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An efficient and novel technology for the extraction of parasite genomic DNA from whole blood or culture

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Abstract

The aim of this study was to assess pathogen DNA extraction with a new spin column-based method (DNA-XT). DNA from either whole-blood samples spiked with *Plasmodium falciparum* or *Leishmania donovani* amastigote culture was extracted with DNA-XT and compared with that produced by a commercial extraction kit (DNeasy®). Eluates from large and small sample volumes were assessed by PCR and spectroscopy. Using a small volume (5 µl) of blood, the DNA-XT and DNeasy methods produced eluates with similar DNA concentrations (0.63 vs 1.06 ng/µl, respectively). The DNA-XT method produced DNA with lower PCR inhibition than DNeasy. The new technique was also twice as fast and required fewer plastics and manipulations but had reduced total recovered DNA compared with DNeasy.

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Author Contributions

Experimentation was designed by D Clark, C Moore, S Croft, J Tyson, S Whitehouse, J O'Halloran, S Krishna and H Staines and was undertaken by D Clark, C Moore, M Flanagan, K Van Bocxlaer, E-T Piperaki and V Yardley. The manuscript was prepared by D Clark, C Moore, V Yardley, S Krishna and H Staines. All authors had the opportunity to read and approve the manuscript.

Financial & Competing Interests Disclosure

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Keywords

blood; diagnostics; DNA extraction; *Leishmania*; malaria; PCR; *Plasmodium*

DNA extraction is an essential starting point for methodologies such as PCR, which is used in the laboratory for molecular biology and clinical diagnostics. It was first achieved in 1869 by Friedrich Meischer [1], but a routine laboratory procedure was not developed until 1958 [2]. Today, a variety of methodologies are available (reviewed by Griffiths and Chacon-Cortes [3]); however, commercial spin column purification of DNA from blood and other tissues and fluids is the most common approach used in modern laboratories. The technique, which originated in 1979 [4], uses silica matrices to selectively bind DNA, allowing washing before elution of the purified product.

NanoMal was an EU-funded industrial-academic consortium that was brought together with the aim of developing a simple-to-use, affordable, handheld diagnostic device to detect malaria infection and the drug resistance status of the parasite by identifying associated genetic mutations. The cartridge-based assay was designed to use only a finger prick of blood, with results being obtained approximately 20 min after sampling. The diagnostics platform technology developed by our industrial partner, QuantuMDx Group Ltd (Newcastle upon Tyne, UK), incorporates a novel DNA extraction technique that allows DNA from lysed whole blood (or other sample types) to flow straight through a proprietary material, but cellular debris is retained. The resulting eluate contains DNA that is of a quality that can be used directly in PCR; therefore, the technique removes the need for additional washing and elution steps, which greatly simplifies the extraction procedure. During the NanoMal project, QuantuMDx created a standalone, spin column-based version of this easy-to-use extraction methodology called DNA-XT, which is optimized for human whole blood. Small-volume samples are lysed and placed over a column of the proprietary material, which is housed within a microcentrifuge collection tube. Following centrifugation, the resulting eluate contains purified, high-quality gDNA.

Here, we examine the performance characteristics of DNA-XT in parallel with a widely used commercial DNA extraction spin column kit for the isolation of parasite DNA from small volumes of *Plasmodium falciparum*-infected erythrocytes spiked into human whole blood (as a model for blood infections) and purified *Leishmania donovani* amastigotes (as might be required in a laboratory setting). The two primary objectives were to determine the efficiency of gDNA extraction and the quality of the isolated gDNA by undertaking parasite-specific PCR. Secondary objectives were to assess the level of operator input, the time required for extractions and the use of reagents or consumables.

Materials & Methods

Parasites

P. falciparum 3D7 parasites were cultured in human red blood cell (RBC) suspensions using RPMI 1640 medium (Sigma-Aldrich, MO, USA; cat. no. R0883-500ML) supplemented with 2-mM l-glutamine, 35-mM HEPES, 0.5% (w/v) AlbuMAX™ I (Thermo Fisher

Scientific, MA, USA), 0.2-mM hypoxanthine and 50- μ g/ml gentamycin, and were maintained at 37°C under 5% CO₂. Parasite growth was followed by microscopic examination of Giemsa-stained thin blood smears and maintained at 10% parasitemia, with an approximately 2% hematocrit. Synchronization of early trophozoite stages was achieved by incubating infected RBCs (iRBCs) in 5% (w/v) sorbitol for 10-20 min at room temperature [5]. *L. donovani* amastigotes were harvested from the spleens of donor mice. Briefly, female RAG1B6 KO mice, infected with *L. donovani* at least 60 days before, were humanely killed. At necropsy, spleens were dissected and homogenized, and the amastigotes harvested by differential centrifugation [6].

Sample preparation

For fresh whole blood spiked with *P. falciparum* iRBCs, stock iRBCs with 8-10% parasitemia were diluted in fresh whole blood (Cambridge Biosciences, Cambridge, UK) to attain a parasitemia of 1%. Two additional tenfold dilutions were performed to obtain samples with parasitemias of 0.1 and 0.01%. The parasitemias of the 1 and 0.1 % samples were confirmed by microscopy (Supplementary file 1). Nonspiked fresh whole blood was used as a negative control. As a reference, levels of parasitemia of 0.1-0.2% (5000-10,000 parasites per μ l of blood) are generally accepted to be the point at which fever begins and a patient becomes symptomatic for *P. falciparum* malaria [7]. For *Leishmania* parasite preparations, parasites (amastigotes) were washed twice in RPMI 1640 without serum prior to counting and were used at a concentration of 1×10^7 /ml.

Column purification

For iRBC-spiked fresh whole blood, the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) and DNA-XT™ DNA extraction kit (QuantuMDx) were used to process samples. Samples (iRBC-spiked or nonspiked fresh whole blood) were processed following the manufacturer's instructions (blood protocols). A 10- μ l sample volume was used for the DNA-XT kit and a 100- μ l sample volume for the DNeasy kit (termed Q100). Notably, only 40 μ l of the 80- μ l lysis step is passed through DNA-XT columns after initial processing; thus, only 5 μ l of the original blood sample passes through the column (Figure 1). In light of this, an additional arm of the study used DNeasy columns with a 5- μ l sample volume (termed Q5) to allow a more direct comparison of the two extraction methodologies.

For *Leishmania* parasite preparations, the same two DNA extraction kits were used to process samples. For the DNA-XT kit, 10 μ l of sample was used, following the manufacturer's instructions (blood protocol); for the DNeasy kit, 5 μ l of sample was used, again following the manufacturer's instructions (nucleated blood protocol).

DNA quantitation

The volume of the eluate (although technically flow-through in the case of the DNA-XT kit) was noted and the DNA concentration determined by fluorescence using a Qubit™ spectrophotometer (Thermo Fisher Scientific). The two kits have a fivefold difference in final eluate/flow-through volume, with DNeasy recommending eluting in 200 μ l and DNA-XT having a flow-through of approximately 40 μ l.

PCR

PCR targeting *P. falciparum* β tubulin (*PfBetaT*) was performed using Phire™ Hot Start II DNA Polymerase (Thermo Fisher Scientific). Two volumes of template (1 and 10 μ l) were used in parallel reactions with primers (*PfBetaT* F, 5'-TTGGGGTCCTTCCCCTTTATTGTAT-3'; *PfBetaT* R, 5'-CAAAGGGGCCAGCAC-GAACACT-3') at final concentrations of 200 nM. Cycling conditions were 95°C for 30 s, then 35 cycles of 95°C for 30 s, 55°C for 30 s and 68°C for 30 s, followed by 68°C for 10 min.

PCR targeting *L. donovani* kinetoplast DNA (high copy number) and DNA polymerase I (Pol I; low copy number) was performed using Phusion™ polymerase (Thermo Fisher Scientific). Volumes of 1 and 10 μ l *L. donovani* eluate were used, respectively, with primers for the kinetoplast DNA (*LdKinetoplast* F2, 5'-CCAAT-GAAGCCAAGCCAGTG-3'; *LdKinetoplast* R2, 5'-GGCTGGTTTTAGATGTGGGC-3') and DNA Pol I (*LdDNApol* I F, 5'-TGTCGCTT-GCAGACCAGATG-3'; *LdDNApol* I R, 5'-GCATCGCAGGTGTGAGCAC-3') [8] at final concentrations of 200 nM. Cycling conditions were 98°C for 30 s, then 10 cycles of 10 s at 98°C, 10 s at 69-59°C touchdown and 2 s at 72°C, followed by 20 cycles of 10 s at 98°C, 10 s at 59°C and 2s at 72°C, which were followed by 72°C for 5 min. PCR products were electrophoresed on 1% (w/v) agarose gels and visualized with UV.

Statistical analysis

For the eluate DNA concentrations, a Student's *t*-test (unpaired, equal variance) was performed using Prism Mac 6.0h (GraphPad Software, CA, USA).

Results & Discussion

Extraction

Process timings—The time to complete extractions from eight samples using each of the kits was recorded. For the DNA-XT kit, the extractions took between 50 and 60 min, compared with 120-130 min for the DNeasy kit.

Manipulations

The number of manipulations required to perform the extractions was also tallied. For an individual sample, the DNA-XT kit required 14 manipulations, whereas the DNeasy kit required 25. A manipulation was considered to be any change of tip or tube/wash column or any action (e.g., centrifugation or incubation step). In terms of plastics usage for a single sample, the DNA-XT kit used five tips and four tubes (including the column), and the DNeasy kit required eight tips and seven tubes.

Eluate characteristics

P. falciparum—All three column extraction arms (Q100, Q5 and DNA-XT) had eluates that contained DNA, as measured by fluorescence spectrometry (Table 1). Given that the theoretical amount of human DNA in each sample far exceeds that of any parasite DNA, samples spiked with different levels of iRBCs were analyzed both alone and together when

comparing yields. Unsurprisingly, in the Q100 arm there was a greater yield of DNA than in the Q5 arm. This difference was 21-fold (mean \pm SEM, 4420 ± 340 vs 205 ± 11 ng; $n = 12$), which is in line with the different volumes of blood processed in the Q100 and Q5 arms (100 and 5 μ l, respectively). Assessing the Q5 and DNA-XT arms where the same amount of sample was passed through the respective column, similar concentrations of DNA were obtained (mean \pm SEM, 0.63 ± 0.04 vs 1.06 ± 0.06 ng/ μ l, respectively; $n = 12$), with the Q5 arm performing significantly better ($p = 0.013$), albeit by 1.7-fold (only a single batch of each column type was tested here). Notably, the volume of eluate produced when using each manufacturer's protocol is different: ~ 200 μ l for Q100 and Q5 and ~ 40 μ l for DNA-XT. Thus, the total DNA yield from the Q5 column was higher than that of the DNA-XT column (7.8-fold).

L. donovani—For the *L. donovani* extractions, only the Q5 and DNA-XT protocols were used (i.e., those that use 5 μ l of sample). Both Q5 and DNA-XT had eluates that contained detectable, albeit low, levels of DNA, with the exception of one replicate of the Q5 arm (Table 2). Again, similar concentrations of DNA were obtained in the Q5 and DNA-XT arms (mean \pm SEM, 0.06 ± 0.002 vs 0.17 ± 0.01 ng/ μ l, respectively; $n = 2$ or 3), with the DNA-XT arm performing slightly better by 2.8-fold. Due to the different eluate volumes, the total DNA yield from the Q5 column was slightly higher than that of the DNA-XT column (1.5-fold).

Parasite DNA quality

P. falciparum—While the DNA extracted in these experiments reflects human DNA predominantly, parasite DNA was collected from spiked samples. Its presence and quality in eluates were determined by PCR detection of *Pf*BetaT. Two different volumes of eluate were used, a low volume (1 μ l) and a high volume (10 μ l). In most cases, amplicon bands of the appropriate size (388 bp) could be detected in samples in which parasite DNA should be present (Figure 2). When using the low volume of eluate, the strongest bands were produced from the Q100 eluates. This was to be expected, as the Q100 eluates contain the highest total DNA concentrations (Table 1). Bands were also visible, though fainter, in lanes using Q5 and DNA-XT samples, even at the lowest (0.01%) parasitemia used. However, when a greater (tenfold) volume of eluate was used, only the DNA-XT-derived samples resulted in positive amplicon bands in a parasitemia-dependent manner. This suggests an apparent inhibition of PCR when using high volumes of the Q5 and Q100 samples. The findings presented in Figure 2 are supported by data from three independent repeats of the experiment (with duplicate technical repeats within each). The number of positive bands in each (see Supplementary file 2 for PCR gel images not present in the main text) are presented in Table 3.

L. donovani—DNA was successfully amplified from low-volume (1 μ l) eluates of purified *L. donovani* amastigotes derived from Q5 and DNA-XT extraction technologies when targeting the kinetoplast, a high copy number DNA sequence (Figure 3A). Targeting a single copy number gene, *Leishmania* spp. DNA Pol I, a PCR amplicon, was obtained with a larger volume (10 μ l) of DNA-XT-derived eluate, but not with Q5-derived eluate (Figure 3B).

Here, a simple process for the extraction of DNA from small, parasite-containing culture samples was assessed against a standard commercial, spin column-based extraction process that is widely used in research and diagnostic laboratories. A considerable difference between the two techniques is that DNA is retained on the Qiagen DNeasy mini spin column prior to elution, but the QuantuMDx DNA-XT column retains contaminants and only the DNA is eluted in one centrifugation step. The advantages of this process, as demonstrated in this study, are time savings, reduced plastics use, reduced reagent use (including removing the necessity to use guanidinium chloride) and a reduction in PCR-inhibiting contaminants. However, total yields of DNA are much lower, generally, when using the DNA-XT protocol.

The DNA-XT methodology is similar to a previously described DNA isolation method that uses a polyaniline-containing silica sorbent [9]. In terms of ease of use, the DNA-XT methodology is also comparable with DNA extraction protocols that use Chelex® 100 (Bio-Rad Laboratories, CA, USA). Both of these alternative DNA extraction techniques have been used for forensics applications and perform well with samples that have low DNA concentrations [10–12]. In this study, DNA-XT was used to extract DNA from whole blood spiked with *P. falciparum*-infected RBCs, simulating a clinical diagnostic sample, and purified *L. donovani* amastigotes, as might be used in a research laboratory. In both cases, it was possible to amplify parasite DNA, although the majority of DNA in whole-blood extracts would be expected to be human DNA. Furthermore, with comparable input volumes, the DNA-XT technology compared favorably with the Qiagen extraction kit. Thus, DNA-XT has potential uses within laboratories where DNA extraction is required from small sample volumes.

The technology behind DNA-XT was designed for microfluidic-based, point-of-care, molecular diagnostics platforms, where cell lysis and DNA extraction occur within a cassette to provide DNA for microfluidic PCR and subsequent amplicon sensing. In the case of malaria diagnosis at point-of-care (and other diseases caused by blood-borne pathogens), capillary sampling from finger or heel pricks are the preferred options to generate test samples for parasite detection. Capillary sampling can be safely performed by staff with basic training and is often quicker and less stressful when compared with venepuncture [13]. Capillary sampling has also been used extensively for epidemiological studies of malaria with the use of blood spot collection on filter paper [14–16].

Future Perspective

Because DNA-XT has been demonstrated here to produce high-quality parasite gDNA (seemingly free of the types of PCR inhibitors that frequently contaminate whole-blood extracts [17–19]), this technology may also be useful for extracting blood from dried blood spots, as has been demonstrated for other DNA extraction methodologies [20]. Further applications for high-quality DNA extraction include genotyping for therapeutic diagnostics (e.g., parasite drug resistance/susceptibility) and epidemiology for monitoring asymptomatic individuals [21,22] and those with submicroscopic parasitemia within a population [23]. These could be particularly useful in potential eradication programs [24–26]. Future work for diagnostic applications for the DNA-XT technology should include patient samples and a larger sample size to field-test this new technology. In addition, a more accurate method of

DNA quantification should be used in these proof-of-concept studies, namely quantitative PCR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Method Summary

DNA-Xt, which is designed for small sample volumes, uses a 5-min detergent and enzymatic lysis step to release DNA from cells. Contaminating proteins and lipids are then bound to a matrix within a spin column during a 1-min centrifugation step while DNA passes directly through.

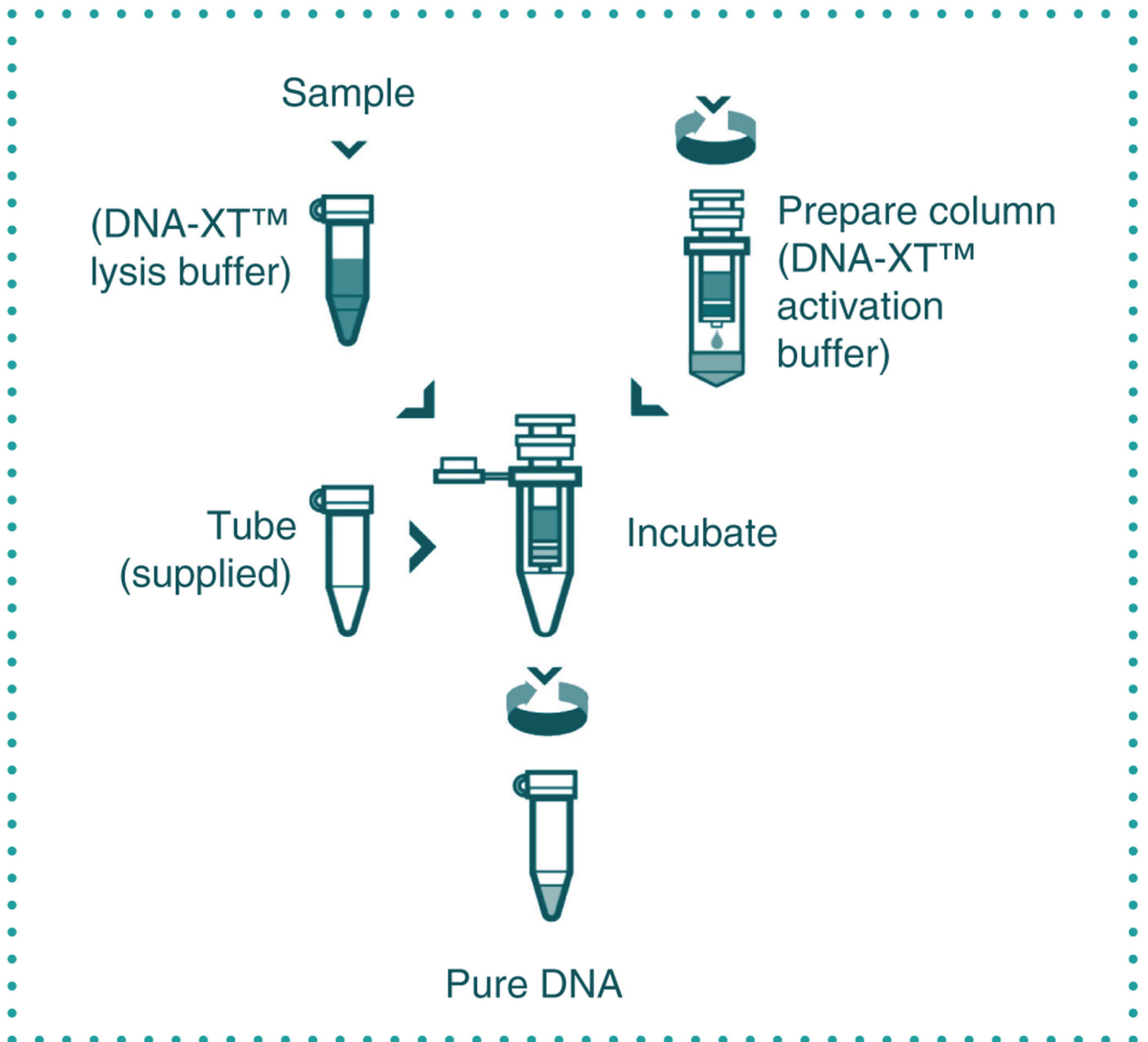


Figure 1.

DNA-XT protocol. Sample preparation: 10 μl of blood/sample was added to 70 μl of lysis buffer, and the mixture was incubated at 55°C for 10 min. Column preparation: 350 μl of buffer was added prior to centrifugation (1400 $\times g$, 3 min), and 40 μl of incubated lysis buffer/sample was added to the prepared column and incubated at room temperature for 3 min prior to centrifugation (1400 $\times g$, 3 min).

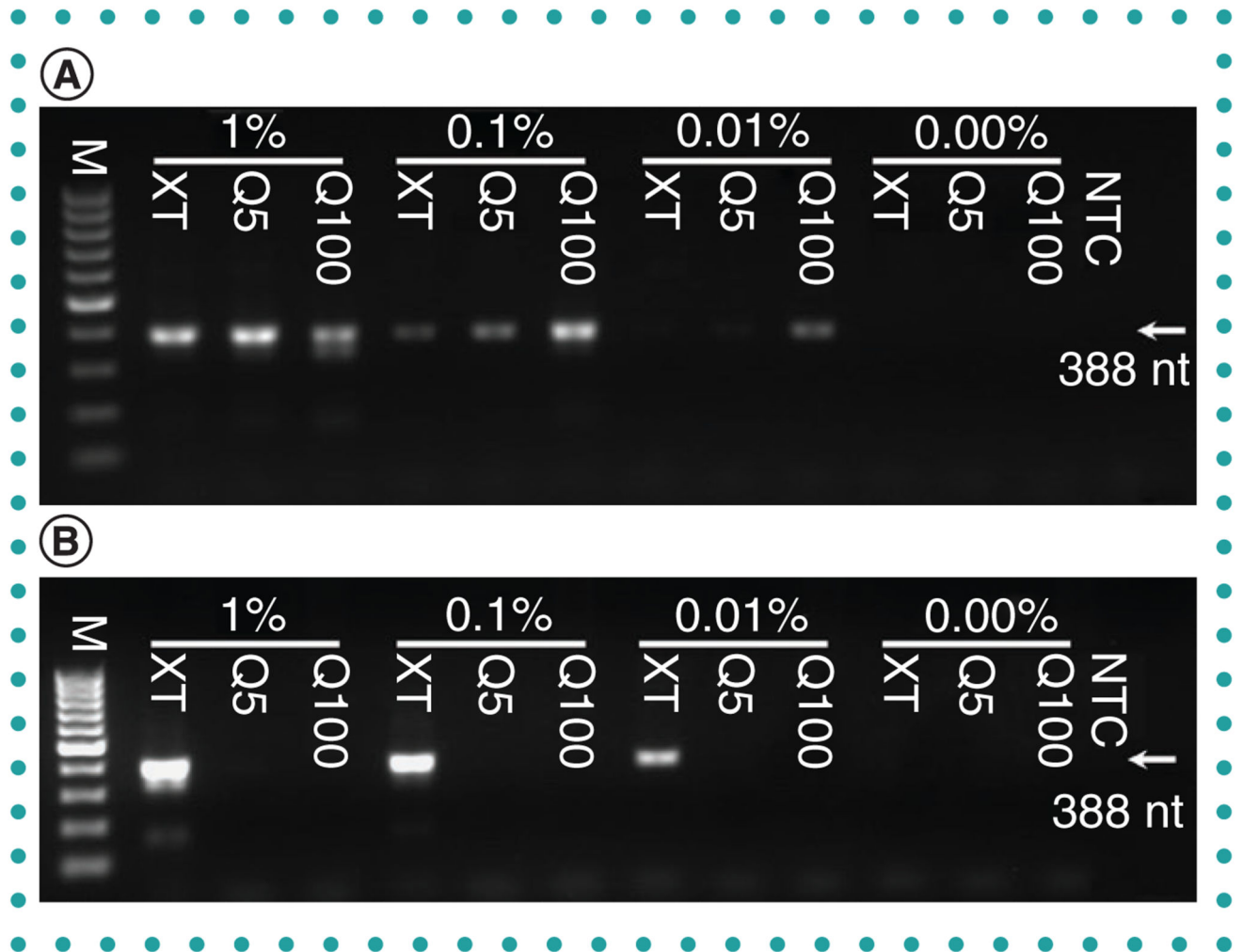


Figure 2.
Plasmodium falciparum DNA from spiked whole blood determined by *Pf*Beta tubulin PCR.
 (A) Low-volume eluate template, 1 µl. (B) High-volume eluate template, 10 µl. Percentage denotes the parasitemia after spiking.
 M: 100-bp marker; NTC: No template control.

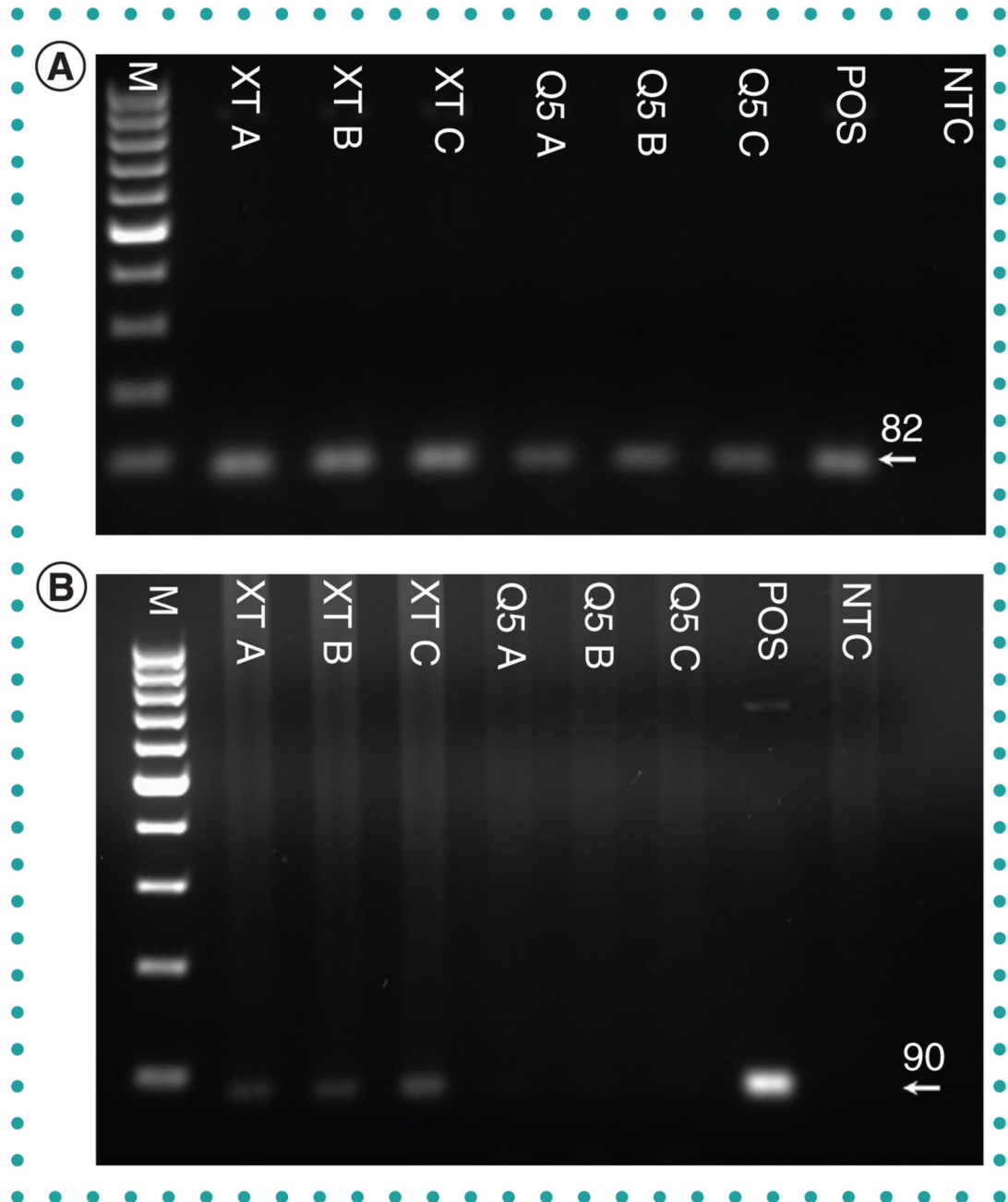


Figure 3.

Leishmania donovani DNA extracted from purified amastigotes and determined by PCR targeting the kinetoplast sequence. (A) 1 µl of eluate from each manufacturer's column was used as a template. (B) 10 µl of eluate from each manufacturer's column was used as a template. Eluates from three separate columns for each manufacturer were used. M: 100-bp marker; NTC: No template control; POS: Positive control.

Table 1
Eluate characteristics for *Plasmodium falciparum*-spiked whole blood-derived samples

Column	Parasitemia	Eluate volume (mean \pm SEM, μ l)	[DNA] (mean \pm SEM, ng/ μ l)	Total DNA yield (mean \pm SEM, ng)	n
Q100	Overall	197 \pm 1.4	22.4 \pm 1.6	4420 \pm 340	12
	1%		22.1 \pm 1.5	4320 \pm 290	3
	0.1%		21.4 \pm 2.2	4260 \pm 500	3
	0.01%		24.5 \pm 0.5	4880 \pm 170	3
	0%		21.5 \pm 1.1	4210 \pm 210	3
Q5	Overall	194 \pm 0.4	1.06 \pm 0.06	205 \pm 11	12
	1%		1.06 \pm 0.02	204 \pm 4.5	3
	0.1%		1.09 \pm 0.12	211 \pm 23	3
	0.01%		1.08 \pm 0.03	209 \pm 5.6	3
	0%		1.02 \pm 0.05	197 \pm 10	3
DNA-XT	Overall	42 \pm 0.4	0.63 \pm 0.04	26.3 \pm 1.7	12
	1%		0.65 \pm 0.03	28.4 \pm 1.1	3
	0.1%		0.59 \pm 0.05	24.8 \pm 2.3	3
	0.01%		0.68 \pm 0.05	28.9 \pm 2.6	3
	0%		0.55 \pm 0.07	22.8 \pm 3.7	3

Table 2
Eluate characteristics for purified *Leishmania donovani* amastigote samples

Column		Eluate volume (µl)	[DNA] (ng/µl)	Total DNA yield (ng)
Q5	Mean ± SEM	193 ± 1.2	0.06 ± 0.002	11 ± 0.4
	A	191	Too low	nd
	B	195	0.06	11
	C	193	0.05	10
DNA-XT	Mean ± SEM	42 ± 0.3	0.17 ± 0.01	7.3 ± 0.2
	A	42	0.16	6.6
	B	42	0.11	7.2
	C	43	0.19	8.1

nd: Not determined.

Table 3
PCR positive outcomes for *Plasmodium falciparum*-spiked whole blood-derived samples

Human whole blood	1% parasitemia (pos/ total)	0.1% parasitemia (pos/ total)	0.01% parasitemia (pos/total)	0% unspiked (pos/ total)
High-volume template Q5	2/6	2/6	2/6	0/6
High-volume template Q100	0/6	1/6	0/6	0/6
High-volume template DNA-XT	6/6	6/6	6/6	0/6
Low-volume template Q100	6/6	6/6	5/6	0/6
Low-volume template Q5	5/6	6/6	6/6	0/6
Low-volume template DNA-XT	6/6	6/6	5/6	0/6

pos: Gel band positive.