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## **Analytical sensitivity of Loopamp and quantitative real-time PCR on dried blood spots and their potential role in monitoring human African trypanosomiasis elimination**

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## **Abstract**

The objective set by WHO to reach elimination of human African trypanosomiasis (HAT) as a public health problem by 2020 is being achieved. The next target is the interruption of *gambiense*-HAT transmission in humans by 2030. To monitor progress towards this target, in areas where specialized local HAT control capacities will disappear, is a major challenge. Test specimens should be easily collectable and safely transportable such as dried blood spots (DBS). Monitoring tests performed in regional reference centres should be reliable, cheap and allow analysis of large numbers of specimens in a high-throughput format. The aim of this study was to assess the analytical sensitivity of Loopamp, M18S quantitative real-time PCR (M18S qPCR) and TgsGP qPCR as molecular diagnostic tests for the presence of *Trypanosoma brucei gambiense* in DBS. The sensitivity of the Loopamp test, with a detection limit of 100 trypanosomes/mL, was in the range of parasitaemias commonly observed in HAT patients, while detection limits for M18S and TgsGP qPCR were respectively 1,000 and 10,000 trypanosomes/mL. None of the tests was entirely suitable for high-throughput use and further development and implementation of sensitive high-throughput molecular tools for monitoring HAT elimination are needed.

## **Key words**

*Trypanosoma brucei gambiense*; diagnosis; dried blood spots; Loopamp; quantitative real-time PCR; sensitivity; feasibility

## 1. Introduction

Human African trypanosomiasis (HAT) or sleeping sickness is a parasitic disease caused by two subspecies of trypanosomes transmitted by tsetse flies (Buscher et al., 2017). *Trypanosoma brucei gambiense* (*Tbg*) is responsible for a chronic disease (*gambiense*-HAT) in Central and West Africa and represents 98% of all reported HAT cases. The remaining 2% of HAT cases are due to *Tb rhodesiense* (*Tbr*) that causes an acute infection in East Africa (Franco et al., 2018). Thanks to control efforts conducted in the last two decades (case detection, treatment and vector control), the number of reported cases has gradually decreased to fewer than 1000 cases in 2018 ([https://www.who.int/trypanosomiasis\\_african/en/](https://www.who.int/trypanosomiasis_african/en/)).

HAT elimination is monitored through two primary global indicators: the annual number of reported cases and the area at risk reporting  $\geq 1$  case/10,000 people/year (calculated over a 5-year period) (Franco et al., 2018). The objective set by World Health Organization (WHO) to reach the elimination of HAT as a public health problem by 2020 (WHO, 2012), with fewer than 2,000 reported cases annually and a reduction of 90% of the area at risk as compared to the 2000–2004 WHO baseline, is being achieved (Franco et al., 2020). The indicator at country level for *gambiense*-HAT is the number of cases/10,000 people/year, in each health district of the country averaged over the previous 5- year period. For formal validation by WHO of the elimination of HAT as a public health problem in a country, this indicator should be  $< 1$ , on condition that sufficient control and surveillance activities have been undertaken. This target has probably been met in some countries (Franco et al., 2020), which can request WHO to validate their status.

Adapted strategies and tools should be implemented to at least maintain this status, i.e. avoid re-emergence, and to reach the next objective set by WHO for *gambiense*-HAT: the interruption of transmission by 2030 (Franco et al., 2018). It will therefore be crucial to monitor for potential transmission. As specialized local HAT control capacities will decrease or disappear, the use of highly specialized methods, such as parasitological tests, which are adapted to field condition but time-consuming, is excluded. In the future, monitoring tests will increasingly be performed in regional reference centres. Test specimens should therefore be easily collectable and safely transportable, such as dried blood spots (DBS) (Sarkar et al., 2015), while tests should be reliable, cheap and allow analysis of large numbers of samples in a high-throughput format.

Remote molecular testing of DBS has been proposed to assess the prevalence of HAT in a population, and a variety of molecular diagnostic tests is available (Buscher and Deborggraeve, 2015). The only commercialized method is the Loopamp *Trypanosoma brucei*

Detection Kit (Eiken Chemical, Tokyo, Japan) (Matovu et al., 2012; Njiru, 2012), based on the loop-mediated isothermal amplification technology (LAMP) (Notomi et al., 2000), and adapted for HAT diagnosis by amplifying the random insertion mobile element (RIME) (Njiru et al., 2008). The quantitative real-time PCR (qPCR) technology (Heid et al., 1996) was also applied to HAT diagnosis (Becker et al., 2004; Mumba et al., 2011) and primers targeting the 18S ribosomal RNA gene were designed (M18S qPCR) (Benfodil et al., 2020). Both Loopamp and M18S qPCR methods are *Trypanozoon*-specific. A *Tbg* specific method would be preferable to exclude the detection of *Tb brucei* (*Tbb*) that can potentially transiently infects humans in HAT endemic areas where *Tbg* and *Tbb* are sympatric (Garcia et al., 2000; Koffi et al., 2006). This is possible through detection of the *Tbg*-specific glycoprotein gene (*TgsGP*) (Berberof et al., 2001; Radwanska et al., 2002), for which a qPCR has been developed (Wagemans et al., 2011).

We compared the analytical sensitivity of the Loopamp, M18S qPCR and *TgsGP* qPCR, applied on DBS in the context of an upcoming diagnostic clinical trial evaluating diagnostic performance and feasibility of different diagnostic methods and algorithms for post-elimination monitoring (DiTECT-HAT-WP3, ClinicalTrials.gov Identifier: NCT04099628).

## 2. Material and Methods

### 2.1 Parasites

B4\_C191 and B4\_F303 *Tbg* strains, both isolated during a medical survey in the Bonon HAT focus (Côte d'Ivoire) in 2004 (Kabore et al., 2018; Koffi et al., 2007) were used throughout the study. The two strains were propagated in Naval Medical Research Institute (NMRI) mice (bred from paternal strains purchased from Charles River laboratories, France) until reaching approximately  $7.5 \times 10^7$  trypanosomes per mL of blood, as determined using an Uriglass® disposable counting chamber. Mouse blood was collected by cardiac puncture using a heparinized 1 mL syringe and diluted into phosphate buffered saline glucose (PSG, 38 mM  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 2 mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 83 mM glucose, 29 mM NaCl, pH 8.0) to achieve a stock concentration of  $1 \times 10^7$  parasites/mL, which was kept on ice. Ten-fold serial dilutions were prepared in PSG to get concentrations ranging from  $1 \times 10^6$  to 10 trypanosomes per mL in a total volume of 1 mL.

### 2.2 Preparation of dried blood spots

Sixteen mLs of blood were collected from a human volunteer in Vacutainer heparinized tubes. Volumes of 450  $\mu$ Ls of blood were aliquoted in 32 microtubes. For each strain, 50  $\mu$ L of each trypanosome suspension ( $1 \times 10^7$  to 10 trypanosomes per mL) were added in each tube. This was performed in duplicate, to obtain for each trypanosome strain, two blood sample series (A and B) with concentrations ranging from  $1 \times 10^6$  to 1 trypanosomes per mL. For each series, a negative control was prepared by mixing 50  $\mu$ L PSG with 450  $\mu$ L human blood.

For both M18S and TgsGP qPCR, 30  $\mu$ L of each trypanosome suspension and sampling control were spotted on Whatman 4 filter paper. For Loopamp, the sampling procedure described by Matovu et al. (2016) was followed with some modifications ([https://www.finddx.org/wp-content/uploads/2016/06/HAT-LAMP-SOP\\_13JUN16.pdf](https://www.finddx.org/wp-content/uploads/2016/06/HAT-LAMP-SOP_13JUN16.pdf)).

Briefly, 90  $\mu$ L of blood was mixed with 10  $\mu$ L of 5% sodium dodecyl sulphate (SDS, Sigma) and incubated for 5 minutes at ambient temperature followed by spotting 40  $\mu$ L on Whatman 1001 filter paper. All blood spots were air dried for one hour in a dry environment out of direct sunlight, and stored separately at room temperature for a maximum of 48 hours in closed plastic bags containing silica gel until DNA extraction.

### *2.3 DNA extraction*

With a perforator, three discs of 5 mm diameter were punched out of each Whatman 4 DBS and out of one blank filter paper used as negative extraction control. Between each sample, the perforator was decontaminated with 1% hypochlorite, ultra-pure water and absolute ethanol. Each set of three discs were processed using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) following the instructions provided by the manufacturer. Extracted DNA eluted in 50  $\mu$ L elution buffer was stored at  $-20^{\circ}\text{C}$  until qPCR analysis. For the Whatman 1001 DBS and a blank filter paper, DNA was extracted from one 5 mm diameter disc by adding 100  $\mu$ L molecular biology grade water, mixing and incubating at  $90^{\circ}\text{C}$  for 10 minutes followed by centrifugation at 18,000 g for 3 minutes. These solutions containing the DNA were used for Loopamp reactions.

### *2.4 Quantitative real-time PCR and Loopamp reactions*

All primers and probes are detailed in Table 1. qPCR reactions were performed using the TaqMan Probe assays. For M18S, forward and reverse primers were described in Deborggraeve et al (2011) and the TaqMan probe was described in Benfodil et al. (2020). For TgsGP, primers and probe were described in Wagemans et al. (2011). Amplifications were

carried out in 25 µl final volume containing 5 µl of extracted DNA and 20 µl mix (1x FastMix II, 0.2µM each primer and TaqMan probe, 6.75 µL PCR grade water). The amplification conditions were as follows: polymerase activation at 95°C for 5 min, 45 cycles at 94°C for 10 sec, 60°C for 30 sec and 72°C for 30 sec. PCR assays were performed using the AriaMix Real-Time PCR system (Agilent Technologies) and the cycle threshold (Ct) values were automatically obtained. A result was considered positive if Ct < 40. A positive control (*Tbg* DNA) and negative control (water) were included in each reaction.

For Loopamp we followed the instructions provided with the kit. Positive and negative controls provided in the kit were included for each run. Reaction tubes were incubated at 65°C for 40 min in the LAMP Genie III (OptiGene Ltd) in which eight tubes can be processed per run. A result was considered positive when fluorescence was observed in the reaction tubes during exposure to a blue LED illuminator with a 470 nm ultra-violet light and confirmed by the kinetics of the fluorescence appearance detection by the integrated analysis system in the LAMP Genie® III instrument.

### 3. Results

M18S qPCR and TgsGP qPCR results for the ten-fold serial dilutions of strains B4\_C191 and B4\_F303 in duplicate are given in Table 2. No Ct value was obtained for the human blood without trypanosomes (negative sample controls), the extraction controls (blank filter paper) and the amplification reaction controls (water). For both strains, the lower detection limit in the duplicate runs was 1,000 and 10,000 trypanosomes/mL for M18S qPCR and TgsGP qPCR respectively.

For Loopamp, negative results were also obtained for negative controls (reaction, extraction and sampling) and positive results were obtained with positive controls validating the tests. The lower detection limit was 100 trypanosomes/mL for the two strains (Table 2). Figure 1 illustrates the results obtained for B4\_C191 Series B with fluorescence observed with ultra-violet light (Figure 1A and B) and with the kinetics of the fluorescence appearance (Figure 1C and D).

### 4. Discussion

Monitoring HAT elimination and post-elimination will probably rely on regional reference centers to overcome (i) the inevitable decrease of HAT control capacities in low endemic to zero transmission areas and (ii) the increasing non-adherence to the active HAT control activities of the communities who will no longer consider the disease as a threat. This

requires the use of high-throughput methods applied on large numbers of samples easily collectable by non-specialized teams and easily transportable to be tested remotely. The qPCR and LAMP technologies that previously showed high sensitivities in experimental and field studies (Buscher and Deborggraeve, 2015; Matovu et al., 2012; Mugasa et al., 2012) seemed promising tools for this purpose. In this study, we evaluated the analytical sensitivity of the *Trypanozoon*-specific M18S qPCR and Loopamp and the *Tbg*-specific TgsGP qPCR applied on DBS.

In *gambiense*-HAT, blood parasitaemia is fluctuating and may be below the detection limit of 10 to 100 trypanosomes/mL of the most sensitive field-adapted parasitological test, the mini Anion Exchange Centrifugation Technique (mAECT) (Buscher et al., 2009; Camara et al., 2010). With a detection limit of 10,000 trypanosomes/mL, the TgsGP qPCR on DBS seems not sufficiently sensitive for screening for HAT. The limited sensitivity of PCR targeting the *Tbg*-specific single-copy gene TgsGP was previously observed in experimental and field studies (Matovu et al., 2010; Morrison et al., 2007) and we show that the qPCR technology is not able to improve the detection limit. Our study confirms the better sensitivity of the PCR targeting the *Trypanozoon* specific multi-copy 18S ribosomal gene (Deborggraeve et al., 2011; Mumba et al., 2014), which in a previous study, had an analytic sensitivity of 100 trypanosomes/mL blood (Mitashi et al., 2013). In the present study, the detection limit using M18S qPCR was only 1,000 trypanosomes/mL, which is probably due to the use of DBS as a decrease in sensitivity has been observed earlier when using such specimens (Mumba et al., 2014). The limited volume of blood spotted in the DBS (30  $\mu$ L) might explain this decrease. Considering the low number of trypanosomes in blood of HAT patients, this analytic sensitivity casts doubt about the diagnostic sensitivity to be expected when analyzing clinical samples.

In the present study, the best analytical sensitivity was observed for the Loopamp test with a detection limit of 100 trypanosomes/mL. This is in line with the high sensitivity previously observed using RIME-LAMP methods for HAT diagnosis using freshly prepared samples (Grab et al., 2011; Kuboki et al., 2003; Mitashi et al., 2013; Mugasa et al., 2014; Njiru et al., 2008), attributed to the high copy number of the targeted RIME sequence (500 copies/haploid genome) (Bhattacharya et al., 2002). This is also in line with results obtained when using DBS of trypanosome-spiked blood samples treated with SDS in a recent experimental study carried out to optimize the procedures for Loopamp sample preparation that gave detection limit of 10 to 100 trypanosomes/mL depending on the storage time of DBS (Matovu et al., 2020). Despite the fact that we adapted the original protocol, by reducing



the amount of blood from 450  $\mu$ L in the original protocol to 90  $\mu$ L, envisaging fingerpick collection, the analytical sensitivity we observed is still in accordance with the low parasitaemia observed in HAT patients. Thanks to its simplicity, rapidity and good sensitivity, Loopamp appears to be the most promising DNA detection tool for HAT diagnosis performed on DBS.

However, the performance of a diagnostic method for large scale monitoring, also depends on its capacity to test a large number of specimens. Loopamp allows only eight specimens to be tested per run on the LAMP Genie III including the positive and negative control. The test duration for one reaction was around 1 hour and 30 minutes in our hands, including extraction, mix preparation and amplification time. We estimated the number of days to test 1,000 samples, for a trained lab technician working 7 hours per day, between 35 to 40 days. However, the analysis time could be reduced by using for example the Eiken LF-160 LAMP incubator that can take 16 tubes in a single run, or by changing for a 96 samples format such as for the Eiken Malaria LAMP kit (Perera et al., 2017), or by testing in multiple machines in parallel. Another drawback of Loopamp for a high-throughput use is the sampling procedure: a blood lysis step with SDS is required before spotting the specimen on Whatman 1001 filter paper. Similarly, the time-consuming DNA extraction step considerably reduces the speed of qPCR to maximum 1,000 samples per 10 days. The applicability of qPCR for high-throughput testing is therefore also questionable in its present form. Including HAT diagnosis in high-throughput platforms could overcome this limitation.

In the present study, we investigated the analytical sensitivity of the assays, known to be a potential challenge with DBS. However, collection of DBS in field conditions should take into account measures to mitigate risk of sample-to-sample contamination. Neither did we assess specificity of the assays for *Trypanozoon* or *Tbg* as this was already performed for both qPCRs and RIME LAMP elsewhere (Njiru et al., 2008; Wagemans et al., 2011). The effect of DBS storage time was not assessed. A decrease of sensitivity in Loopamp was recently observed with increasing storage time of the DBS, probably due to a progressive DNA degradation (Matovu et al., 2020). Within an elimination monitoring context, storage time will largely be influenced by exterior logistic limitations, like time needed for DBS collection and for dispatching them to the reference laboratory, and is not entirely intrinsic to the assay. It can indeed be expected that performance of the assays under real life conditions, for several reasons, will be inferior than under ideal laboratory conditions. DNA degradation as observed elsewhere (Matovu et al., 2020) may have a serious impact on the accuracy of

prevalence estimations with molecular tools. Alternatives, such as blood collected on DNA stabilization buffer, may also be considered (Mumba et al., 2014).

Other DNA detection methods previously described and applicable on DBS were not selected. For example, we did not choose the highly sensitive TBR primers targeting the 177-bp repeat satellite DNA previously used in PCR (Moser et al., 1989) and qPCR (Becker et al., 2004; Mumba et al., 2011) because of doubts about their specificity and the risk of contamination, in particular when many specimens need to be collected, transported and analyzed. The *Tbg*-specific methods such as 5.8S rRNA-internal transcribed spacer 2 LAMP (Thekisoe et al., 2007) or the TgsGP LAMP (Njiru, 2011) were suspected not to be sufficiently sensitive. Also, the nucleic acid sequence-based amplification technology (NASBA) which is highly sensitive for HAT diagnosis (Mugasa et al., 2014; Mugasa et al., 2008), was not selected because of the need for RNA purification. Our results show that the challenge and the necessity of developing a molecular tool adapted to monitor HAT elimination still remains. The 7SL-sRNA target recently described as highly sensitive, and applicable to DBS might be a potential candidate although it is not *Tbg*-specific neither (Chiweshe et al., 2019).

Developing a DBS based molecular tool adapted for monitoring HAT elimination is a challenge that relies on the sensitivity/specificity balance and the feasibility to test large numbers of samples. The three methods chosen for this study do not meet these requirements. The TgsGP qPCR is in theory the most specific method but has low sensitivity. This was not completely unexpected, but as it follows the same methodology as the M18S qPCR, it was added to this study. The M18S qPCR is not *Tbg*-specific and its sensitivity is still not sufficient considering the parasitaemia observed in HAT patients. The high-throughput potential of both qPCR methods was disappointing. Loopamp has a better sensitivity but is not *Tbg*-specific and in its present form is also not entirely suitable for high-throughput use.

In the past, several serological methods for screening for HAT through DBS testing have been proposed. Several versions of the Card Agglutination Test for Trypanosomiasis (Magnus et al., 1978) adapted to DBS have been developed (Chappuis et al., 2002; Miezán et al., 1991). However, an important decrease in sensitivity was observed after DBS storage, and the methodology was not entirely suited for high-throughput use (Chappuis et al., 2005). For high-throughput screening for HAT on DBS, ELISA (Geerts et al., 2020; Hasker et al., 2010; Lejon et al., 1998), and immune trypanolysis (Mumba et al., 2011; Van Meirvenne et al., 1995) seem more satisfactory. However, serological tests detect trypanosome specific antibodies, which indicate potential contact with *Tbg*, but not necessarily active infection

since antibody responses in general disappear very slowly (several years) after resolution of the infection (Jamonneau et al., 2010). Combination of antibody detection tests followed by nucleic acid detection tests to confirm (or not) an active infection would take into account the limitations of both methods. We therefore recommend to investigate the diagnostic performance and feasibility of different algorithms, combining serological and molecular tests for elimination monitoring.

### **Declaration of competing interest**

The authors declare that they have no conflicts of interests.

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**Table 1. Primers and probes used in M18S qPCR, TgsGP qPCR and Loopamp**

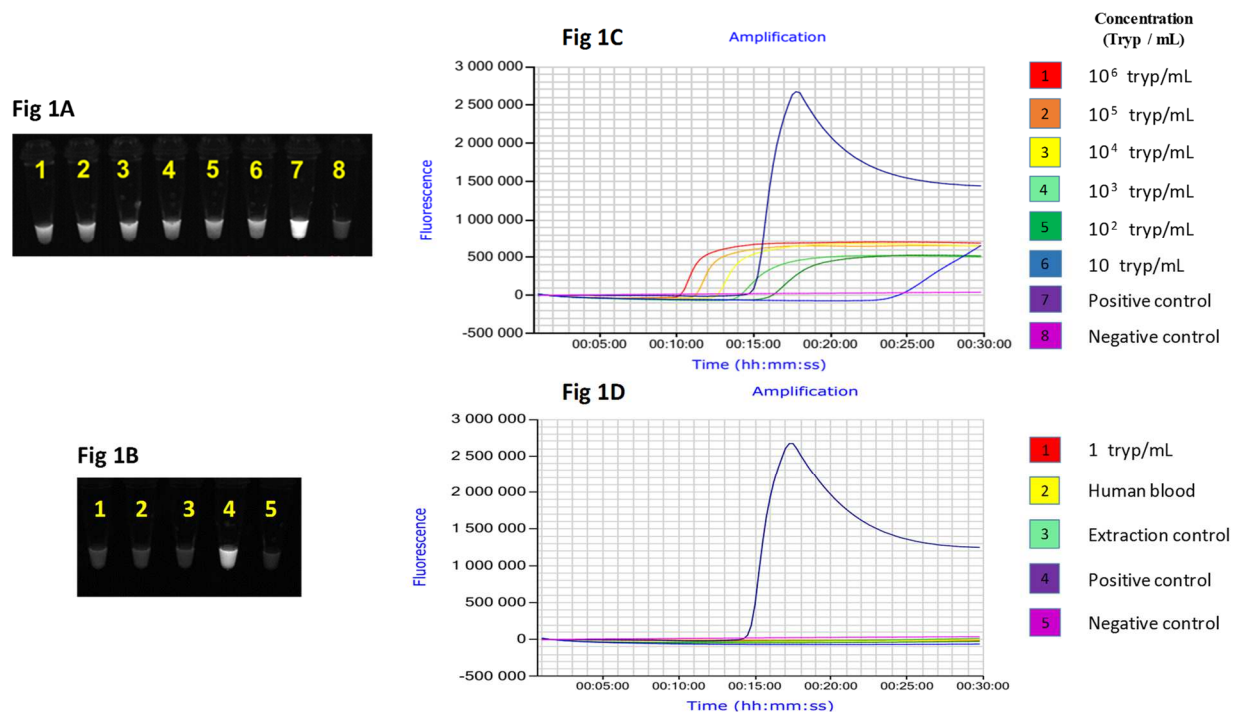
Method	Primers/probe	Gene	Target group	Sequences (5'-3')
M18S qPCR	m18S-II-F-Tb	18S	Trypanozoon	CGTAGTTGAACTGTGGGCCACGT
M18S qPCR	m18S-II-R-Tb	18S	Trypanozoon	ATGCATGACATGCGTGAAAGTGAG
M18S qPCR	m18S-II-probe	18S	Trypanozoon	FAM -TCGGACGTGTTTTGACCCACGC-BHQ1
TgsGP qPCR	Tgs-F	TgsGP	Tbg	GGCTGTCACGGACTTGCTCA
TgsGP qPCR	Tgs-R	TgsGP	Tbg	CTTGCAGATGCCTCGCACAC
TgsGP qPCR	Tgs-probe	TgsGP	Tbg	FAM-ACGGCGGTATCGACGGCGGCAGTGA-BHQ1
Loopamp	RIME-F3	RIME	Trypanozoon	CTGTCCGGTGATGTGGAAC
Loopamp	RIME-B3	RIME	Trypanozoon	CGTGCCTTCGTGAGAGTTTC
Loopamp	RIME-FIP	RIME	Trypanozoon	GGAATACAGCAGATGGGGCGAGGCCAATTGGCATCTTTGGGA
Loopamp	RIME-BIP	RIME	Trypanozoon	AAGGGAGACTCTGCCACAGTCGTCAGCCATCACCGTAGAGC
Loopamp	RIME-LF	RIME	Trypanozoon	GCCTCCCACCCTGGACTC
Loopamp	RIME-LB	RIME	Trypanozoon	AGACCGATAGCATCTCAG

**Table 2. 18S qPCR, TgsGP qPCR and Loopamp results**

Strain	M18S qPCR			TgsGP qPCR		Loopamp	
	C <sup>a</sup>	Ct A <sup>b</sup>	Ct B	Ct A	Ct B	A	B
B4_C191	10 <sup>6</sup>	27,23	26,38	28,74	28,02	Positive	Positive
B4_C191	10 <sup>5</sup>	29,86	29,63	31,1	30,99	Positive	Positive
B4_C191	10 <sup>4</sup>	34,35	33,13	35,12	33,96	Positive	Positive
B4_C191	10 <sup>3</sup>	37,7	37,46	No Ct	No Ct	Positive	Positive
B4_C191	10 <sup>2</sup>	No Ct	No Ct	No Ct	No Ct	Positive	Positive
B4_C191	10	No Ct	No Ct	No Ct	No Ct	Negative	Positive
B4_C191	1	No Ct	No Ct	No Ct	No Ct	Negative	Negative
B4_F303	10 <sup>6</sup>	27,33	26,3	27,59	27,54	Positive	Positive
B4_F303	10 <sup>5</sup>	30,25	29,89	31,15	30,82	Positive	Positive
B4_F303	10 <sup>4</sup>	35,29	33,44	35,38	34,3	Positive	Positive
B4_F303	10 <sup>3</sup>	37,53	37,99	No Ct	No Ct	Positive	Positive
B4_F303	10 <sup>2</sup>	No Ct	No Ct	No Ct	No Ct	Positive	Positive
B4_F303	10	No Ct	No Ct	No Ct	No Ct	Negative	Positive
B4_F303	1	No Ct	No Ct	No Ct	No Ct	Negative	Negative
Human blood	0	No Ct	No Ct	No Ct	No Ct	Negative	Negative
Extraction control		No Ct	No Ct	No Ct	No Ct	Negative	Negative
Positive control		28,6	28,6	27,94	27,94	Positive	Positive
Negative control		No Ct	No Ct	No Ct	No Ct	Negative	Negative

<sup>a</sup> C = Initial concentration of trypanosomes in blood (number of trypanosomes per mL) before spotting on filter paper

<sup>b</sup> Ct A = Cycle threshold for series A. Ct B = Cycle threshold for series B.



**Figure 1. Loopamp results for B4\_C191 ten-fold serial dilutions (Series B) and controls**

Fig 1A and 1B illustrate the results observed with ultra-violet light. Fig 1C and 1D illustrate the kinetics of the fluorescence appearance. Results were obtained in two runs. Results obtained for  $10^6$  to 10 trypanosomes/mL (tryp/mL) are given in Fig 1A and Fig 1C and results obtained for 1 tryp/mL, human blood (sampling control) and extraction control are given in Fig B and Fig D. Positive and negative reaction controls were added for each run.

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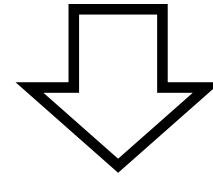
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## Monitoring HAT elimination requires:

Easy collection and  
safe transport of large numbers  
of specimens in the field



Testing with reliable, cheap  
and high throughput methods  
in regional reference centre