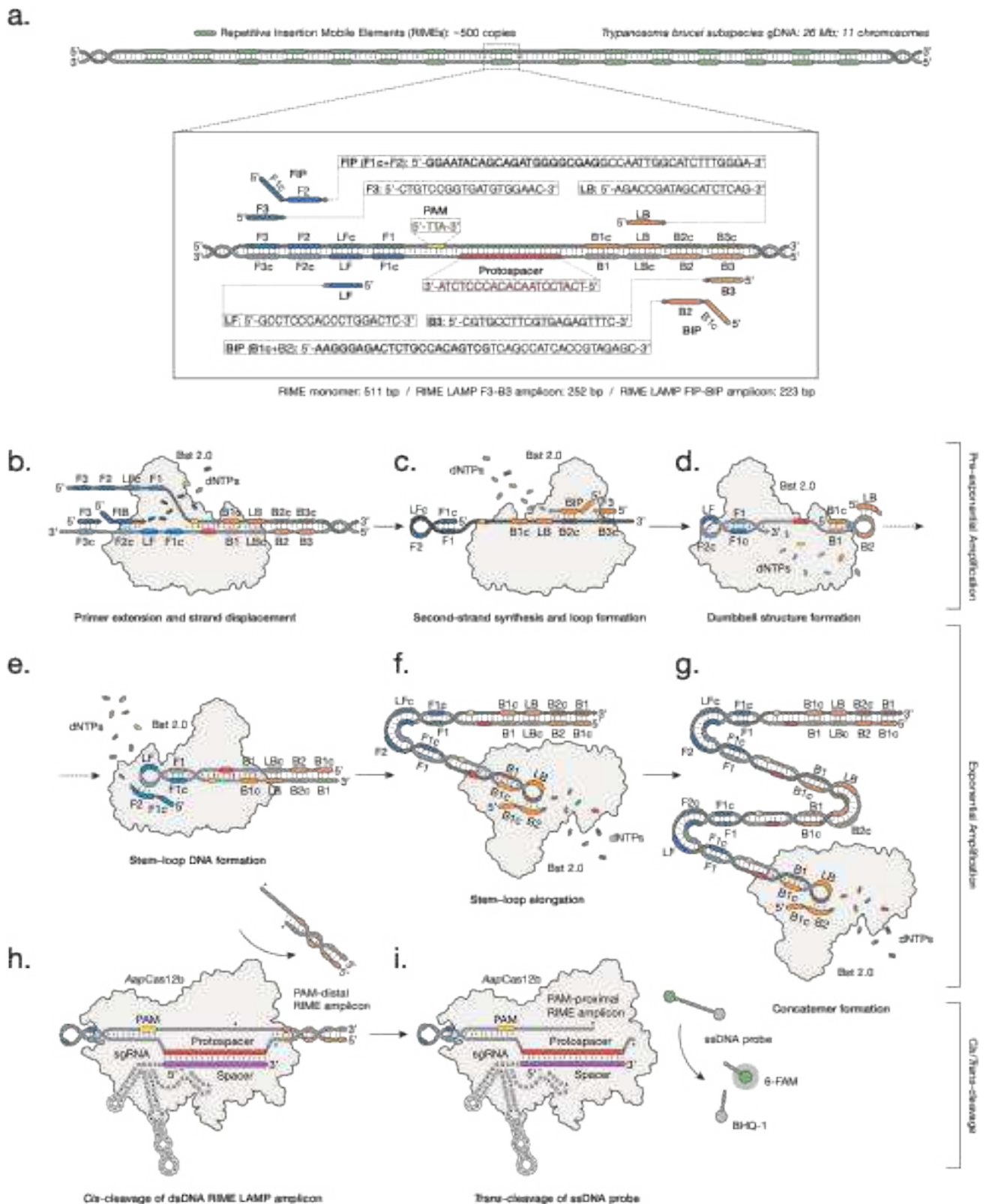


Figure 1. Molecular design and mechanistic principle of the *TrypOne* assay. (a) Architecture of the RIME region amplified by RIME LAMP and coupled to *AapCas12b*-mediated detection within the *TrypOne* assay. RIME is a 511-bp high-copy repetitive insertion mobile element present at approximately 500 copies in the *Trypanosoma brucei* subspecies genome [18, 24]. The RIME LAMP assay targets a 252-bp region within this RIME region, using the primer set designed by Z. K. Njiru et al. (2007), which includes F3, B3, FIP, BIP, LF, and LB primers [18]. These are shown together with the

AapCas12b protospacer and PAM, required for *AapCas12b* recognition and selected in this study for CRISPR-*AapCas12b* detection of RIME LAMP products. (b) Initiation of LAMP amplification by primer extension and strand displacement. FIP anneals to the target sequence and initiates DNA synthesis. Binding of F3 promotes strand displacement, releasing a linear single-stranded DNA intermediate. (c) Second-strand synthesis and loop formation. The displaced strand serves as a template for BIP-primed synthesis. The resulting product contains self-complementary sequences that fold to form a loop. (d) Dumbbell structure formation. Terminal complementary regions anneal to form a dumbbell-shaped intermediate, and loop primers anneal to single-stranded loops to accelerate amplification. (e) Stem-loop DNA formation. The dumbbell intermediate self-primers to generate a stem-loop template that serves as the template for exponential amplification. (f) Stem-loop elongation. Inner and loop primers initiate strand-displacement synthesis, producing extended stem-loop intermediates. (g) Concatemer formation. Repeated cycles of primer annealing, strand displacement, and self-primed synthesis generate high-molecular-weight concatemers. These fold into complex, multi-looped, cauliflower-like structures characteristic of LAMP products [16]. (h) RIME LAMP amplicon detection by CRISPR-*AapCas12b*. RIME LAMP amplicon detection by CRISPR-*AapCas12b*. The *AapCas12b*-sgRNA RNP binds the RIME LAMP amplicon through recognition of the 5'-TTN-3' PAM, promoting local DNA unwinding. The exposed target strand hybridizes with the sgRNA spacer sequence at the complementary protospacer region, initiating from the seed region and extending to form a complete R-loop [25]. R-loop formation induces conformational rearrangements that activate the RuvC catalytic domain [26]. The activated nuclease performs specific *cis*-cleavage of the non-target strand followed by the target strand. *AapCas12b* cleaves the complementary DNA strand 23 bp upstream of the PAM sequence and the non-complementary DNA strand 17 bp upstream of the PAM sequence [27]. This releases the PAM-distal fragment while the PAM-proximal fragment remains bound, maintaining the enzyme in an active conformation [25, 28]. (i) *Trans*-cleavage and fluorescence signal generation. The activated *AapCas12b* exhibits non-specific *trans*-cleavage of nearby ssDNA, including 6-FAM-(T)₅-BHQ-1 probes. Cleavage separates FAM fluorophore and quencher, restoring FAM fluorescence. This activity is sustained by the activated conformation of the RuvC domain and does not require continued target *cis*-cleavage [28].

In recent years, clustered regularly interspaced short palindromic repeats (CRISPR)-Cas systems have expanded the molecular diagnostics toolbox by enabling programmable, sequence-specific nucleic-acid detection. Diagnostic platforms such as specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) [29] and one-hour low-cost multipurpose highly efficient system (HOLMES) [30] use Cas13- and Cas12-mediated collateral nuclease activity, respectively, to convert single guide RNA (sgRNA)-directed recognition of nucleic acid targets into reporter cleavage and signal generation [31]. When coupled to LAMP, CRISPR-Cas detection complements target amplification with sequence-specific verification, distinguishing target-derived amplicons from non-specific amplification products while preserving analytical sensitivity [32]. These CRISPR-Cas12-based isothermal assays have recently been applied to kinetoplastid parasites, including *T. cruzi* [33] and *Leishmania* spp. [34]. However, their reliance on two-pot workflows requires the transfer of amplified products into a separate reaction for Cas12-based detection. This post-amplification handling increases contamination risk, operational complexity and reaction time, limiting their suitability for PON deployment.

In this study, we describe the development of *TrypOne*, the first one-pot CRISPR-Cas12b-based LAMP assay for the detection of active pan-*T. brucei* subspecies infections. We demonstrate how *AapCas12b*-mediated amplicon verification improves RIME-LAMP specificity while preserving analytical sensitivity. We further show how *TrypOne* enables molecular confirmation of active infection, supports monitoring of treatment response, and can be translated into a field-compatible format based on portable heat-pack incubation and visual fluorescence readout.

Results

RIME-LAMP assay optimization and analytical characterization for *T. brucei* detection

The development of a LAMP-CRISPR assay for detection of *T. brucei* required the pre-amplification of a parasite-specific double stranded (ds)DNA target compatible with CRISPR-*Alicyclobacillus acidiphilus* (*Aap*)Cas12b recognition and cleavage (Figure 1a-i). For this purpose, the RIME-LAMP

assay originally described by Njiru et al. [18] was selected. This assay targets RIMEs, present at approximately 500 copies per *T. brucei* genome (Figure 1a; Supplementary Figure 1).

Given the reported occurrence of non-specific amplification in RIME-LAMP, this limitation was first evaluated under the reaction conditions used in this study. Non-specific amplification resulting in false-positive (FP) signals observed in 44% of true-negative (TN) reactions after 120 min (Figure 2a-c). This corresponded to a positive predictive value (PPV) of 31.25%. While endpoint relative fluorescence units (RFU) values could not discriminate FP from TP reactions, melt-curve analysis identified the expected RIME-LAMP amplicon by a peak at 82.5°C and FP products by higher melting peaks at 85.5-87.0°C (Figure 2d). Time-to-threshold analysis showed that FP reactions occurred mainly after 60 min, with 81.8% appearing between 60 and 120 min and 18.2% appearing before 60 min. A 60 min cutoff therefore reduced FP amplification from 44% to 18.2% while maintaining detection of all TP reactions (Figure 2e).

The temperature profile of RIME-LAMP amplification was then established to guide future one-pot integration with CRISPR-*AapCas12b*. Amplification was sustained across 54-62°C (Figure 2f). Relative amplification rates were calculated against the 62°C condition, set as 100% (Figure 2g). No significant reduction was observed at 60°C, which retained 97.7% relative amplification. In contrast, relative amplification rates decreased significantly at 58°C, 56°C and 54°C, corresponding to 91.9%, 79.9% and 67.9% of the 62°C condition, respectively. Therefore, 62°C was retained as the optimal RIME-LAMP temperature for subsequent analytical characterization.

Analytical sensitivity was then assessed using serial dilutions of *T. brucei* genomic (g)DNA. RIME-LAMP detected 10^4 , 10^3 and 10^2 parasite equivalents mL^{-1} in all technical replicates, defining 10^2 parasite equivalents mL^{-1} as the limit of detection (LOD) (Figure 2h, i). This concentration corresponded to 67.65 fg of total gDNA, equivalent to 0.5 parasite equivalents per reaction. At 10^1 parasite equivalents mL^{-1} , the positivity rate decreased to 60%. Melt-curve analysis identified one replicate at 10^1 parasite equivalents mL^{-1} and one no-template control (NTC) replicate as non-specific amplification, both showing higher melting peaks of 85.75-86.5°C (Supplementary Figure 2).

Analytical specificity was evaluated using gDNA from *T. b. gambiense*, *T. b. rhodesiense*, *T. b. brucei*, *T. b. evansi*, *T. b. equiperdum*, *T. congolense*, *T. vivax* and *T. cruzi*. All *T. brucei* subspecies were classified as positive by RIME-LAMP (Figure 2j, k). FP reactions were also observed in two NTC replicates, one *T. congolense* replicate and one *T. cruzi* replicate. Melt-curve analysis identified these as non-specific amplification, with melting peaks in the FP range of 84.5-86.5°C rather than the target-specific 82.5°C profile (Supplementary Figure 2).

CRISPR-*AapCas12b* verification of RIME-LAMP products

Following RIME-LAMP optimization, CRISPR-*AapCas12b* cleavage was evaluated as a sequence-specific readout of RIME-LAMP amplicons. The assay was designed to discriminate target-specific amplification from non-specific products without requiring melt-curve analysis, thereby supporting a simple, field-compatible endpoint readout.

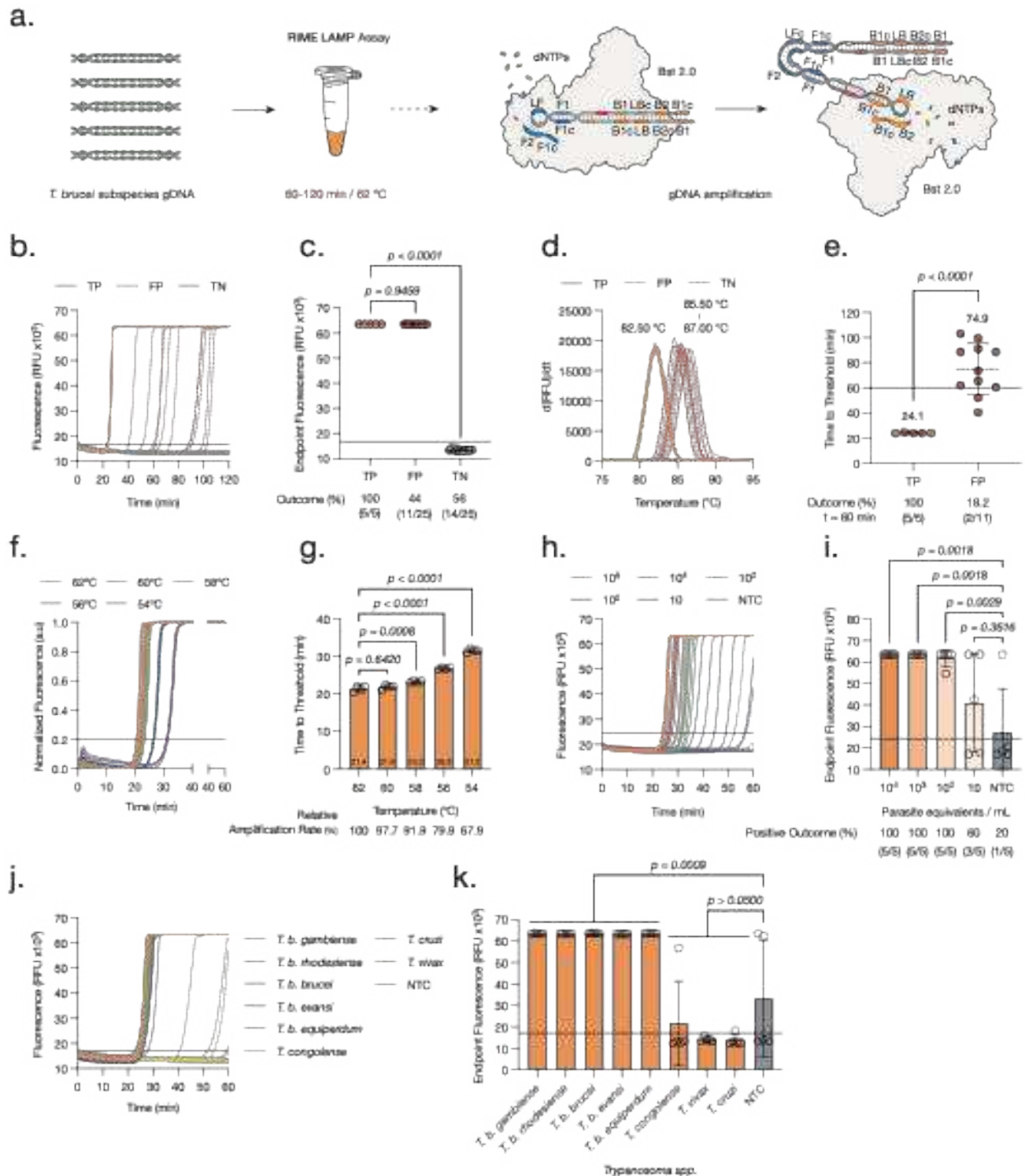


Figure 2. Optimization and analytical performance of the RIME LAMP assay. (a) RIME LAMP workflow. Genomic DNA from *T. brucei* subspecies is amplified by RIME LAMP under isothermal conditions at 62°C for 60-120 min. (b-e) Evaluation of nonspecific RIME LAMP amplification. Fluorescence kinetics (b), endpoint fluorescence (c), melt curves (d), and time-to-threshold analysis (e) distinguish true-positive (TP) amplification from nonspecific late false-positive (FP) amplification and true-negative (TN) outcomes. Reactions were monitored for 120 min to define the optimal cutoff time. TP and TN controls were tested with 5 and 25 technical replicates, respectively. A 60-min cutoff reduced late FP amplification from 44% to 18.2%. (f, g) Temperature optimization of RIME LAMP at 54-62°C using 4 technical replicates per condition, shown as normalized kinetics (f) and time-to-threshold with amplification rates relative to 62°C (g). (h, i)

Analytical sensitivity of RIME LAMP using serially diluted samples from 10^4 to 10 parasite equivalents/mL and a no-template control (NTC), shown as fluorescence kinetics (h) and endpoint fluorescence (i) from 5 technical replicates per condition. (j, k) Analytical specificity of RIME LAMP against *T. b. gambiense* ANTAT 9.1, *T. b. rhodesiense* STIB 850, *T. b. brucei* ANTAT 1.8, *T. b. evansi* ROTAT 1.2, *T. b. equiperdum* BOTAT 1.1, *T. congolense* TRT 17, *T. vivax* ILRAD 700, *T. cruzi* TULAHUEN, and NTC, shown as fluorescence kinetics (j) and endpoint fluorescence (k) from 5 technical replicates. False positive outcomes are indicated by dotted lines and dotted symbols in the corresponding panels. Positivity thresholds were defined as TN or NTC mean + $10 \times$ SD, except in (f), where the threshold was manually selected within the linear phase of exponential amplification. Individual replicates in (c, e, g, i, k) are shown with mean (dotted line) $\pm$ SD. Statistical analyses in (c, g, i, k) used one-way ANOVA with Dunnett's multiple-comparisons test; (e) used Welch's t-test. *P* values are indicated in the panels.

To guide sgRNA design, the 511 bp RIME monomer sequences from *T. b. evansi* (GenBank accession EF567425.1), *T. b. rhodesiense* (GenBank accession EF567424.1) and *T. b. brucei* (GenBank accession EF567426.1), originally reported by Njiru et al. [18], were aligned and annotated with the RIME-LAMP primer-binding sites (Supplementary Figure 1). The RIME-LAMP amplicon was then screened for *AapCas12b*-compatible target sites, defined by a 5'-TTN-3' protospacer adjacent motif (PAM) upstream of a 20-nt protospacer, as required for *AapCas12b* target recognition [27, 35]. Candidate target sites overlapping LAMP primer-binding regions, or located within primer-derived sequences, were excluded to minimize primer-gRNA overlap and reduce primer-derived cross-reactivity. Three *AapCas12b*-compatible 5'-TTN-3' PAM-protospacer target sites were identified on the plus DNA strand, all located within a region showing 100% sequence identity across the three aligned RIME sequences. As a result, three RIME-targeting sgRNAs were designed and evaluated in *cis*- and *trans*-cleavage assays (Supplementary Table 1). All three candidates supported *AapCas12b*-mediated *cis*-cleavage, with sgRNA-dependent cleavage efficiencies of 47.64%, 87.10% and 72.71% for sgRNA1, sgRNA2 and sgRNA3, respectively (Supplementary Figure 3a). *Trans*-cleavage assays further identified sgRNA2 as the best-performing guide, producing the strongest reporter signal at both 15 and 60 min of incubation (Supplementary Figure 3b, c). In addition, sgRNA2 showed comparable *trans*-cleavage activity against RIME amplicons from all tested *T. brucei* subspecies (Supplementary Figure 3d, e). Based on its superior *cis*- and *trans*-cleavage performance, sgRNA2 was selected for subsequent assay development and is hereafter referred to as RIMEsgRNA.

RIMEsgRNA was next evaluated in a two-pot *TbLAMP*-CRISPR format. RIME-LAMP products classified as TP, FP or TN were transferred directly into the CRISPR-*AapCas12b* cleavage reaction to determine whether sequence-specific cleavage could distinguish target-specific from non-specific amplification (Figure 3a). CRISPR-*AapCas12b* detection retained all TP reactions as positive and all TN reactions as negative, while reclassifying all FP RIME-LAMP products as negative (Figure 3b, c). The analytical sensitivity and specificity of the two-pot *TbLAMP*-CRISPR assay were subsequently evaluated. CRISPR-*AapCas12b* detection maintained the sensitivity of RIME-LAMP, detecting 10^4 , 10^3 and 10^2 parasite equivalents mL^{-1} in all technical replicates and retaining a 40% detection rate at 10 parasite equivalents mL^{-1} (Figure 3d, e). The FP RIME-LAMP signals previously observed at 10 parasite equivalents mL^{-1} and in the NTC were reclassified as negative. Analytical specificity for all tested *T. brucei* subspecies was retained, and the non-specific signals previously observed in *T. congolense*, *T. cruzi* and NTC reactions were eliminated (Figure 3f, g).

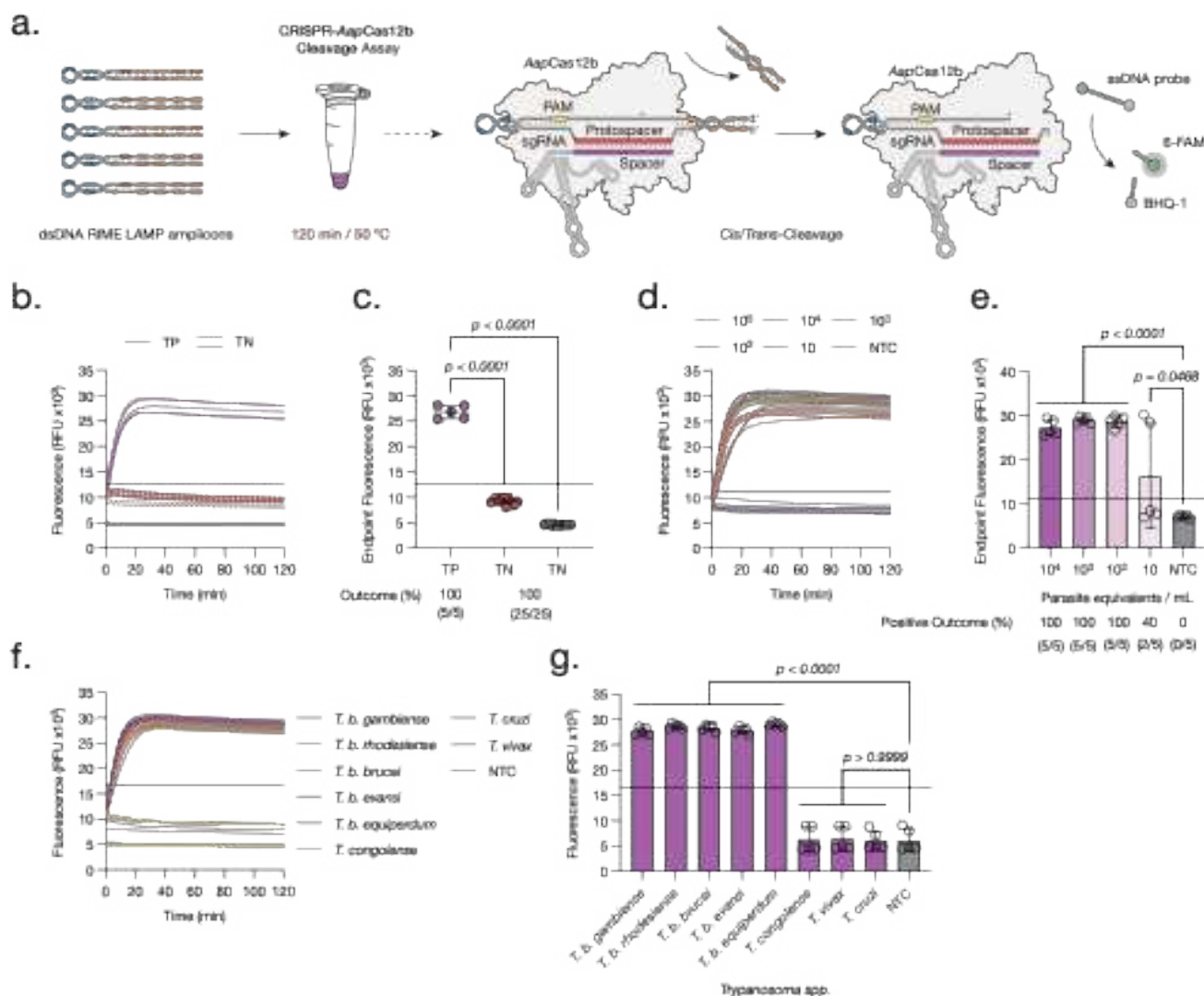


Figure 3. Analytical performance of the CRISPR-AapCas12b cleavage assay. RIME LAMP amplicons generated in Figure 2 were used to evaluate CRISPR-AapCas12b cleavage in a two-pot format. (a) Two-pot *Tb*LAMP-CRISPR workflow. Pre-amplified dsDNA RIME LAMP products were transferred to a CRISPR-AapCas12b cleavage reaction and incubated at 50°C for 120 min. Target recognition induced the *cis*-cleavage of the RIME amplicon and *trans*-cleavage of a 6-FAM/BHQ-1 ssDNA probe. (b, c) CRISPR-AapCas12b verification of RIME LAMP outcomes. Fluorescence kinetics (b) and endpoint fluorescence (c) show that CRISPR-AapCas12b preserves TP and TN classification while resolving RIME LAMP FP outcomes. TP, FP, and TN RIME LAMP products were tested with 5, 11, and 14 technical replicates, respectively. (d, e) Analytical sensitivity of Two-pot *Tb*LAMP-CRISPR using RIME LAMP products generated from serially diluted samples from 10⁴ to 10 parasite equivalents/mL and a NTC, shown as fluorescence kinetics (d) and endpoint fluorescence (e) from 5 technical replicates per condition. (f, g) Analytical specificity of Two-pot *Tb*LAMP-CRISPR using RIME LAMP products generated from *T. b. gambiense* ANTAT 9.1, *T. b. rhodesiense* STIB 850, *T. b. brucei* ANTAT 1.8, *T. b. evansi* ROTAT 1.2, *T. b. equiperdum* BOTAT 1.1, *T. congolense* TRT 17, *T. vivax* ILRAD 700, *T. cruzi* TULAHUEN, and NTC, shown as fluorescence kinetics (f) and endpoint fluorescence (g) from 5 technical replicates. Positivity thresholds were defined as NTC mean + 10 × SD. Individual replicates in (c, e, g) are shown with mean (dotted line) ± SD. Statistical analyses in (c, e, g) used one-way ANOVA with Dunnett's multiple-comparisons test. *P* values are indicated in the panels.

One-pot *TrypOne* assay optimization and analytical characterization

Following two-pot validation, RIME-LAMP amplification and CRISPR-AapCas12b detection were integrated into a single one-pot reaction, termed *TrypOne* (Figure 4a). Since RIME-LAMP amplification and CRISPR-AapCas12b cleavage were independently optimized at 62°C and 50°C,

respectively, *TrypOne* was evaluated across a temperature range of 50-62°C to identify a single incubation temperature compatible with both enzymatic reactions. *TrypOne* real-time (RT) fluorescence readout revealed a temperature-dependent response, with maximal normalized signals between 52°C and 55°C and reduced signals at temperatures outside this range (Figure 4b, f; Supplementary Figure 4).

To determine whether assay performance at different temperatures was influenced by parasite input, *TrypOne* was evaluated at 54°C, 55°C and 56°C using 10^4 , 10^3 and 10^2 parasite equivalents mL⁻¹ and NTC. Fluorescence kinetic profiles also differed among temperatures, with steeper curves at 54°C, flatter curves at 55°C, and further signal attenuation at 56°C (Figure 4c-e). Using the RT readout, positive detection rates were 100%, 87.5% and 62.5% at 54°C; 100%, 100% and 100% at 55°C; and 100%, 100% and 50% at 56°C for 10^4 , 10^3 and 10^2 parasite equivalents mL⁻¹, respectively (Figure 4g-i). Considering both endpoint fluorescence intensity and RT positive detection rates across all parasite inputs, 55°C was selected for subsequent *TrypOne* optimization.

TrypOne reaction conditions were subsequently optimized by evaluating buffer composition and reagent concentrations. Among the reaction buffer tested, Isothermal Amplification Buffer provided improved detection at 10^2 parasite equivalents mL⁻¹ compared with ThermoPol® Reaction Buffer and was therefore selected for all subsequent assays (Supplementary Figure 5). Optimization of individual reaction components identified 62.5 nM *AapCas12b*, 250 nM RIMEsgRNA, 2 mM MgSO₄, and 0.67 U µL⁻¹ Bst 2.0 DNA Polymerase as the optimal conditions. Compared with the alternative concentrations tested, these conditions improved detection at low parasite-equivalent inputs, particularly at 10^2 parasite equivalents mL⁻¹ (Supplementary Figure 6-9).

The optimized *TrypOne* assay was then evaluated for analytical performance. Using RT fluorescence readout, *TrypOne* detected 10^4 , 10^3 and 10^2 parasite equivalents mL⁻¹ in all technical replicates, establishing a LOD of 10^2 parasite equivalents mL⁻¹, corresponding to 67.65 fg of total gDNA, equivalent to 0.5 parasite equivalents per reaction (Figure 4k, l). At 10 parasite equivalents mL⁻¹, the RT positive detection rate was 37.5%. Visual fluorescence readout was then assessed using reaction-tube images and the empirically defined naked-eye (NE) threshold of 10×10^3 RFU (Figure 4n). NE fluorescence readout detected 10^4 and 10^3 parasite equivalents mL⁻¹ in all technical replicates, establishing an LOD of 10^3 parasite equivalents mL⁻¹, corresponding to 676.5 fg gDNA, equivalent to 5 parasite equivalents per reaction (Figure 4o). Detection was also observed at 10^2 and 10 parasite equivalents mL⁻¹, with positive detection rates of 75% and 37.5%, respectively. Both RT and NE positivity thresholds were reached within 60 min on average (Supplementary Figure 10b, c).

TrypOne analytical specificity was assessed using gDNA from *T. b. gambiense*, *T. b. rhodesiense*, *T. b. brucei*, *T. b. evansi*, *T. b. equiperdum*, *T. congolense*, *T. vivax* and *T. cruzi*. All tested *T. brucei* subspecies were positive by *TrypOne*, whereas non-target *Trypanosoma* species remained negative (Figure 4j, m, p). The breadth of strain detection was further evaluated using 51 *T. brucei* isolates derived from different countries, host species and years of isolation (Supplementary Table 2). All isolates were classified as positive by *TrypOne* (Supplementary Figure 10).

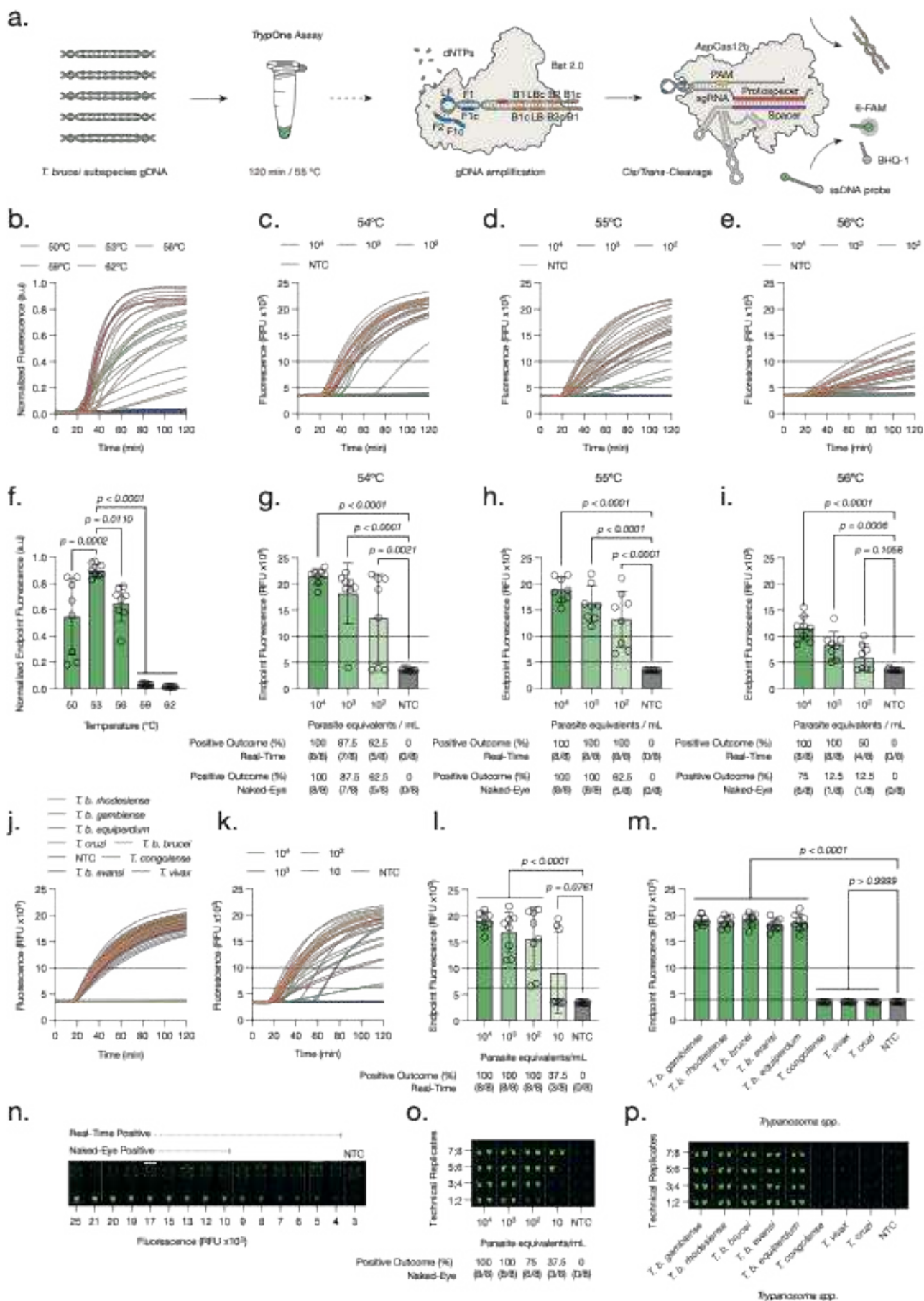


Figure 4. Optimization and analytical performance of the one-pot *TrypOne* assay. (a) Genomic DNA from *T. brucei* subspecies was used to evaluate the one-pot *TrypOne* assay, integrating RIME LAMP amplification and CRISPR-*AapCas12b* cleavage in a single closed reaction. Target recognition by the *AapCas12b*-sgRNA RNP complex induces the *cis*-cleavage of the RIME amplicon and *trans*-cleavage of a 6-FAM/BHQ-1 ssDNA probe. (b, f) Temperature optimization of *TrypOne* at 50-62°C, shown as normalized fluorescence kinetics (b) and endpoint fluorescence (f) from 8 technical replicates per condition. (c-e, g-i) Analytical sensitivity of *TrypOne* at 54°C, 55°C, and 56°C using serially diluted samples from 10⁴ to 10² parasite equivalents/mL and a NTC, shown as fluorescence kinetics (c-e) and endpoint fluorescence (g-i) from 8 technical replicates per condition. (j, m) Analytical specificity of *TrypOne* at 55°C against *T. b. gambiense*, *T. b. rhodesiense*, *T. b. brucei*, *T. b. evansi*, *T. b. equiperdum*, *T. congolense*, *T. vivax*, *T. cruzi*, and NTC, shown as fluorescence kinetics (j) and endpoint fluorescence (m) from 8 technical replicates. (k, l) Analytical sensitivity of optimized *TrypOne* at 55°C using serially diluted samples from 10⁴ to 10 parasite equivalents/mL and NTC, shown as fluorescence kinetics (k) and endpoint fluorescence (l) from 8 technical replicates per condition. (n) Empirical determination of the naked-eye (NE) fluorescence threshold. The NE positivity threshold was defined as 10×10³ RFU. Real-time (RT) positivity thresholds were defined as the NTC mean + 10 × SD. (o, p) Endpoint fluorescence tube images from the analytical sensitivity (o) and specificity (p) experiments after 120 min, visualized by NE readout. Positive detection rates, defined as the percentage of technical replicates classified as positive, are indicated for RT and NE readouts in (g-i, l, o) and were calculated using the corresponding RT and NE positivity thresholds. Individual replicates in (f-i, l, m) are shown with mean (dotted line) ± SD. Statistical analyses in (f-i, l, m) used one-way ANOVA with Dunnett's multiple-comparisons test. *P* values are indicated in the panels.

Previous studies reported inhibition of RIME-LAMP at DNA concentrations ≥200 ng [18]; therefore, the effect of host-derived background gDNA on *TrypOne* was evaluated. To this end, purified parasite gDNA was spiked with increasing amounts of non-infected mouse gDNA. Across 5, 50 and 500 parasite equivalents, increasing background gDNA from 100 to 400 ng produced a dose-dependent reduction in endpoint fluorescence, with inhibition becoming more pronounced at lower parasite-equivalent inputs (Supplementary Figure 11).

TrypOne performance in longitudinal mouse infection samples

C57BL/6 mice infected with *T. b. brucei* ANTAT 1.1 were monitored over 36 days post-infection (dpi) by microscopy, reference *Trypanosoma brucei* repeat (TBR) PCR, RIME LAMP and *TrypOne* with both RT and NE readouts. Untreated and melarsoprol-treated mice were analysed in parallel, with an uninfected control included in each group (Figure 5a-d; Supplementary Figures 13-24). In untreated mice, microscopy detected patent parasitaemia at 6 dpi and from 12 to 36 dpi, whereas reference TBR PCR classified infected mice as positive from 3 to 36 dpi (Figure 5e-h). In melarsoprol-treated mice, microscopy was positive at 6 dpi and became negative after treatment from 9 dpi onward, whereas TBR PCR was only positive from 3 to 6 dpi and negative thereafter (Figure 5e-h). Microscopy failed to detect early infections at 3 and 9 dpi, resulting in only moderate agreement with TBR PCR (Cohen's κ = 0.58; Table 1).

Table 1. Agreement of RIME LAMP, *TrypOne* RT and *TrypOne* NE with TBR PCR

Cohen's Kappa Coefficient	TBR PCR and Microscopy	TBR PCR and RIME LAMP	TBR PCR and <i>TrypOne</i> NE	TBR PCR and <i>TrypOne</i> RT
Value	0.58	0.82	0.98	1
Agreement	Moderate	Strong	Very strong	Perfect

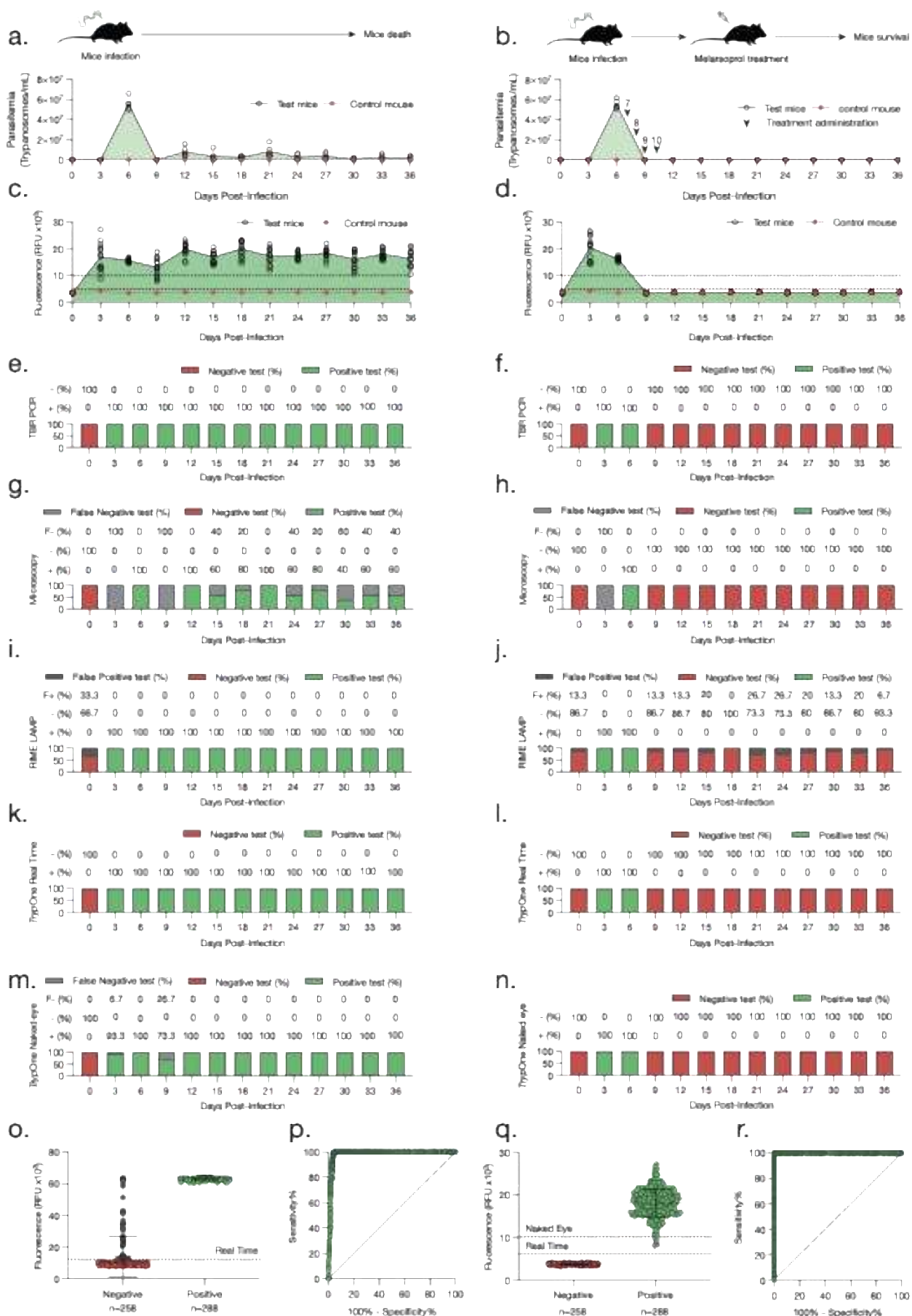


Figure 5. Evaluation of *TrypOne* for longitudinal monitoring of active and treated *T. brucei* infections *in vivo*. C57BL/6 mice (n = 5 per group) were infected with *T. b. brucei* AnTAt 1.1E and monitored for 36 days post-infection (dpi) by microscopy, reference TBR PCR, RIME LAMP, and *TrypOne* with RT and NE readouts. Untreated and melarsoprol-treated mice were analyzed in parallel, with an uninfected control included in each group. (a, b) Microscopy-based parasitemia profiles in untreated (a) and treated (b) mice. Individual infected mice and the uninfected control are shown over time. Black arrows indicate the time points at which the treatment was administered. (c, d) Corresponding *TrypOne* fluorescence profiles in untreated (c) and treated (d) mice. Dashed lines indicate the RT and NE positivity thresholds. (e-n) Longitudinal detection profiles for TBR PCR (e, f), microscopy (g, h), RIME LAMP (i, j), *TrypOne* RT readout (k, l), and *TrypOne* NE readout (m, n) in untreated and treated mice, respectively. Bars show the percentage of mouse samples classified as positive or negative at each time point according to method-specific detection criteria. FP and FN outcomes were then assigned relative to the reference TBR PCR. (o-r) Endpoint fluorescence distributions and ROC analyses for RIME LAMP (o, p) and *TrypOne* (q, r), respectively. Fluorescence values include all technical replicates from mouse samples and positive and negative controls, grouped as TBR PCR-negative or TBR PCR-positive according to the reference result. ROC curves were generated across alternative positivity thresholds. For RIME LAMP, the selected threshold retained PCR-discordant false positive signals as LAMP-positive outcomes. For *TrypOne*, the NE threshold was fixed at 10×10^3 RFU, whereas the RT threshold was selected to maximize separation between TBR PCR-negative and TBR PCR-positive samples.

Table 2. Intrinsic properties of RIME LAMP, *TrypOne* RT and *TrypOne* NE

Mice Infection Samples	RIME LAMP		<i>TrypOne</i> RT		<i>TrypOne</i> NE		Total
	Negative	Positive	Negative	Positive	Negative	Positive	
Positive confirmed by TBR PCR	0	282	0	282	5	277	282
Negative confirmed by TBR PCR	210	48	258	0	258	0	258
Total	210	330	258	282	263	277	540
Statistics	Value	95% Confidence Interval	Value	95% Confidence Interval	Value	95% Confidence Interval	Best Performance
Sensitivity	100 %	98.68 % – 100.0 %	100 %	98.68 % – 100.0 %	98.26 %	96.00 % – 99.26 %	<i>TrypOne</i> RT / LAMP
Specificity	80.62 %	75.36 % – 84.98 %	100 %	98.53 % – 100.0 %	100 %	98.53 % – 100.0 %	<i>TrypOne</i> RT
Likelihood Ratio (+)	5.16	4.00 – 6.66	ND	67.13 – ND	ND	65.30 – ND	<i>TrypOne</i> RT
Likelihood Ratio (-)	0	0.018 – 0	0	0.013 – 0	0.017	0.04 – 0.007	<i>TrypOne</i> RT
PPV	85.45 %	81.86 % – 88.61 %	100 %	98.70 % – 100 %	100 %	98.66 % – 100 %	<i>TrypOne</i> RT
NPV	100 %	98.17 % – 100 %	100 %	98.58 % – 100 %	98.1 %	95.66 % – 99.37 %	<i>TrypOne</i> RT
Accuracy	91.11 %	87.54 % – 92.82 %	100 %	98.61 % – 100 %	99.07 %	97.21 % – 99.61 %	<i>TrypOne</i> RT

RIME LAMP classified all TBR PCR-positive samples as positive in both untreated and treated mice, including early infection time points missed by microscopy (Figure 5i, j). However, RIME LAMP also generated PCR-discordant FP classifications among TBR PCR-negative samples in both groups. Overall, RIME LAMP showed 100% sensitivity but classified 48 of 258 TBR PCR-negative samples as positive, reducing specificity to 80.62% (Figure 5o, p; Table 2). This yielded 85.45% PPV, 100% negative predictive value (NPV), 91.11% accuracy, a positive likelihood ratio of 5.16

and a negative likelihood ratio of 0, with strong agreement with TBR PCR (Cohen's $\kappa = 0.82$; Table 1; Table 2).

TrypOne RT matched reference TBR PCR classifications across untreated and melarsoprol-treated mice (Figure 5k, l). All 288 TBR PCR-positive and all 258 TBR PCR-negative technical replicates were correctly classified, resulting in 100% sensitivity, 100% specificity, 100% PPV, 100% NPV and 100% accuracy (Figure 5q, r; Table 2). The positive likelihood ratio was not finite as no FP classifications were observed, while the negative likelihood ratio was 0. Agreement with TBR PCR was perfect (Cohen's $\kappa = 1$; Table 1; Table 2).

TrypOne NE eliminated the PCR-discordant FP classifications observed with RIME LAMP (Figure 5m, n). However, five TBR PCR-positive technical replicates were classified as negative, leading to 98.26% sensitivity, while all 258 TBR PCR-negative technical replicates remained negative (Table 2). This resulted in 100% specificity, 100% PPV, 98.10% NPV, 99.07% accuracy and very strong agreement with TBR PCR (Cohen's $\kappa = 0.98$; Table 1; Table 2). The positive likelihood ratio was not finite as no FP classifications were observed, and the negative likelihood ratio was 0.017. Endpoint fluorescence values and ROC analyses confirmed the improved discrimination achieved by *TrypOne* relative to RIME LAMP (Figure 5o-r).

Field-compatible implementation and optimization of *TrypOne*

Field-compatible implementation of *TrypOne* was evaluated by adapting both assay incubation and visual fluorescence readout to a portable, reusable and low-cost workflow. Assay incubation was performed using sodium acetate trihydrate (SAT)-based phase-change (PC) heat packs, and fluorescence was visualized using a battery-powered blue-light torch, convex lens and orange high-pass filter (Figure 6a, b; Supplementary Figure 25). The portable visual fluorescence readout reproduced the laboratory NE classification, with reactions above the NE positivity threshold remaining distinguishable from negative reactions (Figure 6c).

Across five independent SAT heat-pack runs, the incubation temperature ranged from 45.9°C to 53.5°C over 60 min (Figure 6d). Under these field-compatible temperature conditions, *AapCas12b*-RIMEsgRNA ribonucleoprotein (RNP) complexation was evaluated using either in situ RNP formation (i.e., during the reaction) or pre-complexed RNP (i.e., before addition to the reaction mix). *TrypOne* showed positive detection rates of 100% at 10^4 parasite equivalents mL^{-1} with both RNP formats by NE readout. At 10^3 parasite equivalents mL^{-1} , positive detection rates were 100% with in situ RNP formation and declined to 75% with pre-complexed RNP. At 10^2 parasite equivalents mL^{-1} , positive detection rates further decreased to 62.5% and 25%, respectively (Figure 6e, f). Overall, in situ RNP formation yielded the highest field-compatible *TrypOne* detection rates, establishing 10^3 parasite equivalents mL^{-1} as the NE LOD under 60 min SAT heat-pack incubation.

Under laboratory-optimized isothermal conditions at 55°C, *TrypOne* performance was not affected by the RNP complexation format, with comparable NE detection observed for in situ RNP formation and pre-complexed RNP (Supplementary Figure 26).

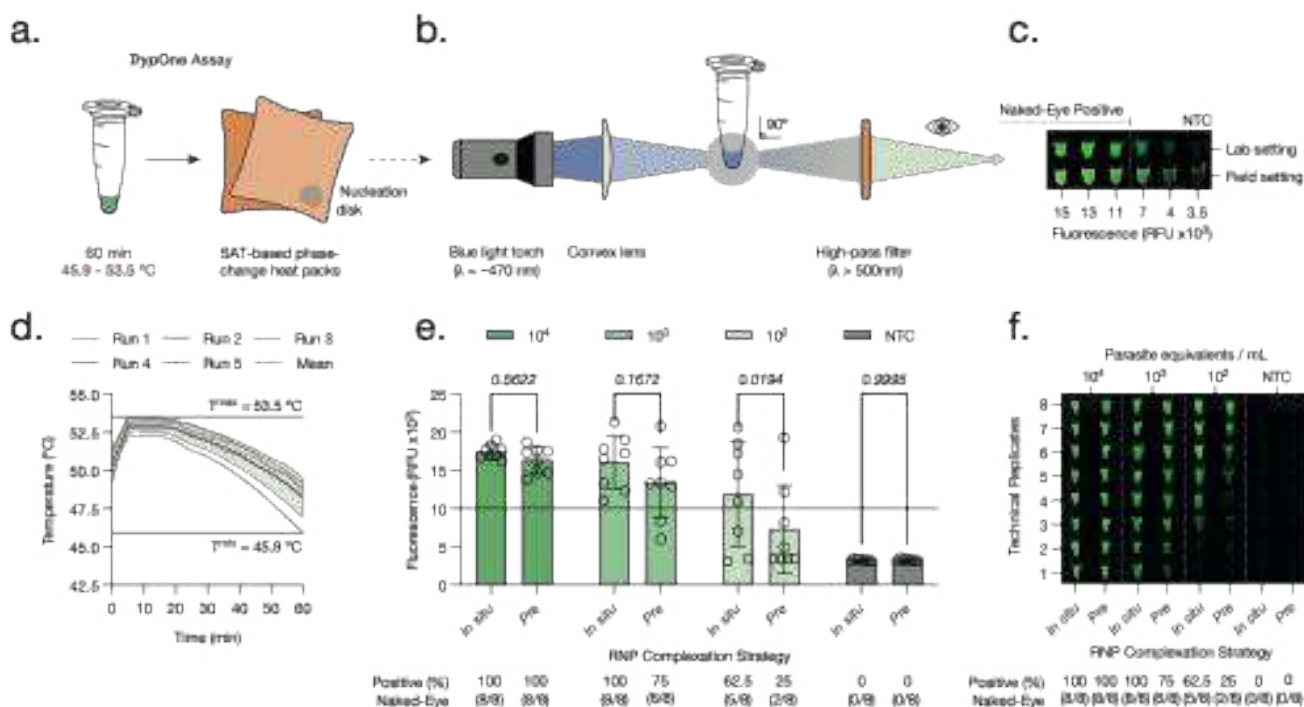


Figure 6. Field-compatible optimization and implementation of *TrypOne*. The field-compatible *TrypOne* workflow starts with assay incubation using sodium acetate trihydrate (SAT)-based phase-change heat packs for 60 min (a). Samples are then excited in a dark room using a battery-powered blue-light torch, approximately $\lambda = 470$ nm, focused through a convex lens (b). Fluorescence is visualized through a 3-mm orange-tinted poly(methyl methacrylate) (PMMA) high-pass filter with a 500-nm cutoff and captured using a standard mobile phone camera. (c) Endpoint fluorescence tube images comparing laboratory and field-compatible readouts. Laboratory images were acquired using a controlled blue-light imaging system, whereas field-compatible images were acquired with the portable setup in (b). The dashed line indicates the NE positivity cutoff. (d) Temperature profiles from five independent SAT heat-pack runs over 60 min; individual runs and the mean are shown, with a recorded range of 45.9-53.5°C. (e, f) Optimization of *AapCas12b* ribonucleoprotein complex (RNP) formation under field-compatible *TrypOne* conditions. In situ RNP complexation, occurring directly in the master mix during incubation, was compared with pre-complexed RNP, generated by incubating *AapCas12b* with sgRNA for 10 min before addition to the master mix. Serially diluted samples from 10^4 to 10^2 parasite equivalents/mL and a NTC were tested, shown as endpoint fluorescence (e) and endpoint tube images (f). Each condition included 8 technical replicates. Positive detection rates by NE readout are indicated below the panels and were calculated using the NE positivity threshold. Individual replicates in (e) are shown with mean $\pm$ SD. Statistical analysis in (e) was performed using two-way ANOVA with Tukey's multiple-comparisons test. *P* values are indicated.

Discussion

In this study, we developed and optimized *TrypOne*, a fluorescence-based one-pot CRISPR-Cas12b-LAMP assay for point-of-need detection of human and animal trypanosomosis. By coupling RIME-based isothermal amplification with *AapCas12b*-mediated sequence verification in a single closed tube, *TrypOne* enables pan-*T. brucei* DNA detection within 60 min while distinguishing target-specific amplification from non-specific products.

As the amplification module of *TrypOne*, RIME-LAMP consistently amplified RIME target sequences from DNA of all tested *T. brucei* subspecies and strains, supporting the suitability of RIME as a multicopy target for broad pan-*T. brucei* detection. RIME-LAMP detected as few as 10 parasite equivalents mL^{-1} , but detection at this concentration was stochastic, with positive rates below 100% across technical replicates. This behaviour has been reported previously and is consistent with Poisson target partitioning and variable low-copy LAMP initiation, mainly driven by the FIP/BIP

inner primers [22]. Nevertheless, the analytical performance of RIME-LAMP may be overestimated when diagnostic interpretation relies solely on endpoint amplification readouts, which cannot distinguish target-specific from non-specific products. Indeed, RIME-LAMP is prone to non-specific amplification, as previously reported [16, 22, 36] and observed in this study, likely due to primer-dimer formation and intrinsic Bst polymerase activities, including the lack of 3'-5' exonuclease proofreading [16, 37]. Although melting-curve analysis clearly distinguished non-specific from true RIME products in the laboratory setting, field-oriented fluorescence, colorimetric or turbidity readouts rely on total DNA accumulation and therefore cannot provide this discrimination [19, 21]. Accordingly, limiting the reaction time to 60 min is commonly used to suppress late non-specific amplification during prolonged LAMP incubation [18, 22]. In our assay, however, this temporal restriction was insufficient to fully prevent FP amplification.

AapCas12b was therefore incorporated as an orthogonal sequence-specific verification layer for RIME amplicons. In the two-pot format, Cas12b-mediated recognition reclassified non-specific RIME-LAMP products as negative without compromising analytical sensitivity or specificity. However, the requirement for tube opening and post-amplification transfer increases operational complexity and contamination risk, limiting the suitability of this format for decentralized testing [28]. Integrating RIME-LAMP amplification and CRISPR-Cas12b verification into a single closed-tube reaction overcame these constraints and established the one-pot *TrypOne* assay.

The performance of *TrypOne* depended on the kinetic balance between target amplification and Cas12b-mediated cleavage. In one-pot LAMP-CRISPR systems, polymerase-driven amplification and nuclease-mediated target recognition occur simultaneously and compete for related DNA substrates; premature target cleavage can therefore restrict amplicon accumulation and reduce downstream *trans*-cleavage signal generation [28]. Among the parameters evaluated, temperature was the principal determinant of this balance. At lower temperatures, close to the reported optimal temperature for CRISPR-Cas12b cleavage (50°C [38]), *TrypOne* showed delayed and flattened fluorescence kinetics with a reduced endpoint signal, an effect also reported by Li et al. [32]. This behaviour is consistent with the reduced LAMP efficiency under these conditions, which may limit amplicon accumulation and allow Cas12b-mediated cleavage to deplete the target DNA pool before sufficient active *trans*-cleaving sgRNA-Cas12b RNP complexes are formed. At higher temperatures, approaching the reported melting temperature of the Cas12b-sgRNA complex (58.3°C [39]), partial RNP dissociation would be expected, limiting active nuclease availability and thereby reducing endpoint fluorescence and flattening the kinetic profile. Consequently, optimal performance was confined to an intermediate temperature window, with 55°C providing the most effective balance between amplicon generation and Cas12b-mediated cleavage. Under these conditions, *TrypOne* retained the specificity conferred by CRISPR verification while matching the sensitivity of the two-pot assay, indicating that sensitivity loss in one-pot LAMP-CRISPR formats can be mitigated by optimizing the balance between amplification and cleavage kinetics rather than physically separating amplification and detection [28, 40].

After analytical optimization, *TrypOne* performance was evaluated in experimental mouse infections. The RT *TrypOne* readout detected active infection and treatment-associated parasite clearance with complete concordance with reference TBR PCR, the World Organisation for Animal Health (WOAH)-recommended molecular reference for *Trypanozoon* diagnosis [3]. CRISPR-mediated verification corrected the main limitation of RIME-LAMP by eliminating PCR-discordant FP while preserving detection of PCR-positive samples, thereby improving assay specificity without compromising analytical sensitivity. This increase in specificity is particularly relevant in low-prevalence HAT settings, where FP results can substantially reduce the PPV [13]. In the experimental

infection model, RIME-LAMP showed 100% sensitivity but only 80.62% specificity, corresponding to a PPV of 85.45%. When extrapolated to a low-prevalence HAT screening scenario (<0.1% in most foci [13]), this value would fall to 0.54% (Supplementary Table 3). In contrast, the CRISPR-verified *TrypOne* assay maintained a PPV of 100% under both experimental and low-prevalence scenarios, highlighting the diagnostic value of sequence-confirmed amplification for field interpretation. Although the *TrypOne* NE format showed occasional false negative (FN) outcomes in some technical replicates, these were attributable to the deliberately conservative visual threshold used for result classification and are therefore likely to underestimate, rather than reflect, the true visual sensitivity of the assay. Nevertheless, both *TrypOne* readout formats nevertheless exceeded the WHO target product profile for field-use diagnostics, which recommends >95% sensitivity and specificity, with specificity ideally exceeding 99% [10].

Evaluation of the optimized *TrypOne* assay in infected samples highlighted matrix-associated inhibition as a critical consideration for assay implementation. This limitation is well recognized for nucleic acid amplification tests and has also been reported for RIME-LAMP [18]. In *TrypOne*, inhibition was associated not only with high background DNA loads but also with residual carryover from the DNA extraction workflow, emphasizing the strong influence of matrix composition and extraction conditions on assay performance. These findings underscore the need to optimize sample preparation and input conditions before field use.

Together with sample-processing optimization, field deployment of *TrypOne* also requires incubation and readout formats that are independent of laboratory instrumentation. To address this, *TrypOne* was adapted to reusable SAT-based PC heat packs, which provide electricity-free incubation through nucleation-induced exothermic crystallization and maintain temperatures within the 53.5-45.9°C range for 1 h [41]. Fluorescence detection was coupled to a simple low-cost optical readout based on blue-light excitation, lens-directed illumination and high-pass filtering, enabling direct NE interpretation or mobile-phone documentation.

Implementation of *TrypOne* using SAT heat packs required operation under a thermal profile distinct from the laboratory-optimized 55°C condition. In line with the temperature-dependent amplification-cleavage balance described above, analytical sensitivity decreased under these conditions. As the heat-pack temperature could not be precisely controlled, optimization focused on modulating the timing of Cas12b activation. Consistent with the findings of Lesinski et al. [42], in situ RNP complexation restored the *TrypOne* LOD in the heat-pack format, supporting delayed Cas12b activation as a mechanism to preserve early LAMP amplification before extensive *cis*-cleavage of newly generated amplicons while maintaining sufficient *trans*-cleavage-dependent fluorescence generation. Together, these findings further support temporal control of nuclease activation as a central strategy for preserving sensitivity in field-compatible one-pot LAMP-CRISPR diagnostics.

Beyond analytical performance, field implementation will also depend on cost, reagent stability and sample-processing requirements. The reusable hardware cost for incubation and readout was limited to approximately €20. As such, this field-deployable *TrypOne* format provides a practical and portable alternative to dedicated fluorescence readers for PON testing, which can approach €1000. However, reagent costs remain a major constraint, with the optimized field-adapted *TrypOne* NE format costing €5.406 per 50 µL reaction compared with €1.625 for the lower-volume *TrypOne* RT format. This remains above the sub-US\$1 benchmark generally considered desirable for HAT/AT field-screening tools [43]. At this stage, *TrypOne* remains a proof-of-concept assay and relies on commercially sourced enzymes and sgRNAs. These costs could be reduced through scale-up and local manufacture of key reagents, particularly recombinant LAMP and CRISPR enzymes [34]. Full

field deployment will also require dry or lyophilized reagent formulations to overcome cold-chain dependence, PON compatible DNA extraction, and diagnostic validation in representative field samples under intended-use conditions, in accordance with WOA principles for diagnostic test validation [44, 45].

In conclusion, *TrypOne* provides a one-pot LAMP-CRISPR-Cas12b assay for pan-*T. brucei* DNA detection that couples RIME amplification with sequence-specific molecular verification. This design preserves RIME-LAMP sensitivity while improving diagnostic specificity, enabling direct confirmation of active infection and treatment-associated parasite clearance. Its translation into a field-compatible workflow will support PON testing across HAT and AT contexts and strengthen the potential for coordinated surveillance and control within a One Health framework.

Methods

Nucleic acid preparations

In this study, *T. brucei* refers to the *T. brucei* subspecies, including *T. b. gambiense*, *T. b. rhodesiense*, *T. b. brucei*, *T. b. evansi* and *T. b. equiperdum*, reflecting their close phylogenetic relationship and the unresolved taxonomic status of dyskinetoplastic animal trypanosomes [46-48].

Total gDNA from the *Trypanosoma* species (Table 3) and *T. brucei* strains (Supplementary table 2) used in this study was extracted and purified from infected mouse whole blood using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. Purified gDNA was eluted in UltraPure DNase/RNase-Free Distilled Water (Invitrogen, United States), diluted to 1 ng μL^{-1} and stored at -20°C until further use. The concentration and quality of the purified total gDNA were assessed by gel electrophoresis and spectrophotometric analysis using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, United States).

For generation of RIME amplicon (a)DNA, gDNA from *T. brucei* strains (Table 3) was amplified using the external RIME-LAMP primers F3 and B3 (Table 4). Each 50 μL PCR reaction contained 10 μL of extracted gDNA and 40 μL of PCR master mix composed of 1X Green GoTaq Reaction Buffer (Promega, United States), 0.4 mM dNTPs (Thermo Fisher Scientific, United States), 0.8 μM F3 primer, 0.8 μM B3 primer (Integrated DNA Technologies, United States) and 0.08 U μL^{-1} GoTaq G2 DNA Polymerase (Promega, United States). Amplifications were performed in a Biometra Trio-block thermocycler (Analytik Jena, Germany) with an initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 40 s, 55°C for 40 s and 72°C for 40 s, and a final extension at 72°C for 5 min. PCR products were resolved by electrophoresis on a 2% ethidium bromide-stained agarose gel at 100 V for 50 min. The 223 bp FIP-BIP RIME fragment, corresponding to the shortest predicted RIME-LAMP amplicon (Figure 1a), was gel-excised under UV visualization and purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. Purified amplicon DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, United States) and diluted to 30 ng μL^{-1} in UltraPure DNase/RNase-Free Distilled Water before storage at -20°C .

For analytical sensitivity experiments, total gDNA from *T. b. brucei* ANTAT 1.1-EATRO pleomorphic was extracted from infected mouse blood containing 10^8 parasites mL^{-1} using the DNeasy Blood & Tissue Kit and eluted in a volume equal to the sample input. Ten-fold serial dilutions were prepared in UltraPure DNase/RNase-Free Distilled Water to generate samples corresponding to 10^8 to 10 parasite equivalents mL^{-1} . The resulting DNA concentrations were 13.56

562 ng μL^{-1} , 1.356 ng μL^{-1} , 135.6 pg μL^{-1} , 13.56 pg μL^{-1} , 1.356 pg μL^{-1} , 135.6 fg μL^{-1} , 13.56 fg μL^{-1} and
 563 1.356 fg μL^{-1} for 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 and 10 parasite equivalents mL^{-1} , respectively.

564 **Table 3.** *Trypanosoma* parasites employed in this study

Strain	Host	Country
<i>T. b. gambiense</i> ANTAT 9.1	Human	Cameroon
<i>T. b. rhodesiense</i> STIB 850	Human	Uganda
<i>T. b. brucei</i> ANTAT 1.8	Bushbuck	Uganda
<i>T. b. equiperdum</i> BOTAT 1.1	Horse	Cameroon
<i>T. b. evansi</i> ROTAT 1.2	Water Buffalo	Indonesia
<i>T. congolense</i> TRT 17	Cattle	Zambia
<i>T. vivax</i> ILRAD 700	Cattle	Nigeria
<i>T. cruzi</i> TULAHUEN	Arthropod	Chile

565

566 **Table 4.** Primers, probes and sgRNAs employed in this study.

Assay type	Primer name	Sequence (5'-3')	Reference
PCR	TBR1	GAATATTAAACAATGCGCAG	[49]
PCR	TBR2	CCATTTATTAGCTTTGTTGC	[49]
PCR	F3	CTGTCCGGTGATGTGGAAC	[18]
PCR	B3	CGTGCCTTCGTGAGAGTTTC	[18]
LAMP	F3	CTGTCCGGTGATGTGGAAC	[18]
LAMP	B3	CGTGCCTTCGTGAGAGTTTC	[18]
LAMP	FIP (F1c + F2)	GGAATACAGCAGATGGGGCGAGGCCAATTGGCATCTTTGGGA	[18]
LAMP	BIP (B1c + B2)	AAGGGAGACTCTGCCACAGTCGTCAGCCATCACCGTAGAGC	[18]
LAMP	LF	GCCTCCCACCCTGGACTC	[18]
LAMP	LB	AGACCGATAGCATCTCAG	[18]
TrypOne	RIMEsgRNA	GUCUAGAGGACAGAAUUUUUCAACGGGUGUGCCAAUGGCCAC UUUCCAGGUGGCAAAGCCCGUUGAGCUUCUCAAUCUGAGAA GUGGCACUAGAGGGUGUGUUAGGAUGA	This study
TrypOne	FAM-Q Probe	[6-FAM]TTTTT[BHQ-1]	This study

567

568 For mouse sample dilution experiments, gDNA was extracted from *T. b. brucei* ANTAT 1.1-EATRO
 569 pleomorphic parasite-infected mouse blood containing 4×10^6 parasites mL^{-1} using the DNeasy
 570 Blood & Tissue Kit. The extracted DNA concentration was 48.82 ng μL^{-1} . Serial dilutions were
 571 prepared in UltraPure DNase/RNase-Free Distilled Water at 1/2, 1/5, 1/10, 1/20, 1/50 and 1/100,
 572 corresponding to 24.41, 9.76, 4.89, 2.44, 0.97 and 0.49 ng μL^{-1} , respectively.

573 For subsequent background DNA inhibition experiments, parasite gDNA and non-infected mouse
 574 background gDNA were prepared independently. Parasite gDNA was obtained from purified *T. b.*
 575 *brucei* ANTAT 1.1-EATRO pleomorphic parasites. Briefly, heparinized blood was collected at peak
 576 parasitaemia from a C57BL/6 female mouse inoculated intraperitoneally with 5,000 parasites in 200
 577 μL HBSS (140 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 0.4 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$,
 578 0.3 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.4 mM KH_2PO_4 , 6 mM D-glucose and 4 mM sodium bicarbonate; Thermo

579 Fisher Scientific, United States). Blood was centrifuged at 805g for 7 min, and trypanosomes present
580 in the buffy coat fraction were purified by anion-exchange chromatography using a 10 mL DEAE
581 column pre-equilibrated with PSG buffer (36.5 mM NaCl, 3.6 mM NaH₂PO₄, 59.5 mM Na₂HPO₄,
582 88.8 mM D-glucose, pH 8.0). Eluted parasites were collected, centrifuged at 805g for 7 min, washed
583 twice with PSG buffer and resuspended in PSG buffer. Parasite concentration was determined by
584 light microscopy using a VisiScope IT415 PH microscope (VWR International, United States). Total
585 gDNA from purified parasites was extracted using the DNeasy Blood & Tissue Kit. Parasite gDNA
586 yield was normalized to the microscopically determined parasite count to generate parasite-
587 equivalent DNA stocks, which were diluted in UltraPure DNase/RNase-Free Distilled Water to 5, 50
588 and 500 parasite equivalents per *TrypOne* reaction, corresponding to 0.75 pg, 7.5 pg and 75 pg of
589 parasite gDNA, respectively.

590 In parallel, background gDNA was extracted from non-infected mouse blood using the DNeasy
591 Blood & Tissue Kit and diluted in UltraPure DNase/RNase-Free Distilled Water to generate stocks
592 providing 50, 100, 200 or 400 ng total mouse gDNA per *TrypOne* reaction.

593 RIME-LAMP assay

594 The RIME-LAMP assay originally described by Njiru et al.[18] was performed as follows. Reactions
595 were performed in a final volume of 25 μ L containing 5 μ L input gDNA, 200 μ M dNTPs, 0.2 μ M
596 each F3 and B3 primer, 2 μ M each FIP and BIP primer, 0.8 μ M each LF and LB primer (Table 4), 8
597 U Bst 2.0 DNA Polymerase (New England Biolabs, United States), 1X ThermoPol® Reaction Buffer
598 (New England Biolabs, United States) and 3.34 μ M SYTO 9 Green fluorescent nucleic acid stain
599 (Thermo Fisher Scientific, United States). Reactions were assembled in Hard-Shell thin-wall 96-well
600 PCR plates (Bio-Rad, United States) and run 62°C for 60-120 min on a CFX Connect RT PCR
601 Detection System (Bio-Rad, United States). Fluorescence was acquired every 30 s in the FAM
602 channel, with excitation and emission wavelengths of 493 nm and 517 nm, respectively. For
603 temperature optimization, reactions were incubated isothermally at 54-62°C for 60 min. Melt-curve
604 analysis (75-95°C) was performed after amplification to distinguish specific RIME-LAMP products
605 from non-specific products. Analytical sensitivity was determined using ten-fold serial dilutions of *T.*
606 *b. brucei* ANTAT 1.1-EATRO pleomorphic gDNA corresponding to 10⁴, 10³, 10² and 10 parasite
607 equivalents mL⁻¹, together with a NTC. Analytical specificity was assessed using gDNA from *T. b.*
608 *gambiense*, *T. b. rhodesiense*, *T. b. brucei*, *T. b. evansi*, *T. b. equiperdum*, *T. congolense*, *T. vivax* and
609 *T. cruzi* (Table 3), each diluted to 1 ng μ L⁻¹.

610 *Tb*CRISPR-*AapCas12b* cis-cleavage reactions

611 Recombinant *AapCas12b* (SignalChem Diagnostics, Canada) was used for *Tb*CRISPR-*AapCas12b*
612 cleavage assays. Candidate RIME-targeting sgRNAs were designed using the *Alicyclobacillus*
613 *acidoterrestris* Cas12b (*AacCas12b*) sgRNA scaffold, which has previously been shown to support
614 robust and specific *AapCas12b* activity and was therefore selected in the absence of an established
615 native, CRISPR-array-derived *AapCas12b* guide scaffold [23]. All sgRNAs were synthesized by
616 Integrated DNA Technologies (United States) (Supplementary table 1).

617 *Tb*CRISPR-*AapCas12b* cis-cleavage reactions were performed as previously described by Álvarez-
618 Rodríguez et al. [38]. Each 15 μ L reaction contained 250 nM *AapCas12b*, 500 nM RIMEsgRNA, 5
619 μ L purified RIME aDNA from *T. b. brucei* ANTAT 1.1-EATRO pleomorphic and 1X ThermoPol®
620 Reaction Buffer (New England Biolabs, United States). The purified RIME aDNA was prepared as
621 described in the Nucleic acid preparations section. Reactions were incubated for 1 h at 50°C in a

622 preset Biometra Trio-block thermocycler (Analytik Jena, Germany). Reactions were stopped by
623 adding 2.75 μL stop solution containing 16.9 mM EDTA, 84.5 $\mu\text{g mL}^{-1}$ RNase A and 67.6 mAU mL^{-1}
624 proteinase K, followed by incubation at 56°C for 10 min. Reaction products were resolved by
625 electrophoresis on a 1% ethidium bromide-stained agarose gel in TBE buffer (90 mM Tris, 90 mM
626 borate, 2.5 mM EDTA) at 100 V for 30 min. *Cis*-cleavage efficiency was quantified from uncleaved
627 and cleaved DNA bands by densitometric analysis using ImageJ.

628 *Tb*CRISPR-*Aap*Cas12b *trans*-cleavage reactions

629 *Tb*CRISPR-*Aap*Cas12b *trans*-cleavage reactions were performed in a 15 μL final volume containing
630 62.5 nM *Aap*Cas12b, 250 nM RIME-targeting sgRNAs, 250 nM 6-FAM/5T/BHQ-1 ssDNA reporter
631 probe (Integrated DNA Technologies, United States) (Table 4), 1X ThermoPol® Reaction Buffer
632 (New England Biolabs, United States) and DNA input. *Aap*Cas12b and RIMEsgRNA were mixed
633 and incubated for 10 min at room temperature before addition to the master mix to allow formation
634 of RNP complexes before addition to the reaction master mix. Reactions were assembled in Hard-
635 Shell thin-wall 96-well PCR plates (Bio-Rad, United States) and run at 50°C for 60-120 min on a
636 CFX Connect RT PCR Detection System (Bio-Rad, United States). Fluorescence was acquired every
637 30 s in the FAM channel, with excitation and emission wavelengths of 493 nm and 517 nm,
638 respectively.

639 For direct *Tb*CRISPR-*Aap*Cas12b *trans*-cleavage assays, 5 μL purified RIME aDNA, prepared as
640 described in the Nucleic acid preparations section, was used as input. Candidate sgRNA1-3 were
641 evaluated using *T. b. brucei* ANTAT 1.1-EATRO pleomorphic RIME aDNA. RIMEsgRNA
642 (sgRNA_2) (Table 4) was further tested using RIME aDNA from *T. b. evansi*, *T. b. gambiense*, *T. b.*
643 *rhodesiense*, *T. b. brucei* and *T. b. equiperdum* (Table 3).

644 For the two-pot *Tb*LAMP-CRISPR assays, 1.5 μL unpurified RIME-LAMP product was transferred
645 directly to the *Tb*CRISPR-*Aap*Cas12b cleavage reaction. Analytical sensitivity was assessed using
646 RIME LAMP products generated from *T. b. brucei* ANTAT 1.1-EATRO pleomorphic gDNA
647 dilutions from 10^4 to 10 parasite equivalents mL^{-1} and a NTC. Analytical specificity was assessed
648 using RIME LAMP products generated from gDNA of *T. b. gambiense*, *T. b. rhodesiense*, *T. b.*
649 *brucei*, *T. b. evansi*, *T. b. equiperdum*, *T. congolense*, *T. vivax* and *T. cruzi* (Table 3), with NTC
650 included.

651 *Tryp*One optimization assays

652 *Tryp*One was established as a one-pot reaction combining RIME-LAMP amplification and
653 *Tb*CRISPR-*Aap*Cas12b cleavage in a single closed tube. For the initial *Tryp*One optimization
654 reaction, assays were performed in a final volume of 15 μL containing 5 μL input gDNA from *T. b.*
655 *brucei* ANTAT 1.1-EATRO pleomorphic at 10^4 parasite equivalents mL^{-1} or NTC, 200 μM dNTPs,
656 0.2 μM each F3 and B3 primer, 2 μM each FIP and BIP primer, 0.8 μM each LF and LB primer, 0.32
657 U μL^{-1} Bst 2.0 DNA Polymerase (New England Biolabs, United States), 1X ThermoPol® Reaction
658 Buffer (New England Biolabs, United States), 62.5 nM *Aap*Cas12b (SignalChem Diagnostics,
659 Canada), 250 nM RIMEsgRNA (Integrated DNA Technologies, United States) and 250 nM 6-
660 FAM/BHQ-1 ssDNA reporter probe (Integrated DNA Technologies, United States). *Aap*Cas12b and
661 RIMEsgRNA were mixed and incubated for 10 min at room temperature before addition to the
662 master mix to allow formation of RNP complexes. Reactions were assembled in Hard-Shell thin-wall
663 96-well PCR plates (Bio-Rad, United States) and run on a CFX Connect RT PCR Detection System
664 (Bio-Rad, United States). Fluorescence was acquired every 30 s in the FAM channel, with excitation

665 and emission wavelengths of 493 nm and 517 nm, respectively. Temperature optimization was
666 performed across 50-62°C for 120 min using gDNA from *T. b. brucei* ANTAT 1.1-EATRO
667 pleomorphic inputs corresponding to 10⁴, 10³ or 10² parasite equivalents mL⁻¹ and NTC.

668 Subsequent optimization assays were performed by varying a single reaction parameter at a time
669 while maintaining all other components unchanged, at 55°C for 120 min. Buffer optimization
670 compared 1X ThermoPol® Reaction Buffer and 1X Isothermal Amplification Buffer (New England
671 Biolabs, United States) using 10⁴ and 10³ parasite equivalents mL⁻¹ and NTC. *AapCas12b* was
672 evaluated at 125, 62.5 and 31.25 nM; RIMEsgRNA at 250, 125 and 62.5 nM; MgSO₄ at 2, 4 and 8
673 mM; and Bst 2.0 DNA Polymerase at 0.67, 0.32 and 0.17 U µL⁻¹.

674 TrypOne validation assays

675 *TrypOne* validation assays were performed in a final volume of 15 µL containing 5 µL input gDNA,
676 200 µM dNTPs, 0.2 µM each F3 and B3 primer, 2 µM each FIP and BIP primer, 0.8 µM each LF and
677 LB primer, 0.67 U µL⁻¹ Bst 2.0 DNA Polymerase (New England Biolabs, United States), 1X
678 ThermoPol® Reaction Buffer (New England Biolabs, United States), 62.5 nM *AapCas12b*
679 (SignalChem Diagnostics, Canada), 250 nM RIMEsgRNA (Integrated DNA Technologies, United
680 States) and 250 nM 6-FAM/BHQ-1 ssDNA reporter probe (Integrated DNA Technologies, United
681 States). *AapCas12b* and RIMEsgRNA were mixed and incubated for 10 min at room temperature
682 before addition to the master mix to allow formation of RNP complexes. Reactions were assembled
683 in Hard-Shell thin-wall 96-well PCR plates (Bio-Rad, United States) and run at 55°C for 120 min on
684 a CFX Connect RT PCR Detection System (Bio-Rad, United States). Fluorescence was acquired
685 every 30 s in the FAM channel, with excitation and emission wavelengths of 493 nm and 517 nm,
686 respectively. Endpoint fluorescence tube images were acquired using a Gel Doc™ EZ Gel
687 Documentation System (Bio-Rad Laboratories, United States) with a 10 s exposure time.

688 Analytical sensitivity was assessed using *T. b. brucei* ANTAT 1.1-EATRO pleomorphic
689 corresponding to 10⁴, 10³, 10² and 10 parasite equivalents mL⁻¹, together with a NTC. Analytical
690 specificity was evaluated using gDNA from the *Trypanosoma* species listed in table 3 and from
691 additional *T. brucei* strains listed in supplementary table 2. Background DNA inhibition assays were
692 performed using different input ratios of *T. b. brucei* ANTAT 1.1-EATRO pleomorphic parasite
693 gDNA and non-infected mouse background gDNA, prepared as described in the Nucleic acid
694 preparations section.

695 Experimental mouse infections

696 Eight-weeks-old C57BL/6J mice (Janvier, France) were randomly divided into two experimental
697 groups (n = 6 per group). In each group, five mice were inoculated intraperitoneally with 5,000 *T. b.*
698 *brucei* ANTAT 1.1-EATRO pleomorphic parasites (originally obtained from N. Van Meirvenne
699 (Institute for Tropical Medicine, Antwerpen, Belgium)) in 200 µL HBSS buffer (140 mM NaCl, 5
700 mM KCl, 1 mM CaCl₂, 0.4 mM MgSO₄·7H₂O, 0.5 mM MgCl₂·6H₂O, 0.3 mM Na₂HPO₄·2H₂O,
701 0.4 mM KH₂PO₄, 6 mM D-glucose and 4 mM sodium bicarbonate; Thermo Fisher Scientific, United
702 States), whereas one mouse remained uninfected and served as a negative control. Bloodstream
703 trypanosomes used for mouse inoculation were stored at -80°C as blood stabilates containing 50%
704 Alsever's solution (Sigma-Aldrich, United States) and 10% glycerol (final v/v).

705 Mice were weighed and tail-bled every 3 days from 0 to 36 dpi. In Group 2, melarsoprol treatment,
706 provided by the World Health Organization (WHO)/Sanofi, was administered intraperitoneally once

707 daily from 7 to 10 DPI at 10-12 mg kg⁻¹, as previously described [50]. Tail-vein blood samples of
708 100 µL were collected and immediately mixed with heparinized saline (Sigma-Aldrich, United
709 States) at a 10:1 blood-to-heparin ratio to prevent coagulation, resulting in a final heparin
710 concentration of 10 units mL⁻¹.

711 Parasitaemia was monitored by light microscopy at each sampling point. Briefly, 2.5 µL of blood was
712 diluted 1:200 in HBSS buffer and examined using a VisiScope IT415 PH microscope (VWR
713 International, United States). The remaining blood was processed for total nucleic acid extraction
714 using the DNeasy Blood & Tissue Kit (Qiagen, Germany), according to the manufacturer's
715 instructions and with the modifications described in the Nucleic acid preparations section. DNA
716 concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher
717 Scientific, United States). Samples were stored at -80°C until further analysis. Before longitudinal
718 testing, a dilution series prepared from a representative infected mouse blood gDNA extract, as
719 described in the Nucleic acid preparations section, was analyzed by *TrypOne* to determine the
720 dilution required to reduce extraction-associated inhibition while maximizing fluorescence signal.
721 Based on this analysis, all gDNA samples from the untreated and treated mouse groups were diluted
722 1/50 before testing (Supplementary figure 12).

723 Longitudinal mouse blood gDNA samples were analyzed by reference TBR PCR, RIME-LAMP and
724 *TrypOne* to monitor active and melarsoprol-treated *T. b. brucei* infections in vivo. RIME-LAMP and
725 *TrypOne* were performed as described in the RIME-LAMP assay and *TrypOne* validation assays
726 sections, respectively. TBR PCR was performed as follows: each 50 µL reaction contained 10 µL
727 DNA sample or NTC, 1X Green GoTaq Reaction Buffer containing 1.5 mM MgCl₂ (Promega,
728 United States), 0.2 mM dNTPs, 1 µM TBR1 primer, 1 µM TBR2 primer (Table 4), 0.05 U µL⁻¹
729 GoTaq G2 DNA Polymerase and nuclease-free water. Amplifications were performed in a Biometra
730 Trio-block thermocycler (Analytik Jena, Germany) with an initial denaturation at 94°C for 1 min,
731 followed by 30 cycles of 94°C for 30 s, 60°C for 2 min and 72°C for 30 s, and a final extension at
732 72°C for 2 min. PCR products were resolved by electrophoresis on a 2% ethidium bromide-stained
733 agarose gel at 110 V for 40 min.

734 Field-compatible *TrypOne* assays

735 Field-compatible *TrypOne* assays were developed to enable incubation and fluorescence visualization
736 without laboratory instrumentation. Assays were performed in a final volume of 50 µL containing
737 16.5 µL input gDNA from *T. b. brucei* ANTAT 1.1-EATRO pleomorphic corresponding to 10⁴, 10³
738 or 10² parasite equivalents mL⁻¹ or NTC, 200 µM dNTPs, 0.2 µM each F3 and B3 primer, 2 µM each
739 FIP and BIP primer, 0.8 µM each LF and LB primer, 0.67 U µL⁻¹ Bst 2.0 DNA Polymerase (New
740 England Biolabs, United States), 1X ThermoPol® Reaction Buffer (New England Biolabs, United
741 States), 62.5 nM *AapCas12b* (SignalChem Diagnostics, Canada), 250 nM RIMEsgRNA (Integrated
742 DNA Technologies, United States) and 250 nM 6-FAM/BHQ-1 ssDNA reporter probe (Integrated
743 DNA Technologies, United States). For pre-complexed RNP conditions, *AapCas12b* and
744 RIMEsgRNA were mixed and incubated for 10 min before addition to the master mix. For in situ
745 complexation conditions, *AapCas12b* and RIMEsgRNA were added directly to the reaction mixture
746 immediately before assay incubation.

747 Reactions were assembled in Hard-Shell thin-wall 96-well PCR plates (Bio-Rad, United States) and
748 incubated for 60 min either under laboratory isothermal conditions at 55°C or under field-compatible
749 conditions using SAT-based PC heat packs. These heat packs operate through exothermic
750 crystallization of metastable supercooled SAT following nucleation-induced liquid-to-solid phase

transition, generating sustained temperatures within the 45.9-53.5°C range [41]. For controlled evaluation of field-compatible conditions, a thermal-gradient incubation program was used to approximate the mean SAT heat-pack temperature profile (Figure 6d). The program started at 50.4°C and increased to 52.9°C during the first 5 min. Temperature was then maintained at 53.0°C from 5 to 15 min, followed by a gradual decrease from 52.9°C to 51.3°C between 15 and 40 min. Temperature then decreased from 50.2°C to 49.1°C between 40 and 55 min, and from 48.8°C to 48.2°C during the final 5 min. Laboratory and field-compatible reactions were monitored on a CFX Connect RT PCR Detection System (Bio-Rad, United States). Fluorescence was acquired every 5 min in the FAM channel, with excitation and emission wavelengths of 493 nm and 517 nm, respectively, and endpoint fluorescence values were used for analysis.

For field-compatible fluorescence visualization, endpoint reactions were illuminated in a dark room using a battery-powered blue-light torch (Weslite, China) emitting approximately 470 nm light through an integrated convex lens. Fluorescence was visualized through a 3-mm orange-tinted poly(methyl methacrylate) high-pass filter (PlaquePlastique, Belgium) with a 500-nm cutoff and captured using an iPhone 15 Pro camera without exposure-time adjustment. For laboratory fluorescence visualization, endpoint tube images were acquired using a Gel Doc™ EZ Gel Documentation System (Bio-Rad Laboratories, United States) with a 10 s exposure time.

Data analysis and statistics

RIME monomer sequences from *T. b. evansi* EF567425.1, *T. b. rhodesiense* EF567424.1 and *T. b. brucei* EF567426.1 were aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation; EMBL-EBI). Sequence identity was calculated across the full 511 bp RIME monomer and the selected *AapCas12b* target region. RIME-LAMP primer-binding sites and positive-strand 5'-TTN-3' *AapCas12b* PAMs were mapped onto the alignment to guide RIMEsgRNA selection. Fluorescence kinetics, endpoint fluorescence and melt-curve data were analyzed using GraphPad Prism 10 (GraphPad Software, United States). Unless otherwise indicated, endpoint fluorescence data are shown as individual technical replicates with mean $\pm$ standard deviation (SD); replicate numbers are indicated in the corresponding figure legends. Positivity thresholds were defined as the TN or NTC mean fluorescence plus $10 \times$ SD, except where specified otherwise. The *TrypOne* NE threshold was fixed at 10×10^3 RFU. Positive detection rates were calculated as the percentage of positive technical replicates among the total replicates tested. RIME-LAMP melt-curve profiles were used to distinguish target-specific amplification from non-specific late amplification.

For RIME-LAMP temperature optimization, normalized fluorescence plots were generated by min-max normalization. For each fluorescence curve, the minimum RFU value was subtracted from all RFU values, and each baseline-corrected value was then divided by the maximum baseline-corrected RFU value of the same curve, scaling fluorescence values from 0 to 1. Ct values were extracted using a fixed fluorescence threshold of 0.2, and relative amplification rates were calculated as the Ct at 62°C divided by the Ct at each tested temperature, multiplied by 100, with 62°C set as 100%. For *TrypOne* temperature optimization, normalized fluorescence plots were generated using a shared normalization across all curves. The minimum RFU value across the dataset was subtracted from all RFU values, and each baseline-corrected value was then divided by the highest baseline-corrected RFU value among all technical replicates, scaling fluorescence values from 0 to 1.

AapCas12b cis-cleavage gels were analyzed using ImageJ 1.53t. Cleavage efficiency was calculated by relative densitometry of the uncleaved RIME amplicon band in paired reactions with and without

sgRNA, using the sgRNA-dependent reduction in uncleaved band intensity relative to the no-sgRNA control. *AapCas12b* trans-cleavage fluorescence values were background-subtracted before analysis.

For longitudinal mouse infection experiments, microscopy, reference TBR PCR, RIME-LAMP, *TrypOne* RT and *TrypOne* NE outcomes were classified using assay-specific criteria. FP and FN outcomes were assigned relative to the corresponding reference TBR PCR result. ROC analyses were performed in GraphPad Prism 10 using endpoint fluorescence values from all technical replicates of mouse samples and controls, grouped as TBR PCR-negative (n = 258) or TBR PCR-positive (n = 288). For RIME-LAMP, the ROC-selected threshold was applied directly to endpoint fluorescence values; therefore, samples above this threshold were classified as RIME-LAMP-positive even when discordant with the reference TBR PCR result. For *TrypOne*, the NE readout was classified using the fixed 10×10^3 RFU threshold. The RT readout was classified using the ROC-selected fluorescence threshold, with samples above this threshold considered *TrypOne*-positive and samples below it considered *TrypOne*-negative relative to reference TBR PCR. Diagnostic performance was calculated relative to reference TBR PCR. Sensitivity was calculated as $TP/(TP + FN)$, specificity as $TN/(TN + FP)$, PPV as $[sensitivity \times prevalence] / ([sensitivity \times prevalence] + [(1 - specificity) \times (1 - prevalence)])$, NPV as $[specificity \times (1 - prevalence)] / ([(1 - sensitivity) \times prevalence] + [specificity \times (1 - prevalence)])$, positive likelihood ratio as $sensitivity/(1 - specificity)$, negative likelihood ratio as $(1 - sensitivity)/specificity$, and accuracy as $(TP + TN)/(TP + TN + FP + FN)$, where TP, TN, FP and FN indicate true-positive, true-negative, false-positive and false-negative results, respectively. Cohen's kappa coefficient was calculated as $(Po - Pe)/(1 - Pe)$, where Po is the observed agreement with reference TBR PCR and Pe is the agreement expected by chance. Pe was calculated from the marginal proportions of the 2×2 contingency table (Table 2) as $((TBR\ PCR\text{-}positive\ proportion \times test\text{-}positive\ proportion) + (TBR\ PCR\text{-}negative\ proportion \times test\text{-}negative\ proportion))$. Statistical analyses were performed in GraphPad Prism 10. Endpoint fluorescence comparisons were analyzed by one-way ANOVA followed by Dunnett's multiple-comparisons test, except field-compatible RNP complexation, which used two-way ANOVA followed by Tukey's multiple-comparisons test. RIME-LAMP time-to-threshold comparisons were analyzed using Welch's t-test. Exact *P* values are indicated in the corresponding figures.

Ethics statement

All experiments, maintenance and care of the mice complied with the European Convention for the Protection of Vertebrate Animals (ECPVA) used for Experimental and Other Scientific Purposes guidelines (CETS No 123) and were approved by the Ethical Committee for Animal Experiments (ECAE) at the Vrije Universiteit Brussel (Permit Number: 20-220-43). Mice were monitored daily, and humane endpoints were defined based on body-weight loss. Animals exhibiting >25% body-weight loss were euthanized by CO₂ inhalation using a TEM SEGA TEG automated euthanasia station (TEM SEGA, France) and a 20-L Type 1 mouse chamber. The programmed cycle, operated at a gas flow rate of 40 L/min, comprised a 30-s induction phase using a 50% CO₂/air mixture, followed by a 120-s euthanasia phase with CO₂ saturation and a 150-s purge phase. All infected animals that did not reach the predefined humane endpoint were euthanized at the end of the study using the same procedure, whereas non-infected animals were returned to housing. The study was conducted in accordance with applicable local legislation and institutional requirements.

Data availability

All data generated in this study are provided in the Supplementary Information within this paper.

837 **Competing Interests**

838 The authors declare that the research was conducted in the absence of any commercial or financial
839 relationships that could be construed as a potential conflict of interest.

840 **Author Contributions**

841 Conceptualization and designation of the study, A.Á.-R. and S.M.; methodology, A.Á.-R., Z.L., B.S.
842 and S.M.; data curation and analysis, A.Á.-R., B.-k.J., P.T., A.M.-A., U.L., Z.L., B.S. and S.M.;
843 writing-original draft preparation, A.Á.-R.; writing-review and editing, A.Á.-R., B.-k.J., P.T.,
844 A.M.-A., U.L., Z.L., B.S., P.G. and S.M.; visualization, A.Á.-R.; supervision, B.S. P.G and S.M.;
845 project administration, A.Á.-R. and S.M.; funding acquisition, A.Á.-R., P.G and S.M. All authors
846 have read and agreed to the published version of the manuscript.

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