



Article

Comparison of the Influence of Different Nucleic Acid Extraction Assays on the Sensitivity of *Trypanosoma cruzi*-Specific Real-Time PCR

Simone Kann ^{1,*}, Wendy Zabala-Monterroza ², Cenia García ², Gustavo Concha ³, Olfert Landt ⁴,
Andreas Hahn ⁵, Felix Weinreich ⁶ and Hagen Frickmann ^{5,6}

¹ Medical Mission Institute, 97074 Würzburg, Germany

² Public Health Laboratory, Secretariat of Health, Department Cesar, Valledupar 20000001, Colombia; wendyzabalam@gmail.com (W.Z.-M.); celigajurado@gmail.com (C.G.)

³ Organization Wiwa Yugumaiun Bunkauanarrua Tayrona (OWYBT), Department Health Advocacy, Valledupar 2000001, Colombia; gustavoconcha16@gmail.com

⁴ TibMolBiol, 10103 Berlin, Germany; olandt@tib-molbiol.de

⁵ Institute for Medical Microbiology, Virology and Hygiene, University Medicine Rostock, 18057 Rostock, Germany; andreas.hahn@uni-rostock.de (A.H.); hagen.frickmann@med.uni-rostock.de (H.F.)

⁶ Department of Microbiology and Hospital Hygiene, Bundeswehr Hospital Hamburg, 20359 Hamburg, Germany; felixweinreich@bundeswehr.org

* Correspondence: simone.kann@medmissio.de



Citation: Kann, S.;

Zabala-Monterroza, W.; García, C.;

Concha, G.; Landt, O.; Hahn, A.;

Weinreich, F.; Frickmann, H.

Comparison of the Influence of
Different Nucleic Acid Extraction

Assays on the Sensitivity of

Trypanosoma cruzi-Specific Real-Time

PCR. *Microorganisms* **2022**, *10*, 1554.

[https://doi.org/10.3390/](https://doi.org/10.3390/microorganisms10081554)

[microorganisms10081554](https://doi.org/10.3390/microorganisms10081554)

Academic Editor: André Talvani

Received: 25 May 2022

Accepted: 29 July 2022

Published: 31 July 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: For the molecular diagnosis of Chagas disease by real-time PCR (polymerase chain reaction), optimization of diagnostic accuracy is desirable. The detection limit of real-time PCR assays for the diagnosis of *Trypanosoma cruzi* in human serum is affected by various influences including the choice of the nucleic acid extraction assay. In this study, three nucleic acid extraction assays were compared regarding their influence on the sensitivity of a *T. cruzi*-specific real-time PCR with 62 reference sera containing *T. cruzi* target DNA (deoxyribonucleotide acid). More than 95% of the positive sera were correctly identified after all three nucleic acid extraction strategies with a detection rate ranging from 96.8% (60/62) for the worst assay to 100% (62/62) for the best one. A matched pairs analysis for the comparison of the cycle threshold (Ct) values obtained with the 59 reference samples with positive real-time PCR results after all three nucleic acid extraction schemes indicated differences in a range of about 3 Ct steps. Summarized, all three compared nucleic acid extraction schemes were basically suitable for *T. cruzi*-specific PCR from serum with some minor differences. However, in the case of low quantities of circulating parasite DNA in the serum of a patient with Chagas disease, even minor effects can make a difference in the individual diagnosis.

Keywords: Chagas; pre-analytics; evaluation; diagnostic accuracy; test comparison

1. Introduction

Poverty-related Chagas disease, which is, e.g., endemic in resource-limited tropical settings in South America [1], has been of increasing interest for the development of molecular diagnostic test assays in the recent two decades [2–15]. However, different from other systemic parasitic diseases such as malaria, for which excellent diagnostic accuracy of molecular diagnostic approaches has been repeatedly shown [16–18], the reliability of molecular techniques for the diagnosis of Chagas disease has often been less convincing in previous studies [8,10–14]. Reasons for the observed varying reliability of *Trypanosoma cruzi*-specific molecular diagnostic assays comprise the pathogen's high genetic variability, but also its close phylogenetic relationship to other parasites [11–14]. As another factor, influencing the diagnostic accuracy with particular relevance for sensitivity, it has been described that the whole diagnostic process needs to be optimized, which also includes the choice of the most suitable nucleic acid extraction technique [8].

The relevance of adequate nucleic acid extraction approaches for the diagnostic reliability of the molecular diagnostic detection of parasitic diseases is not specific to Chagas disease only. For helminth infections, in particular, the diagnostic sensitivity of real-time PCR-based detection strongly depends on the choice of the applied extraction strategy [19–21]. Protocols comprising digestion steps and bead beating-based disruption of pathogen cells to release their target DNA have been described as particularly useful [19–21]. Even the appropriate choice of the beads for the bead beating has been shown to make a difference regarding the yield of target DNA within the sample [20]. Interestingly, the superiority of more robust extraction procedures compared to standard procedures was not found to be consistent for all helminth species [22].

Protozoan parasites are no exemption either. The reliability of their diagnostic detection with molecular assays has been shown to depend on the sufficient release of target DNA from parasite cells. Again, harsh extraction assays containing digestion, freeze-thawing, or bead-beating steps were associated with superior results compared to less robust extraction approaches [23,24].

In this study, three different nucleic acid extraction assays were applied with serum samples from Colombian Indigenous people with known active Chagas disease and subsequent analysis with the NDO (“newly developed one”) real-time PCR, a recently described assay specifically amplifying *T. cruzi* DNA from human serum samples [15]. By doing so, optimization of nucleic acid extraction with the aim to improve sensitivity for *T. cruzi* detection from human serum was intended.

2. Materials and Methods

2.1. Sample Materials

A total of 62 frozen residual serum sample materials from Indigenous people living in remote areas of Colombia with active Chagas disease were included in the assessment. Circulation of *T. cruzi* DNA in serum had been confirmed by PCR in a previously published study [15] as well as in the course of other yet unpublished investigations. More than this, the reference materials had been pre-characterized by multiple approaches as detailed elsewhere [15], including rapid antigen testing, serology based on enzyme-linked immunosorbent assay (ELISA) and/or immunofluorescence testing (IFT), real PCR for kinetoplast DNA (kDNA), the 18 S ribosomal ribonucleotide acid gene (18 S rRNA), and *T. cruzi* nuclear DNA (TCZ), in 9 out of 62 cases even by sequencing of PCR amplicons. So, Chagas disease was considered as well confirmed. The samples had been collected from Chagas patients at the Colombian sites Ashintukwa (n = 20), Ahuyamal (n = 6), Cherua (n = 2), Dungakare (n = 1), Marocaso (n = 3), Sabannah Crespo (n = 2), Sabannah de Higuieron (n = 5), Seminke (n = 14), and Tezhumake (n = 9). The patients’ female:male ratio was nearly balanced with 32 females and 30 males, the mean age \pm standard deviation was 24.5 ± 17.5 , and the median age (interquartile range IQR) was 19.5 (12; 34). Prior to further assessments, the serum samples were stored deep-frozen at $-80\text{ }^{\circ}\text{C}$ to preserve the quality of the DNA.

2.2. Applied Nucleic Acid Extraction Schemes

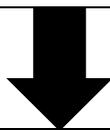
All included frozen serum samples were subjected to nucleic acid extraction as described by the manufacturers’ protocols, applying the following three nucleic acid extraction assays: the RTP (“Ready-to-Prep”) Pathogen kit (Invitex Molecular GmbH, Berlin, Germany; later referred to as RTP assay), the MagaBio plus Virus DNA/RNA purification kit version 2 (Hangzhou Bioer Technology Co., Ltd., Hangzhou, China; later referred to as the MagaBio assay), and the EZ1&2 Virus Mini Kit v2.0 performed on an EZ1 extraction automate (Qiagen, Hilden, Germany; later referred to as the Qiagen assay). The extracted sample volumes and the obtained eluate volumes were in a similar range over the compared extraction schemes with 200 μL and 60 μL for the RTP assay, 300 μL and 80 μL for the MagaBio assay, as well as 200 μL and 60 μL for the Qiagen assay, respectively. The extractions were performed in close temporal association to avoid bias due to different

states of DNA degradation within the samples. The eluates were stored at -80°C prior to further assessment by real-time PCR.

2.3. Applied Real-Time PCR Targeting *T. cruzi* DNA in the Eluates

The Chagas real-time PCR (NDO-PCR, patented, purchased at TibMolBiol, Berlin, Germany: *T. cruzi* LightMix[®], Ref 53-0755-96, Phocid Herpes Virus (PhHV) Extraction control reference 66-0901-96, Lyophilized 1-step RT-(real-time-)PCR Polymerase Mix, Cat-No 90-9999-96) was applied with all nucleic acid eluates as described previously [15,25] on a RotorGene Q cycler (Qiagen, Hilden, Germany). As a minor modification compared to a previous description of the assay, the probe sequence had been slightly altered to 5'-TCG + AACCCC + ACCTCC-3', the "+" symbol marks locked nucleic acid (LNA) bases included to alter the annealing temperature. Positive control samples containing target DNA, as well as PCR-grade water-based negative controls, were included in each run. Extraction, as well as sample inhibition, was controlled, applying the commercial Phocid herpes virus-(PhHV)-DNA-based PhHV spiked extraction control (DNA) assay mentioned above. To simulate routine-like diagnostic conditions, each eluate was only assessed once by the *T. cruzi*-specific PCR. Only samples with negative results in the *T. cruzi*-specific PCR were repeatedly amplified once to exclude random errors during pipetting of the PCR assays. A schematic representation of the diagnostic workflow is indicated in Figure 1.

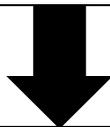
Identification of 62 deep-frozen (-80°C) residual samples of pre-characterized serum containing *T. cruzi* DNA from patients with Chagas disease



Nucleic acid extraction applying the RTP Pathogen kit (Invitek Molecular GmbH, Berlin, Germany; later referred to as RTP assay); extracted sample volumes and the obtained eluate volumes: 200 μL and 60 μL .

Nucleic acid extraction applying the MagaBio plus Virus DNA/RNA purification kit version 2 (Hangzhou Bioer Technology Co., Ltd., Hangzhou, China; later referred to as the MagaBio assay); extracted sample volumes and the obtained eluate volumes: 300 μL and 80 μL .

Nucleic acid extraction applying the EZ1&2 Virus Mini Kit v2.0 performed on an EZ1 extraction automate (Qiagen, Hilden, Germany; later referred to as the Qiagen assay); extracted sample volumes and the obtained eluate volumes: 200 μL and 60 μL .



Diagnostic application of a commercial *T. cruzi* DNA-specific real-time PCR (NDO-PCR, patented, purchased at TibMolBiol, Berlin, Germany: *T. cruzi* LightMix[®], Ref 53-0755-96, PhHV Extraction Ctrl. Ref. 66-0901-96, Lyophilized 1-step RT-PCR Polymerase Mix, Cat-No 90-9999-96) after deep-frozen storage of the eluates at -80°C

Figure 1. Flow-chart of the diagnostic workflow.

2.4. Statistical Assessment

Due to the low number of included positive reference sample materials in the assessment, reaction failures were just descriptively recorded. Samples, for which a positive *T. cruzi*-specific PCR result was recorded from all three nucleic acid extraction attempts, were included in the comparison of the cycle threshold (Ct) values of the real-time PCR. After passing Kolmogorov–Smirnov testing for normality, the Ct values of the paired rows were subjected to repeated measures ANOVA (analysis of variance) with multi comparison post testing according to Tukey–Kramer in case of significant results in the ANOVA assessment. The analysis was performed applying the software GraphPad InStat, version 3.06 (GraphPad Software Inc., La Jolla, San Diego, CA, USA).

2.5. Ethics

Ethical clearances applicable for this study comprised the clearance “2019_HA 163 Acta No 2019-4”, provided by the Ethics Committee for Investigation, Bogota, Colombia in 2019, the clearance “2016_HA 190 Acta No 032018”, provided by the Ethics Committee, St. Marta, Colombia in 2018 and also the clearance “BWF/H/52228/2012/13.10.10-1/3.4.6Tropendiagnostik, Acto No 0022013”, provided by the Ethics committee Valledupar, Colombia, in 2013. Informed consent was obtained from the study participants or next to kin. In addition, ethical clearance for the technical evaluation was granted by the medical association of Hamburg, Germany (reference number: WF-011/19, obtained on 11 March 2019), allowing anonymous use of residual sample materials for test comparison purposes even without informed consent. The assessments were conducted according to the guidelines of the Declaration of Helsinki.

3. Results

3.1. Effects of the Nucleic Acid Extraction Schemes on PCR Failure in the Qualitative Assessment

Positive *T. cruzi*-specific PCR results with 100% (62/62) of the assessed reference materials from patients with active Chagas disease were recorded after nucleic acid extraction with the RTP assay only. The MagaBio assay scored second-best with 98.4% (61/62) correctly identified samples containing *T. cruzi*-specific DNA, closely followed by the Qiagen assay with 96.8% (60/62) correct identifications. All positive samples showed positive results already during the first run, while the failed reactions remained negative in the repetitions. The extraction and inhibition controls did not indicate sample inhibition as a source of interference.

3.2. Effects of the Nucleic Acid Extraction Schemes on the Recorded Cycle Threshold (Ct) Values

Due to positive *T. cruzi*-specific PCR results, 59 samples were included in the matched pair analysis of the measured cycle threshold (Ct) values. As indicated in Table 1, the lowest mean Ct values were calculated for the RTP assay followed by the Qiagen assay and the MagaBio assay, with standard deviations in a comparable range for all three assays (Table 1). The mean difference between the RTP extraction assay and the MagaBio assay was less than 3 Ct values and, thus, less than a decadic logarithmic step. The repeated measures ANOVA confirmed variation between the quantitative results of the three compared assays ($p < 0.0001$). In the Tukey–Kramer multiple comparisons test, the significance of lower Ct values in comparison to the MagaBio assay could be confirmed for the RTP assay ($p < 0.001$) and the Qiagen assay ($p < 0.05$), while no statistically significant difference between the RTP assay and the Qiagen assay was observed. Effective matching was confirmed by the assumption test with a p -value of 0.0078.

Table 1. Mean values and standard deviations of the cycle threshold (Ct) values measured after nucleic acid extraction with the different assays with the 59 samples, which showed positive *T. cruzi*-specific PCR results with eluates from all three assessed nucleic acid extraction schemes.

	Nucleic Acid Extraction with the RTP Assay	Nucleic Acid Extraction with the MagaBio Assay	Nucleic Acid Extraction with the Qiagen Assay
Mean value of the measured Ct values	30.8	33.4	31.8
Standard deviation of the measured Ct value	3.0	2.8	3.6

Focusing on the three reference materials with discordant results after real-time PCR from the eluates obtained with the different nucleic acid extraction assays, the sample missed after extraction with the MagaBio assay showed Ct values of 32.9 and 22.0 in the PCR reactions after extraction with the RTP assay and the Qiagen assay, respectively. For the two samples that were missed by PCR after the Qiagen assay-based extraction, Ct

values of 32.2 and 33.5 were measured after extraction with the RTP assay, while Ct values of 36.2 and 38.4 were recorded after extraction with the MagaBio assay for the two samples missed by the Qiagen assay.

The raw data of the measured Ct values for all 62 samples subjected to all three compared nucleic acid extraction schemes are shown in Appendix A Table A1.

4. Discussion

The study was performed to assess the effects of three different nucleic acid extraction schemes on the diagnostic sensitivity of real-time PCR for the identification of *T. cruzi*-specific DNA in human serum samples. With all three applied nucleic acid extraction schemes, more than 95% of the assessed *T. cruzi* DNA-positive reference samples were correctly identified as positive. However, minor differences were observed and so, only extraction with the RTP assay was associated with the detection of all positive samples, while one sample was missed after extraction with the MagaBio assay and two after extraction with the Qiagen assay. Thereby, the reaction failures affected different samples for both types of nucleic acid extraction. Compared to the calculated mean values obtained with the eluates from the three compared nucleic acid extraction methods, the samples with the failed PCR reactions after individual extraction schemes showed mostly higher cycle threshold (Ct) values with the exemption of a very low Ct value after extraction with the Qiagen extraction for the sample missed after extraction with the MagaBio assay. These higher Ct values are indicative of low amounts of target DNA, explaining the individually failed reactions with target DNA amounts close to the diagnostic detection threshold. However, and as shown in a previously published study, which introduced the performed *T. cruzi*-specific PCR [15], target DNA amounts in serum samples of Chagas patients are often close to the detection threshold. While a previous study had shown good sensitivity of more than 90% [15] for the *T. cruzi*-specific PCR applied in the present investigation, individual samples had gone undetected due to low parasitemia close to the diagnostic detection threshold. Accordingly, even such minor differences as observed with the compared nucleic acid extraction assays may indeed be of relevance for the diagnosis in individual patients.

When focusing on Ct value-based semi-quantification rather than on qualitative PCR results alone, repeated measures ANOVA was performed in order to assess whether the eluates from the different extraction schemes formed a homogenous group. As indicated by the calculation, this was obviously not the case. While there was no statistically significant difference between the RTP assay and the Qiagen assay, significant evidence for later Ct values after nucleic acid extraction with the MagaBio assay compared to both competitor assays could be shown, indicating slightly reduced sensitivity by a mean of less than 3 Ct steps, corresponding to less than a decadic logarithmic step. This phenomenon is even more pronounced by the fact that due to a higher extracted sample volume with a proportionally lower eluate volume compared to the competitor assays, even a slightly higher concentration of *T. cruzi* DNA in the eluates after MagaBio assay-based extraction might have been expected. This observation suggests that the MagaBio assays either resulted in a lower yield of target DNA in the eluates or, alternatively, were associated with a chemical component within the eluates that interfered with the amplification efficiency of the *T. cruzi*-specific PCR.

Although there are considerably more nucleic acid extraction assays available on the market that might have been included in the assessment, the included assays were not arbitrarily chosen. The RTP assay had already been applied for the nucleic acid extractions in the course of the initial study on the evaluation of the applied *T. cruzi*-specific PCR [15] and is the standard procedure included in the diagnostic workflow at the participating laboratory in Würzburg, Germany. The MagaBio assay is the standard nucleic acid extraction scheme applied by the Colombian project partners for the *T. cruzi* PCRs on site in the area of endemicity. The Qiagen assay is the standard approach for nucleic acid extraction from serum samples in the participating laboratory in Hamburg, Germany, and has already been

successfully applied for the diagnosis of visceral leishmaniasis from human serum samples by the German National Reference Center for Tropical Pathogens [26]. Accordingly, the choice of the assays reflects the diagnostic real-life situation in settings where the molecular diagnosis of *T. cruzi* and other parasites from patient sera is indeed applied.

The study has a number of limitations. First, only a limited number of pre-characterized *T. cruzi*-positive reference materials were available for the study. However, considering the good pre-characterization of the reference material, it remains difficult to obtain higher quantities even in the course of multicentric assessments as conducted here. Second, the study was performed with stored residual samples, so DNA degradation in comparison to the time of sample acquisition cannot be excluded despite frozen storage. To circumvent this problem, all three extraction schemes were applied in close temporal association to keep DNA degradation-associated bias as low as possible for the comparison. So, comparable sample conditions can be assumed for all extraction approaches. Third, nucleic acid quantification within the eluates was not performed. The diagnostic workflow in the study mimicked the workflow as performed in the diagnostic routine, in which such a step would not be part of the standard procedures. So, the PCR results were chosen as the only outcome parameter. Fourth, no extraction assay providing particularly harsh extraction conditions especially adapted to parasites [8,19–24] was included in the assessment, as the amplification of freely circulating DNA was intended. As stated above, the choice of the assessed assays was meant to reflect the diagnostic real-life conditions at the participating study sites.

5. Conclusions

The study confirmed acceptable diagnostic sensitivity >95%, as calculated for the tested reference materials, with all compared nucleic acid extraction assays. Focusing on the qualitative results, the ranking order of diagnostic reliability was RTP assay > MagaBio assay > Qiagen assay. Focusing on the comparison of the cycle threshold (Ct) values, the ranking order was RTP assay = Qiagen assay > MagaBio assay. These rankings are associated with a residual uncertainty arising from the fact that limited amounts of residual sample materials did not allow repeated nucleic acid extractions with each assay from the same sample. So, confirmation by testing in replicas was unfeasible. However, even minor differences may be of relevance for individual diagnostic decisions in the case of DNA quantities in serum close to the diagnostic detection threshold [15]. The major strength of the study, despite its abovementioned limitations, is the comparably high number of well-characterized residual sample materials available for the assessment. Accordingly, it can be assumed that the conclusions of the study are empirically well confirmed.

Author Contributions: Conceptualization, S.K. and H.F.; methodology, S.K., A.H., W.Z.-M., C.G.; software, S.K. and A.H.; validation, S.K.; formal analysis, A.H., S.K., W.Z.-M., C.G.; investigation, S.K. and F.W.; resources, S.K., G.C., O.L. and H.F.; data curation, S.K.; writing—original draft preparation, H.F.; writing—review and editing, S.K., G.C., O.L., A.H., F.W. and H.F.; visualization, A.H.; supervision, S.K.; project administration, S.K.; funding acquisition, S.K. and H.F. All authors have read and agreed to the published version of the manuscript.

Funding: Parts of the study (extraction with the Qiagen assay) were funded by grant 36K2-S-45 1922 “Evaluation and optimization of molecular diagnostic tests for tropical parasitic diseases for surveillance and risk assessment purposes in tropical deployment settings—a German–French co-operation project between the German Armed Forces Hospital Hamburg and the Military Hospital Laveran, Marseille” of the German Ministry of Defense (MoD) awarded to Hagen Frickmann. The other parts of the study (extraction with RTP and MagaBio) were funded within the ongoing study 2019_HA163: “Colombia–Germany research program on diagnostics, research, treatment and prevention of Chagas Disease (CD) and emerging infectious diseases in vulnerable groups” of the Else Kröner-Fresenius-Stiftung (EKFS), Germany.

Institutional Review Board Statement: Ethical clearances applicable for this study comprised the clearance “2019_HA 163 Acta No 2019-4”, provided by the Ethics Committee for Investigation, Bogota, Colombia in 2019, the clearance “2016_HA 190 Acta No 032018”, provided by the Ethics

Committee, St. Marta, Colombia in 2018 and also the clearance “BWF/H/52228/2012/13.10.10-1/3.4,6Tropendiagnostik, Acto No 0022013”, provided by the Ethics committee Valledupar, Colombia in 2013. Informed consent was obtained from the study participants or next to kin. In addition, ethical clearance for the technical evaluation was granted by the medical association of Hamburg, Germany (reference number: WF-011/19, obtained on 11 March 2019), allowing anonymous use of residual sample materials for test comparison purposes even without informed consent. The assessments were conducted according to the guidelines of the Declaration of Helsinki.

Informed Consent Statement: Not applicable as granted by the medical association of Hamburg, Germany (reference number: WF-011/19, obtained on 11 March 2019).

Data Availability Statement: All relevant data are provided in the manuscript. Raw data can be provided at reasonable request.

Acknowledgments: Annett Michel is gratefully acknowledged for technical assistance.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. One of the co-authors, Olfert Landt, is the founder of TibMolBiol, Berlin, Germany, the company that contributed the Chagas RT-PCR Kit.

Appendix A

Table A1. Cycle threshold values measured with the different nucleic acid extraction assays.

Sample Number	RTP Assay	MagaBio Assay	Qiagen Assay
0001	30.9	33.5	33.7
0002	28.2	31.6	32.5
0003	26.2	30.9	28.2
0004	30.3	33.1	28.2
0005	27.6	29.2	27.6
0006	26.9	31.3	30.3
0007	31.8	32.1	28.2
0008	33.3	36.7	28.6
0009	30.1	32.0	27.9
0010	29.2	33.6	30.0
0011	30.8	34.6	30.8
0012	27.8	29.4	27.6
0013	28.8	32.3	39.7
0014	30.6	34.8	31.6
0015	36.5	27.7	31.5
0016	29.7	33.3	33.6
0017	30.1	37.6	22.2
0018	39.2	26.7	39.9
0019	31.1	38.0	34.4
0020	32.0	33.5	33.6
0021	30.4	31.8	30.1
0022	28.5	31.8	33.8
0023	32.9	34.8	31.5
0024	31.8	34.6	33.0
0025	30.9	38.8	34.9
0026	34.8	34.2	38.6
0027	29.7	34.3	36.0
0028	30.0	33.0	36.3
0029	20.5	35.7	37.1
0030	32.9	33.6	27.8

Table A1. Cont.

Sample Number	RTP Assay	MagaBio Assay	Qiagen Assay
0031	28.9	33.6	28.0
0032	30.4	34.0	34.4
0033	30.6	28.3	31.1
0034	37.1	40.8	32.6
0035	33.1	35.4	23.8
0036	32.9	repeatedly negative	22.0
0037	35.7	28.9	30.9
0038	27.4	31.3	29.9
0039	34.9	31.1	34.7
0040	31.3	32.8	30.6
0041	33.7	34.2	32.1
0042	29.0	31.3	28.4
0043	32.3	37.1	32.6
0044	27.8	29.8	28.0
0045	29.5	33.3	32.7
0046	28.3	31.8	29.6
0047	31.7	33.1	31.6
0048	31.2	35.8	34.0
0049	27.9	33.5	29.5
0050	31.1	37.8	37.5
0051	29.1	35.3	28.5
0052	28.5	33.1	28.8
0053	29.4	34.6	32.0
0054	35.6	38.9	38.0
0055	36.4	37.5	37.4
0056	32.2	36.2	repeatedly negative
0057	31.2	36.2	33.4
0058	30.0	33.2	32.2
0059	30.3	31.1	34.4
0060	32.8	34.4	34.0
0061	33.5	38.4	repeatedly negative
0062	28.8	29.7	31.3

References

- Andrade, D.V.; Gollob, K.J.; Dutra, W.O. Acute chagas disease: New global challenges for an old neglected disease. *PLoS Negl. Trop. Dis.* **2014**, *8*, e3010. [[CrossRef](#)] [[PubMed](#)]
- Schijman, A.G.; Bisio, M.; Orellana, L.; Sued, M.; Duffy, T.; Mejia Jaramillo, A.M.; Cura, C.; Auter, F.; Veron, V.; Qvarnstrom, Y.; et al. International study to evaluate PCR methods for detection of *Trypanosoma cruzi* DNA in blood samples from Chagas disease patients. *PLoS Negl. Trop. Dis.* **2011**, *5*, e931. [[CrossRef](#)]
- Mattos, E.C.; Meira-Strejevitch, C.D.S.; Marciano, M.A.M.; Faccini, C.C.; Lourenço, A.M.; Pereira-Chioccola, V.L. Molecular detection of *Trypanosoma cruzi* in acai pulp and sugarcane juice. *Acta Trop.* **2017**, *176*, 311–315. [[CrossRef](#)] [[PubMed](#)]
- Finamore-Araujo, P.; Faier-Pereira, A.; Ramon do Nascimento Brito, C.; Gomes Peres, E.; Kazumy de Lima Yamaguchi, K.; Trotta Barroso Ferreira, R.; Moreira, O.C. Validation of a novel multiplex real-time PCR assay for *Trypanosoma cruzi* detection and quantification in acai pulp. *PLoS ONE* **2021**, *16*, e0246435. [[CrossRef](#)]
- Rampazzo, R.C.P.; Graziani, A.C.; Leite, K.K.; Surdi, J.A.; Biondo, C.A.; Costa, M.L.N.; Jacomasso, T.; Cereda, M.; De Fazio, M.; Bianchessi, M.A.; et al. Proof of Concept for a Portable Platform for Molecular Diagnosis of Tropical Diseases: On-Chip Ready-to-Use Real-Time Quantitative PCR for Detection of *Trypanosoma cruzi* or *Plasmodium* spp. *J. Mol. Diagn.* **2019**, *21*, 839–851. [[CrossRef](#)]
- Melo, M.F.; Moreira, O.C.; Tenório, P.; Lorena, V.; Lorena-Rezende, I.; Júnior, W.O.; Gomes, Y.; Britto, C. Usefulness of real time PCR to quantify parasite load in serum samples from chronic Chagas disease patients. *Parasit. Vectors* **2015**, *8*, 154. [[CrossRef](#)] [[PubMed](#)]
- Ferreira Filho, J.C.R.; Braz, L.M.A.; Andriano, M.L.A.; Yamamoto, L.; Kanunfre, K.A.; Okay, T.S. Mitochondrial and satellite real time-PCR for detecting *T. cruzi* DTU II strain in blood and organs of experimentally infected mice presenting different levels of parasite load. *Exp. Parasitol.* **2019**, *200*, 13–15. [[CrossRef](#)] [[PubMed](#)]
- Abras, A.; Ballart, C.; Llovet, T.; Roig, C.; Gutiérrez, C.; Tebar, S.; Berenguer, P.; Pinazo, M.J.; Posada, E.; Gascón, J.; et al. Introducing automation to the molecular diagnosis of *Trypanosoma cruzi* infection: A comparative study of sample treatments, DNA extraction methods and real-time PCR assays. *PLoS ONE* **2018**, *13*, e0195738. [[CrossRef](#)]

9. Duffy, T.; Bisio, M.; Altchek, J.; Burgos, J.M.; Diez, M.; Levin, M.J.; Favalaro, R.R.; Freilij, H.; Schijman, A.G. Accurate real-time PCR strategy for monitoring bloodstream parasitic loads in chagas disease patients. *PLoS Negl. Trop. Dis.* **2009**, *3*, e419. [[CrossRef](#)] [[PubMed](#)]
10. Longoni, S.S.; Pomari, E.; Antonelli, A.; Formenti, F.; Silva, R.; Tais, S.; Scarso, S.; Rossolini, G.M.; Angheben, A.; Perandin, F. Performance Evaluation of a Commercial Real-Time PCR Assay and of an In-House Real-Time PCR for *Trypanosoma cruzi* DNA Detection in a Tropical Medicine Reference Center, Northern Italy. *Microorganisms* **2020**, *8*, 1692. [[CrossRef](#)]
11. Qvarnstrom, Y.; Schijman, A.G.; Veron, V.; Aznar, C.; Steurer, F.; da Silva, A.J. Sensitive and specific detection of *Trypanosoma cruzi* DNA in clinical specimens using a multi-target real-time PCR approach. *PLoS Negl. Trop. Dis.* **2012**, *6*, e1689. [[CrossRef](#)]
12. Piron, M.; Fisa, R.; Casamitjana, N.; López-Chejade, P.; Puig, L.; Vergés, M.; Gascón, J.; Gómez i Prat, J.; Portús, M.; Sauleda, S. Development of a real-time PCR assay for *Trypanosoma cruzi* detection in blood samples. *Acta Trop.* **2007**, *103*, 195–200. [[CrossRef](#)]
13. Ramírez, J.C.; Cura, C.I.; da Cruz Moreira, O.; Lages-Silva, E.; Juiz, N.; Velázquez, E.; Ramírez, J.D.; Alberti, A.; Pavia, P.; Flores-Chávez, M.D.; et al. Analytical Validation of Quantitative Real-Time PCR Methods for Quantification of *Trypanosoma cruzi* DNA in Blood Samples from Chagas Disease Patients. *J. Mol. Diagn.* **2015**, *17*, 605–615. [[CrossRef](#)]
14. De Winne, K.; Büscher, P.; Luquetti, A.O.; Tavares, S.B.; Oliveira, R.A.; Solari, A.; Zulantay, I.; Apt, W.; Diosque, P.; Monje Rumi, M.; et al. The *Trypanosoma cruzi* satellite DNA OligoC-TesT and *Trypanosoma cruzi* kinetoplast DNA OligoC-TesT for diagnosis of Chagas disease: A multi-cohort comparative evaluation study. *PLoS Negl. Trop. Dis.* **2014**, *8*, e2633. [[CrossRef](#)]
15. Kann, S.; Kunz, M.; Hansen, J.; Sievertsen, J.; Crespo, J.J.; Loperena, A.; Arriens, S.; Dandekar, T. Chagas Disease: Detection of *Trypanosoma cruzi* by a New, High-Specific Real Time PCR. *J. Clin. Med.* **2020**, *9*, 1517. [[CrossRef](#)]
16. Altangerel, E.; Frickmann, H. Meta-analysis of the diagnostic performance characteristics of three commercial and one in-house nucleic acid amplification tests for malaria screening. *J. Lab. Med.* **2020**, *44*, 47–53. [[CrossRef](#)]
17. Frickmann, H.; Hinz, R.; Rojak, S.; Bonow, I.; Ruben, S.; Wegner, C.; Zielke, I.; Hagen, R.M.; Tannich, E. Evaluation of automated loop-mediated amplification (LAMP) for routine malaria detection in blood samples of German travelers—A cross-sectional study. *Travel Med. Infect. Dis.* **2018**, *24*, 25–30. [[CrossRef](#)]
18. Frickmann, H.; Wegner, C.; Ruben, S.; Behrens, C.; Kollenda, H.; Hinz, R.; Rojak, S.; Schwarz, N.G.; Hagen, R.M.; Tannich, E. Evaluation of the multiplex real-time PCR assays RealStar malaria S&T PCR kit 1.0 and FTD malaria differentiation for the differentiation of Plasmodium species in clinical samples. *Travel Med. Infect. Dis.* **2019**, *31*, 101442.
19. Moundounga, H.K.; Adegnik, A.A.; Nkoma, A.-M.; Ateba-Ngoa, U.; Mbong, M.; Zinsou, J.; Lell, B.; Verweij, J.J. Impact of Short-Time Urine Freezing on the Sensitivity of an Established Schistosoma Real-Time PCR Assay. *Am. J. Trop. Med. Hyg.* **2014**, *90*, 1153–1155.
20. Kaisar, M.M.M.; Brien, E.A.T.; Djuardi, Y.; Sartono, E.; Yazdanbakhsh, M.; Verweij, J.J.; Supali, T.; Van Lieshout, L. Improved diagnosis of *Trichuris trichiura* by using a bead-beating procedure on ethanol preserved stool samples prior to DNA isolation and the performance of multiplex real-time PCR for intestinal parasites. *Parasitology* **2017**, *144*, 965–974. [[CrossRef](#)]
21. Ayana, M.; Cools, P.; Mekonnen, Z.; Biruksew, A.; Dana, D.; Rashwan, N.; Prichard, R.; Vlaminc, J.; Verweij, J.J.; Levecke, B. Comparison of four DNA extraction and three preservation protocols for the molecular detection and quantification of soil-transmitted helminths in stool. *PLoS Negl. Trop. Dis.* **2019**, *13*, e0007778. [[CrossRef](#)] [[PubMed](#)]
22. Hoffmann, T.; Hahn, A.; Verweij, J.J.; Lebouille, G.; Landt, O.; Strube, C.; Kann, S.; Dekker, D.; May, J.; Frickmann, H.; et al. Differing Effects of Standard and Harsh Nucleic Acid Extraction Procedures on Diagnostic Helminth Real-Time PCRs Applied to Human Stool Samples. *Pathogens* **2021**, *10*, 188. [[CrossRef](#)] [[PubMed](#)]
23. Moss, J.A.; Gordy, J.; Snyder, R.A. Effective concentration and detection of cryptosporidium, giardia, and the microsporidia from environmental matrices. *J. Pathog.* **2014**, *2014*, 408204. [[CrossRef](#)]
24. Menu, E.; Mary, C.; Toga, I.; Raoult, D.; Ranque, S.; Bittar, F. Evaluation of two DNA extraction methods for the PCR-based detection of eukaryotic enteric pathogens in fecal samples. *BMC Res. Notes* **2018**, *11*, 206. [[CrossRef](#)] [[PubMed](#)]
25. Tanida, K.; Balczun, C.; Hahn, A.; Veit, A.; Nickel, B.; Poppert, S.; Scheid, P.L.; Hagen, R.M.; Frickmann, H.; Loderstädt, U.; et al. Comparison of Three In-House Real PCR Assays Targeting Kinetoplast DNA, the Small Subunit Ribosomal RNA Gene and the Glucose-6-Phosphate Isomerase Gene for the Detection of Leishmania spp. in Human Serum. *Pathogens* **2021**, *10*, 826. [[CrossRef](#)] [[PubMed](#)]
26. Hahn, A.; Podbielski, A.; Meyer, T.; Zautner, A.E.; Loderstädt, U.; Schwarz, N.G.; Krüger, A.; Cadar, D.; Frickmann, H. On detection thresholds—a review on diagnostic approaches in the infectious disease laboratory and the interpretation of their results. *Acta Trop.* **2020**, *205*, 105377. [[CrossRef](#)] [[PubMed](#)]