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# A new LAMP-based assay for the molecular diagnosis of toxoplasmosis: comparison with a proficient PCR assay

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## ABSTRACT

Toxoplasmosis is generally a benign infection caused by the protozoan parasite *Toxoplasma gondii* but can have severe consequences in fetuses of mothers infected during pregnancy (congenital toxoplasmosis) and immunocompromised individuals. PCR-based diagnostic tests have become crucial for its diagnosis. However, this molecular diagnosis essentially relies upon laboratory-developed methods and suffers from a lack of standardization, leading to great variation in methods and performance among laboratories. With the need for accreditation of clinical microbiological laboratories, the use of commercial PCR kits has become an attractive alternative; but thorough evaluation of newly commercialized kits by proficient groups is necessary before any recommendation can be made to parasitology laboratories by health authorities or learned societies. Here, we compared the performance of an original commercial method, the *Iam TOXO* Q-LAMP (DiaSorin), using Loop-mediated isothermal amplification (LAMP) technology, with our reference laboratory-developed method using real-time PCR. The kit was first tested using amniotic fluid (AF) and plasma samples (either negative or spiked with live *T. gondii* tachyzoites at different concentrations (from 7 to 10<sup>5</sup> tachyzoites/mL)). It was then assessed using a cohort of 11 AF, five placental and 32 blood clinical samples preserved at -20 °C. For the processing of placental/blood samples, a pretreatment step was used, which did not strictly follow the manufacturer's recommendations. The practical ease of use and compliance with good laboratory practices were also evaluated. Although the LAMP assay was less sensitive than the laboratory-developed method at very low parasite concentrations (0.1 *T. gondii* genome equivalents/mL), the two methods yielded identical results qualitatively and, in some instances, quantitatively, particularly for AF samples.

**Keywords:** *Toxoplasma gondii*, diagnosis, PCR LAMP, Commercial kit

### 1. Introduction

Toxoplasmosis is a global endemic protozoan disease, caused by the ubiquitous protozoan parasite *Toxoplasma gondii* which infects

human via ingestion of undercooked meat containing trophozoites or tissue cysts, or through ingestion of sporulated oocysts found in food, soil and water contaminated with cat feces (Krueger et al., 2014). This infection is generally benign but it can have severe

consequences. When mothers are contaminated during pregnancy, congenital toxoplasmosis ranges from asymptomatic cases to fetal death. In immuno-compromised individuals, it also ranges from pauci-symptomatic cases to fatal visceral involvement. PCR-based diagnostic tests have become crucial for its diagnosis (Bastien, 2002; Sterkers et al., 2012) but essentially rely upon 'in-house' or 'laboratory-developed' methods, hence there is a lack of standardization (Pelloux et al., 1998; Sterkers et al., 2010a,b). Indeed, this leads to important variations in protocols between laboratories (in particular in DNA extraction, choice of DNA target, design of primers, PCR conditions and amplicon detection), and hence in performance (Sterkers et al., 2010a,b). Several turnkey systems for molecular detection of *T. gondii* in humans have been marketed over the past few years; they respond to an increasing demand for (i) standardization and (ii) quality management systems, and need to be evaluated thoroughly in comparison with the laboratory-developed PCR assays used in diagnostic laboratories, particularly the proficient ones.

In recent years, loop-mediated isothermal amplification (LAMP) has increasingly appeared as an alternative molecular method to PCR diagnosis, particularly in malaria diagnosis. LAMP consists in an isothermal amplification of target DNA through a repetition of two successive elongation reactions occurring at loop regions formed from the primer sequences: self-elongation of templates from a stem loop structure formed at the 3'-terminal end; and the binding and elongation of new primers to the loop region. An original commercial method based on LAMP technology (Mori et al., 2013) including rapid real-time amplification and fluorescence detection of *T. gondii* DNA, has been developed by DiaSorin, *lam* TOXO Q-LAMP kit (for *lam* TOXOPLASMOSIS Q-LAMP QUALITATIVE ASSAY (CE/IVD), hereafter termed *lam* TOXO).

The present study aimed to evaluate the performance of the *lam* TOXO assay in *T. gondii* infection diagnosis compared with a routine laboratory-developed real-time PCR assay used in the coordinating center of the Molecular Biology 'pole' of the French National Reference Centre for Toxoplasmosis, France. Both methods were compared using amniotic fluid (AF), plasma, placental and blood samples.

## 2. Materials and methods

### 2.1. Study design

This study was performed in accordance with the regulations of the local ethics committee of the Hospital University Center (CHU), Montpellier, France, in accordance with the revised Helsinki Declaration. All samples were part of the authorized collection of the Biological Resources Center of the Academic Hospital (CHU), Montpellier, France (declaration # DC-2011-1405 and DC-2012-1579). French regulations allow utilization of such samples in retrospective studies. In addition, written informed consent was obtained before any AF sampling. All tests were carried out in the Parasitology-Mycology Department of the Academic Hospital (CHU), Montpellier (France), a proficient laboratory coordinating the Molecular Biology 'Pole' of the National Reference Centre for Toxoplasmosis, France (<http://cnrttoxoplasmosse.chu-reims.fr>). Seventy specimens were tested using the *lam* TOXO assay on the LIAISON *lam* amplification/detection LAMP analyzer (DiaSorin) and the results compared with those obtained using the laboratory-developed real-time *T. gondii* PCR assay which has been routinely used in our laboratory since 2009 (Sterkers et al., 2010b; Morelle et al., 2012). In a first phase (termed 'analytical'), the kit was tested using three AF samples, either negative or spiked with *T. gondii* at different concentrations (see Section 2.2), as well as one panel from the European External Quality Assessment (EQA) of

Quality Control for Medical Diagnostics (QCMD, Glasgow, UK, European controls 2012). In the second phase, the evaluation was based on a patient cohort of 48 clinical samples. All samples were fully processed, i.e. DNA was extracted and the resulting DNA amplified separately, using each method.

### 2.2. Freeze-dried calibrated 'standard' samples and *Toxoplasma* serial dilution assays for analytical sensitivity detection

The preparation of the freeze-dried 'standard' samples was described earlier (Varlet-Marie et al., 2014). Briefly, PCR-negative hydramnios AF samples obtained with written consent were spiked with live *T. gondii* tachyzoites of a type II clinical strain (Ajzenberg et al., 2002) isolated from a placenta in Montpellier, France, in 2010 (code name MTP040-COR/TgH25040) to obtain standard vials at a concentration of  $10^5$  tachyzoites (T)/mL, 50 T/mL and 10 T/mL (Varlet-Marie et al., 2014). Three vials of each of these three concentrations were tested in this study (batch #25040-0611): vials at 50 T/mL and 10 T/mL were DNA-extracted and tested as such, while  $10^5$  T/mL vials were DNA-extracted and then submitted to serial dilutions to obtain the following concentrations:  $10^4, 10^3, 10^2, 10, 1$  and  $0.1$  *T. gondii* genome equivalents (Tgg)/mL. These correspond to 20,000, 2000, 200, 20, 2, 0.2 and 0.02 Tgg per PCR tube, and to 40,000, 4000, 400, 40, 4, 0.4 and 0.04 Tgg per LAMP reaction tube.

Calibrated "TG12" samples from the QCMD EQA for *Toxoplasma* PCR were freeze-dried samples of *T. gondii* tachyzoites provided by QCMD at the following concentrations: three AF samples at 7 T/mL, one AF sample at 20 T/mL; one plasma sample at 11 T/mL, two plasma samples at 23 T/mL, one plasma sample at 80 T/mL; and two negative plasma samples. Two panels comprised of one each of these samples were tested in this study.

### 2.3. Clinical samples

Forty-eight clinical samples preserved at 20 °C were tested using both methods: 11 AF, 32 blood, and five placental samples. For AF and placenta, the European Research Network on Congenital Toxoplasmosis classification system and case definitions of *T. gondii* infection (Lebech et al., 1996) were used. The criteria based on serological, parasitological, and clinical information allowed separation of the cases into two of the five mutually exclusive categories: definitely infected and not infected. AF samples (2 mL) were spun down 10 min at 16,000g, and the pellet was resuspended in either 50 mL of Tween-Noninon-9-NaOH (TNN) (Hohlfeld et al., 1994) for the laboratory-developed method, or in 250 mL of AF before extraction in the LIAISON IXT (DiaSorin) system (see Table 1). Blood samples were centrifuged for 10 min at 1,750g and 300 mL of buffy coat carefully aspirated by pipetting. Half of the buffy coat (150 mL) was diluted in 300 mL of Tris 10 mM pH 7.2-2% SDS Buffer, lysed overnight at 56 °C using 20 mL of 20 mg/mL proteinase K stock solution (EU0090C, Euromedex, France) and preserved at 20 °C. Three hundred grams of placental samples were manually ground using a scalpel, diluted in 300 mL of PBS1X and trypsinized (0.6 g Trypsine T4799, Sigma, France) for 2 h at 37 °C under magnetic agitation. This was filtered, and 100 mL were then washed twice in PBS 1X. The pellet (1 mL) was diluted in 3 mL of Tris 10 mM pH 7.2-2% SDS Buffer and lysed overnight at 56 °C using 80 mL of 20 mg/mL proteinase K stock solution (EU0090C, Euromedex) before preservation at 20 °C. All samples were collected in Montpellier in accordance with the routine practice of the laboratory, either from pregnant women acutely infected with *T. gondii* during gestation or from immuno-suppressed patients.

Table 1  
Description of the methods used for the molecular detection of *Toxoplasma gondii* in this study.

	Local method	Diasorin technology
AF samples pre-treatment	Centrifugation	
Whole blood pre-treatment	Centrifugation; buffy coat diluted 1/2 in Tris-SDS, lysed by PK and preserved at $-20^{\circ}\text{C}$	
Placenta pre-treatment	Digestion by trypsin, centrifugation, pellet diluted 1/2 in Tris-SDS, lysed by PK and preserved at $-20^{\circ}\text{C}$	
Extraction		
Instrument	None	LIAISON IXT
AF protocol	2 mL eluted in 50 $\mu\text{L}$	2 mL eluted in 10 $\mu\text{L}$
Whole blood and placenta protocol	450 $\mu\text{L}$ eluted in 75 $\mu\text{L}$	250 $\mu\text{L}$ eluted in 100 $\mu\text{L}$
Duration	120 min for 8 samples	40 min for 1–12 samples
Amplification		
Method	'In-house' real-time PCR	LAMP
Analyzer	LC 480 (Roche)	LIAISON Iam (Diasorin)
DNA volume per reaction	5 $\mu\text{L}$	20 $\mu\text{L}$
Mix volume	20 $\mu\text{L}$	30 $\mu\text{L}$
Duration of a run	90 min	40 min
Total 'hands-on' technical time	120 min	75 min
Total duration of the process	210 min	115 min

AF, amniotic fluid; PK, Proteinase K; LAMP, Loop mediated isothermal amplification.

#### 2.4. DNA extraction and PCR assays

Protocols used in each method are summarized in Table 1. Freeze-dried samples were reconstituted by adding 2.0 mL of sterile water (Nucleic Acids Tested grade) to each vial, in accordance with the manufacturer's specifications. The supplier recommended the use of a DNA extraction kit using the LIAISON IXT (Diasorin) system. Nucleic acids were thus extracted from each sample/specimen using both the LIAISON IXT (Diasorin) system and the in-house extraction methods. The latter were manual methods based upon (i) TNN (Hohlfeld et al., 1994) for AF and plasma, and (ii) the protein precipitation kit from Promega for the other specimen types; both extraction methods have performed well in previous studies (Sterkers et al., 2010b; Morelle et al., 2012; Filisetti et al., 2015). The LIAISON IXT extractor is a fully automated magnetic beads-based nucleic acids isolation system. The device includes a UV decontamination mechanism.

The laboratory-developed *T. gondii* real-time PCR assay was performed using the DNA primers, fluorescence resonance energy transfer (FRET) probes and conditions already published (Reischl et al., 2003; Varlet-Marie et al., 2014) on a LightCycler 480 (Roche) apparatus according to the supplier's instructions. For *Iam TOXO*, samples were tested using a prototype LAMP Analyser (LIAISON Iam, Diasorin) with temperature control and multi-channel fluorescent detection. Five mL of internal control were added to each sample prior to extraction, and 20  $\mu\text{L}$  of DNA extract were added to 30  $\mu\text{L}$  of reconstituted LAMP reagents before incubation in the LAMP Analyser at  $67^{\circ}\text{C}$ .

#### 2.5. Data and statistical analysis

Crossing point ( $C_p$ ) values were determined using the "absolute quantification/s derivative maximum" algorithm in the LightCycler 480 Software release 1.5.0.39. A spreadsheet (Microsoft Excel) was used for all statistical calculations including mean, S. D. and correlations ( $R^2$ ). The best-fitting line was determined using least-squares analysis.

### 3. Results and discussion

#### 3.1. Rationale of the study and study design

The study, totalling 421 test reactions (264 PCR and 157 LAMP), followed three independent steps (Table 2). In the first step, in order to verify that the LAMP-based assay was performing similarly to our laboratory-developed method, we compared both methods using 'in-house' freeze-dried calibrated 'standard' samples which have been used in national quality assessments (Varlet-Marie et al., 2014). In the second step, in order to test whether the LAMP-based assay complied with European standards, we tested two panels of the QCMD EQA for *Toxoplasma* PCR.

Finally, in order to verify the practicality and performance of the assay in routine laboratory use, we tested 48 clinical samples. It should be noted that, as Diasorin recommended the use of a specific DNA extraction apparatus (Liaison IXT), all analyzed samples were tested following both the extraction and amplification steps specific to each method. Thus, we tested two DNA extraction/PCR combinations: 'in-house' extraction + 'in-house' PCR; and Diasorin (IXT) extraction + *Iam TOXO*. Three notable differences which may influence sensitivity exist between the laboratory-developed method and the LAMP-based method: the tested volume of the primary sample, the elution volume and the DNA input per reaction (see Table 1); these were 450 mL (blood) to 2 mL (AF), 50 mL and 5 mL, respectively, for the laboratory-developed method; and 250 mL (blood) to 2 mL (AF), 100 mL and 20 mL, respectively, for the second. Thus, the volume of primary sample tested in the final PCR was approximately twofold lower in the 'in-house' method than in the LAMP-based method: 200 and 400 mL for AF, and 30 and 50 mL for blood, respectively (Table 1).

#### 3.2. Analytical performance of the LAMP-based assay and the laboratory-developed PCR assay using 'standard' spiked samples

This step used a previously developed common 'standard' (Varlet-Marie et al., 2014), consisting of serial dilutions of spiked AF at six *T. gondii* concentrations. Nine spiked samples were independently extracted: three at a concentration of 50 T/mL, three at 10 T/mL, and three at  $10^5$  T/mL (Table 2). Moreover, two calibrated QCMD "TG12" EQA panels were independently extracted, each totalling 10 vials at five different concentrations (see Section 2.2). Three negative AF samples and two negative plasma samples were included for each molecular detection assay. For the PCR assay, DNA extracts were tested in triplicate. For the LAMP-based assay, DNA extracts were tested in mono, duplicate or triplicate depending on the concentration/sample (Table 2).

Using both the 'in-house' spiked samples and QCMD panels, we observed a total concordance between both methods, including at the lowest concentration tested (0.1 Tg/mL) (Table 3). Taking into account the different DNA volumes per reaction between both methods, the analytical sensitivity threshold was  $<0.02$  and  $<0.04$  Tgg per reaction tube for the laboratory-developed PCR method and LAMP-based method, respectively.

#### 3.3. Comparison of the LAMP-based assay and laboratory-developed PCR assay using clinical samples

Forty-eight clinical samples (11 AF, 32 blood and five placental samples) were then tested using both methods. Again a total concordance was observed between both methods, including for the low concentration samples ( $<1$  Tgg/mL) (Table 3), indicating 100% sensitivity and 100% specificity for the LAMP-based assay. It should be noted, however, that blood (buffy coat) and placental samples

Table 2

Study design: number of planned DNA extractions, *Toxoplasma*-specific PCR tests and Loop mediated isothermal amplification (LAMP) tests, according to the parasite concentration/clinical sample, for each extraction method.

Type of sample and Concentration	No. of samples extracted per DNA extraction method <sup>a</sup>	No. of <i>Toxoplasma</i> -PCR reactions after in-house extraction methods		No. of LAMP reactions after Liaison IXT (Diasorin) extraction <sup>b</sup>	
		Per DNA extract	Total No. of PCRs	Per DNA extract	Total No. of LAMP reactions
<b>In-house AF<sup>c</sup> spiked samples</b>					
10 <sup>5</sup> T/mL	3	3	9	1	3
10 <sup>4</sup> T/mL	NA <sup>c</sup>	3	9	1	3
10 <sup>3</sup> T/mL	NA <sup>c</sup>	3	9	1	3
10 <sup>2</sup> T/mL	NA <sup>c</sup>	3	9	1	3
10 T/mL	NA <sup>c</sup>	3	9	1	3
1 T/mL	NA <sup>c</sup>	3	9	3	9
0.1 T/mL	NA <sup>c</sup>	3	9	3	9
50 T/mL	3	3	9	2	6
10 T/mL	3	3	9	2	6
Negative AF samples	3	3	9	2	6
<b>QCMD EQA panels</b>					
Plasma 80 T/mL	1	3	3	1	1
Plasma 23 T/mL	2	3	6	1	2
AF 20 T/mL	1	3	3	1	1
Plasma 11 T/mL	1	3	3	1	1
AF 7 T/mL	3	3	9	1	3
Negative plasma samples	2	3	6	1	2
<b>Clinical samples</b>					
AF (5 neg, 6 pos)	11	3	33	2	22
Buffy coat (5 neg, 27 pos)	32	3	96	2	64
Placenta (5 pos)	5	3	15	2	10
Total No.	120 <sup>b</sup>	57	264	29	157

AF, amniotic fluid; T/mL, tachyzoites per mL; EQA, external quality assessment; neg, negative; pos, positive.

<sup>a</sup> Two extraction methods were used: Tween-Nonidet-NaOH or Promega for the laboratory-developed method, and Diasorin Liaison IXT.

<sup>b</sup> Due to cost and to follow the recommendations of the supplier, only 1–2 LAMP reactions were performed (instead of three PCRs generally).

<sup>c</sup> NA, not applicable. For the serial dilution assay, only the highest concentration was extracted, and the other concentrations were obtained by 10-fold dilutions.

Table 3

Compared evaluation of the laboratory-developed *Toxoplasma gondii* real-time PCR method and the Diasorin full method (IXT extraction + kit Iam Toxoplasmosis assay).

			Iam TOXO LAMP assay		
			Positive	Negative	Total
QCMD	Positive		8	0	8
	Negative		0	2	2
	Total		8	2	10
In-house real time PCR	Spiked amniotic fluid	Positive	6	0	6
		Negative	0	3	3
		Total	6	3	9
	Amniotic fluid	Positive	6 <sup>a</sup>	0	6
		Negative	0	5 <sup>a</sup>	5
		Total	6	5	11
	Blood	Positive	27	0	27
		Negative	0	5	5
		Total	27	5	32
Placenta	Positive	5 <sup>b</sup>	0	5	
	Total	5	0	5	

LAMP, loop mediated isothermal amplification.

<sup>a</sup> All positive amniotic fluid samples corresponded to infants with congenital toxoplasmosis (CT-positive) and all negative ones to uninfected infants.

<sup>b</sup> The positive placental samples corresponded to three CT-positive infants and two uninfected infants.

had been previously lysed by proteinase K and preserved at -20 °C (instead of a simple proteinase K lysis as recommended by the manufacturer), introducing a bias for this category of samples.

It is noteworthy that very few studies have evaluated commercial assays for the diagnosis of toxoplasmosis using clinical samples. One tested 157 AF samples using the Bio-Evolution kit and found 99% concordance between the kit and 'in-house' reference methods for *T. gondii*-infected samples (Filisetti et al., 2015); another evaluated the *Toxoplasma* ELITE MGB assay using 128 clinical samples (56 AF, 55 placental, and various other samples), and showed that this kit is suitable for the diagnosis of toxoplasmosis from non-cell-rich or non-hemoglobin-rich samples

(Robert-Gangneux et al., 2017). Thus, several commercial kits are now available for the diagnosis of congenital toxoplasmosis using AF, showing equivalent performance to those of the best 'in-house' methods.

### 3.4. Quantification tests

The results obtained from serial dilutions of AF standards at 10<sup>5</sup> T/mL (concentrations ranging from 10<sup>5</sup> to 0.1 Tgg/mL) indicated that the LAMP-based assay detected all concentrations down to 1 Tgg/mL; the concentration 0.1 Tgg/mL was detected in five reactions out of nine compared with eight out of nine with the in-house

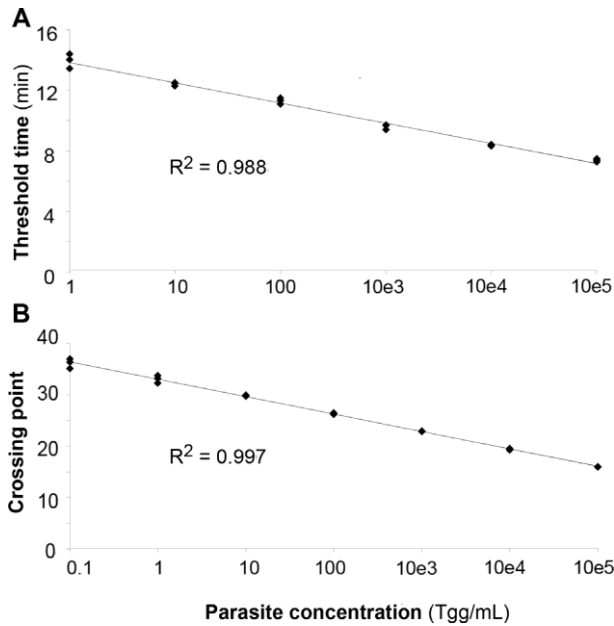


Fig. 1. Comparison of quantitative values obtained by both molecular methods using serial dilutions of amniotic fluid standards at 10<sup>5</sup> *Toxoplasma gondii* genome equivalents (Tgg)/mL (concentrations ranging from 10<sup>5</sup> to 0.1 Tgg/mL). (A) Diasorin extraction method (IXT) + Diasorin LAMP (*lam*); abscissa: parasite concentrations per mL; ordinate: time-to-threshold (Tt). (B) In-house (Tween-Nonineth-NaOH /Promega) extraction method + laboratory-developed real-time PCR; abscissa: parasite concentrations per mL; ordinate: Crossing point (Cp). For both methods, an excellent correlation with parasite concentrations was demonstrated by linear regression in both systems down to 1 Tgg/mL ( $r = 0.99$ ). At 0.1 Tgg/mL, the LAMP assay showed no correlation between the Tt and the parasite concentration, which is still acceptable for a non-quantitative method.

PCR method. A good correlation was seen between the tachyzoite concentrations and the signal time-to-threshold (Tt) values obtained with the *lam TOXO* system for concentrations between 10<sup>5</sup> and 1 Tgg/mL ( $r = 0.99$ ) (Fig. 1A), as was the case for Cp values obtained by PCR (Fig. 1B). The LAMP technology might therefore be applied to quantitation in this range of concentrations, but more studies are needed to confirm this. In any case, as the *lam TOXO* assay is qualitative, a range of concentrations must be quantified in each laboratory in order to be able to provide quantitative results.

### 3.5. Evaluation of practicality and good laboratory practices

The *lam TOXO* assay was felt to be a handy and easily managed system. Positive points included automated homogenization of magnetic beads by the LIAISON IXT extractor, disposable single use pump tips, accurate 100 mL elution volumes, and the presence of negative controls and internal positive (LAMP inhibition) controls in the kit. For eight samples, the effective 'hands-on' technical time was 75 min using the LIAISON IXT extractor, which was clearly shorter than our in-house extraction methods (Table 1); moreover, the run time was only 40 min, as opposed to 90 min for our PCR assay. Thus, the time necessary for complete processing (from DNA extraction to result) for eight samples was 210 min for the 'in-house' PCR method, versus 115 min for the LAMP-based assay.

Negative points of the *lam TOXO* system included (i) the manual opening of pre-filled reagent cartridges within the extractor, (ii) the requirement for ice between DNA denaturation and amplification start, and (iii) the manufacturer's indication to perform a single PCR per patient (we rather recommend duplication of the reaction, due to frequently low parasitic loads). Also, all the com-

ponents of the kit, i.e., positive controls, negative controls, and mixture vials, were stored in the same box, which makes it difficult to comply with the recommended separation of the manipulation into two different rooms. Similarly, transporting the refrigerated tube rack from the DNA extraction room to the amplification room might expose the PCR to carry-over contamination and requires decontamination of the rack.

Similar to PCR assays, it would be useful to include the use of dUTP-uracil-*N*-glycosylase (dUTP-UNG) in the mix to prevent carryover contamination. Finally, one major limitation of the LAMP-based assay is the requirement to buy a specific apparatus for amplification. The fact that only eight reactions (including the two controls) can be carried out in one run, therefore making the apparatus better suited to laboratories with a low daily number of analyses, can be compensated by the connection of four apparatuses to a single computer monitor, allowing processing of >250 reactions/day. The use of a specific extractor is considered by us as a good point in view of the importance of the match between DNA extraction and amplification conditions (unpublished data).

In conclusion, the combined utilization of the LIAISON IXT extractor and the *lam TOXO* assay kit in the LIAISON *lam* apparatus showed excellent analytical performance in terms of sensitivity, comparable to the best laboratory-developed assays in France (Sterkers et al., 2010b). Although the number of negative samples was low (15 in total), analytical specificity appeared to be 100%. Diagnostic (clinical) sensitivity and specificity were not examined here. It thus provides a reliable and sensitive diagnostic solution for the detection of the *T. gondii* genome in AF, which complies with the European In-Vitro Diagnostic Devices Directive 98/79/EC (CE/IVD marking). With respect to blood and placental samples, the results obtained here were highly satisfactory, however the study was not performed using the manufacturer's recommendations but rather using samples that had been frozen and then thawed. As in most comparative studies, both methods being optimized as a whole, any change in the DNA extraction or amplification methods may yield results that differ from those obtained here.

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