Leishmania parasite detection and quantification using PCR-ELISA

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This protocol describes an improved and optimized PCR-ELISA method for detection and quantification of *Leishmania* parasites in host tissues. Unlike other DNA-based assays, this method uses digoxigenin- and biotin-labeled primers. This eliminates the need for a separate step of hybridization of the PCR product with labeled probes. The PCR product is detected using sandwich ELISA with antidigoxigenin-detecting antibodies. Primers are complementary to the kinetoplast minicircle conserved region of parasite DNA, allowing the detection of several *Leishmania* species. For measurement of a wide range of parasite concentrations, ±25 cycles were optimal. The sensitivity of this technique is 0.3 fg of parasite DNA per reaction in 40-cycle PCR-ELISA, corresponding to 0.004 parasites. DNA preparation by a standard TRI reagent procedure takes about 4 h. When DNA is prepared, a single person can test a large number of samples (at least 150) in a maximum of 7 h. This method might also be suitable for detecting and quantifying other pathogens, especially for detecting small differences in pathogen numbers.

INTRODUCTION

Infectious diseases are a major health problem in a number of countries. Detection of pathogens confirms a diagnosis, characterizes the host defense and determines the effectiveness of the applied therapy.

The model of *Leishmania* infection in mice reveals complex interactions between the pathogen and the host[1]. Methods of parasite detection include microscopy[2], cell cultures[3–5], immunodetection[6] and DNA-based techniques[7–17]. Culture and microscopy methods are very slow and laborious, particularly when a large number of samples are analyzed. Immunodetection methods based on estimation of anti-*Leishmania* antibody levels do not reflect the exact parasite load because the relation between the presence of antibodies and parasites depends highly on individual responsiveness and the general health condition of the host organism. DNA-based methods of parasite detection and quantification have therefore been developed to overcome these limitations. PCR-ELISA is cheaper than real-time PCR, has high sensitivity and allows the detection of small differences in parasite load[10–12]; however, it is complicated and laborious because it requires a PCR reaction and a subsequent hybridization step. We improved and optimized the PCR-ELISA method[18] for detection and quantification of *Leishmania major* parasites. The new technique uses two labeled primers; therefore, the step of hybridization with labeled probes[10–12] is omitted. By virtue of its incorporated biotin, the PCR product is attached to the streptavidin-coated plate, and can be visualized by peroxidase-linked antidigoxigenin antibody (**Fig. 1**). The primer molecules that were not engaged in the reaction are not detectable by ELISA. Tissues or DNA can be stored at −70 or −20 °C, which allows performing or repeating the measurement at any time.

The present technique can be helpful both in scientific research and in the diagnosis of leishmaniasis. The principle of the method may be used for detection and quantification of other pathogens after selection of appropriate primers.

MATERIALS

REAGENTS

- Parasites (*L. major* LV 561 (MHOM/IL/67/LRC-L137 JERICHO II) promastigotes; see REAGENT SETUP) **! CAUTION** *L. major* is a causal agent of human cutaneous leishmaniasis. Avoid contact of media containing *Leishmania* parasites with open wounds. Always work with gloves in a biological safety cabinet.
- Organs or tissues (lymph nodes obtained from male BALB/cHeA (BALB/c (n = 14)) and STS/A (STS (n = 17)) mouse strains killed 8 weeks after *L. major* infection)[19]
- PBS (see REAGENT SETUP)
- NaCl (Sigma-Aldrich, cat. no. S7653)
- Na_2HPO_4 (Sigma-Aldrich, cat. no. S9390)
- KH_2PO_4 (Sigma-Aldrich, cat. no. P5655)
- KCl (Sigma-Aldrich, cat. no. P9333) **! CAUTION** Avoid contact with skin and eyes and do not breathe dust.
- Lysis buffer (see REAGENT SETUP)
- Tris(hydroxymethyl)-aminomethane (SERVA Electrophoresis, cat. no. 37190) **! CAUTION** Irritating to eyes, respiratory system and skin. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice.
- EDTA-disodium (SERVA Electrophoresis, cat. no. 11280; pH 8.0)
- SDS (Sigma-Aldrich, cat. no. L5750) **! CAUTION** Highly flammable. Harmful when it comes in contact with skin and if swallowed. Irritating to eyes, respiratory system and skin. Wear suitable gloves and eye/face protection, as well as protective clothing. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice.
- Proteinase K (20 mg ml^{-1}; Sigma-Aldrich, cat. no. P6556)
- Isopropanol (PENTA, cat. no. 59300) **! CAUTION** Highly flammable Irritating to eyes. Vapors may cause drowsiness and dizziness. Keep container tightly closed. Keep away from sources of ignition—no smoking.

Avoid contact with skin and eyes. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice.
- Ethanol (PENTA, cat. no. 32294) **! CAUTION** Highly flammable. Keep the container tightly closed. Keep away from sources of ignition—no smoking.
- Chloroform (PENTA, cat. no. 25692) **! CAUTION** It is harmful and there is danger of serious damage to health by prolonged exposure through inhalation and if swallowed. It is irritating to the skin. There is limited evidence of carcinogenic effect. Wear suitable protective clothing and gloves.
- TRI reagent (Molecular Research Center, cat. no. TR 118) **! CAUTION** Contains phenol and thiocyanate. It causes burns. It is poisonous and can be fatal. It is toxic if inhaled, if it comes in contact with skin and if swallowed. It is harmful and there is danger of serious damage to health by prolonged exposure through inhalation and if swallowed. There is a possible risk of irreversible effects. Avoid contact with skin and eyes. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice. Wear suitable gloves and eye/face protection, and also wear protective clothing. In case of accident or if one feels unwell, seek medical advice immediately. Keep away from food, drink and animal food. Avoid release into the environment.
- DNA washing solution (100 mM sodium citrate tribasic hydrate, Sigma-Aldrich, cat. no. 25114); in 10% (vol/vol) ethanol (PENTA, cat. no. 32294)
- NaOH (8 mM; Sigma, cat. no. S8045) **! CAUTION** It causes severe burns. Wear suitable gloves and eye/face protection. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice. In case of accident or if one feels unwell, seek medical advice immediately.
- TE buffer (see REAGENT SETUP)
- Primers complementary to the 120-bp conserved region present in kinetoplast minicircle DNA (**Fig. 1**): digoxigenin-labeled F 5′-ATTTTACAC-CAACCCCCAGTT-3′ and biotin-labeled R 5′-GTGGGGGAGGGGCGT-TCT-3′ (ref. 8) (VBC-GENOMICS Biosciences research)
- PCR buffer without $MgCl_2$ (10×; Invitrogen, cat. no. Y02028 or Sigma-Aldrich, cat. no. P2317-1.5ML)
- $MgCl_2$ (50 mM; Sigma-Aldrich, cat. no. M8266) **! CAUTION** Do not breathe dust. Avoid contact with skin and eyes **▲ CRITICAL** The concentration of $MgCl_2$ must be exact.
- dNTPs (100 mM, deoxynucleotide set; Sigma-Aldrich, cat. no. DNTP100A-1KT)
- Taq DNA polymerase (5 U μl^{-1}) **▲ CRITICAL** The polymerase from Invitrogen, cat. no. 10342-020, is strongly recommended as the most effective and as lacking nonspecific priming under the described conditions (compared with Taq DNA Polymerase from Sigma, cat. no. D4545; DynaZyme II DNA Polymerase from Finnzymes, cat. no. F-503; or Perfect Taq RED from Central European Biosystems, cat. no. CEB001).
- Streptavidin (Sigma-Aldrich, cat. no. S4762)
- Coating buffer (100 mM $NaHCO_3$ (pH 7.0); Sigma-Aldrich, cat. no. S6297)
- ELISA washing solution (0.5% Tween 20 (Sigma-Aldrich, cat. no. P1379) in PBS)
- FCS/PBS (2% (vol/vol) fetal calf serum (Sigma-Aldrich, cat. no. F 2442) in PBS) **▲ CRITICAL** It must be prepared under sterile conditions.
- Antidigoxigenin-detecting antibody Fab fragments (Roche Diagnostic GmbH, cat. no. 11 207 733 910)
- ABTS substrate solution (150 mg ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt); Sigma-Aldrich, cat. no. A1888) in 500 ml of 100 mM citric acid (pH 4.35) (Sigma-Aldrich, cat. no. C7129) **! CAUTION** It is irritating to respiratory system and skin. There is a risk of serious damage to eyes. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice. Wear suitable gloves and eye/face protection, as well as protective clothing **▲ CRITICAL** It must be stored in the dark at −20 °C in 11-ml aliquots in vials; it is not recommended to be refrozen.
- H_2O_2 (30%; PENTA cat. no. 95313) **! CAUTION** Harmful if swallowed. Risk of serious damage to eyes. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice. Wear suitable gloves and eye/face protection, as well as protective clothing. In case of accident or if one feels unwell, seek medical advice immediately.
- Stop solution (500 mM oxalic acid; Sigma-Aldrich, cat. no. 75688) **! CAUTION** Harmful if it comes in contact with skin and if swallowed. Avoid contact with eyes also.

EQUIPMENT
- Biological Safety Cabinet (Telstar BioUltra)
- Centrifuges (Eppendorf 5810 R and Eppendorf MiniSpin, Eppendorf)

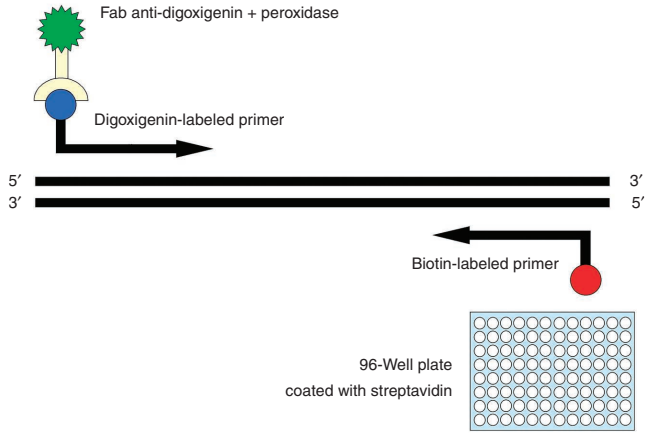

Figure 1 | PCR-ELISA scheme. The method uses two labeled primers for the PCR amplification of target DNA: one is labeled with biotin, the other with digoxigenin. The resulting PCR product (amplicon) with biotin incorporated to one end and dioxigenin to the other is transferred to the microtiter plate coated with streptavidin that binds biotin. The detection of immobilized amplicon is performed by antidigoxigenin antibodies conjugated to peroxidase with final colorimetric development.

- NanoDrop Spectrophotometer (ND-1000, Thermo Scientific)
- DNA Engine DYAD Peltier Thermal Cycler or I-Cycler (Bio-Rad Laboratories)
- ELISA-reader Sunrise (Tecan Group)
- Microtubes (1.5 ml; Axygen, cat. no. MCT-150-C)
- Manual homogenizers (Zymo Research, cat. no H1001-50)
- Pipette tips with filter (Gilson, DF30ST, cat. no. F161933 and DF200ST, cat. no. F161934) for PCR mixture preparation **▲ CRITICAL** Tips with filter should be used to exclude the possibility of contamination.
- 96-well PCR plates (ABgene, cat. no. AB-0600) with cap strips (ABgene, cat. no. AB-0784)
- 96-well ELISA plates (EIA/RIA Clear Flat Bottom Microplate, Corning, cat. no. 3590)
- Curve fitter program KIM-E for Windows (version 5.14, Dan Kittrich, Shoeller Pharma)
- The statistical program Statistica for Windows (version 8.0, StatSoft)

REAGENT SETUP

Cultivate parasites (*L. major* LV 561 (MHOM/IL/67/LRC-L137 JERICHO II) promastigotes) in a diphasic SNB-9 (saline–neopeptone–blood) medium in flat flasks (Nunclon, cat. no. 156340)[20]. Wash stationary-phase promastigotes (7-d-old subculture 2) three times in sterile saline, adjust to 2×10^8 promastigotes per ml in sterile saline and inoculate 50 µl containing 10^7 promastigotes into the mouse tail base[19].

PBS Comprises 140 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and 2.7 mM KCl. To prepare 1 liter of PBS, dissolve 8.18 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4 and 0.2 g KCl in a small volume of distilled water and adjust volume to 1 liter with distilled water. Solution can be stored at room temperature for up to 1 year.

Lysis buffer Comprises 100 mM tris(hydroxymethyl)-aminomethane, 5 mM EDTA-disodium (pH 8.0), 0.2% SDS and 200 mM NaCl. For preparation of 1 liter of lysis buffer, dissolve 10 ml 20% (wt/vol) SDS, 12.12 g Tris, 10 ml 500 mM EDTA (pH 8.0) and 11.68 g NaCl in a small volume of distilled water and adjust to the final volume of 1 liter with distilled water. To prepare 500 mM EDTA, add 372.3 g of EDTA to 500–700 ml of distilled water, mixing and gradually adding NaOH granules until EDTA is dissolved completely. Adjust pH to 8.0; adjust volume to 1 liter with distilled water. Solution can be stored at room temperature for up to 1 year.

TE buffer Comprises 1 mM EDTA-disodium (pH 8.0) and 10 mM tris(hydroxymethyl)-aminomethane. For preparation of 1 liter of TE buffer, dissolve 1.21 g of Tris and 2 ml of 500 mM EDTA in a small volume of distilled water and adjust volume to 1 liter with distilled water. Solution can be stored at room temperature for up to 1 year.

PROCEDURE

DNA preparation

1| Prepare DNA from parasites or from analyzed tissues. DNA can be prepared in one of two alternative ways: DNA isolation using TRI reagent (based on the protocol of RNA, DNA and protein isolation http://www.mrcgene.com/tri.htm; option A) or through a proteinase procedure[21] (option B).

(A) DNA isolation using TRI reagent ● TIMING ~4 h

(i) Homogenize 50–100 mg of the tissue sample (fresh or frozen) with 1 ml of TRI reagent in a microtube using a manual homogenizer.
▲ **CRITICAL STEP** Sample volume should not exceed 10% of the volume of TRI reagent used for homogenization. Leave the homogenate for 5 min at room temperature (21–23 °C).

(ii) Add 0.2 ml of chloroform per 1 ml of TRI reagent and mix vigorously. Leave the resulting mixture for 2–15 min at room temperature and centrifuge at 12,000*g* for 15 min at 4 °C.

(iii) Remove the aqueous phase overlying the interphase.

(iv) Precipitate DNA from the interphase and organic phase with 0.3 ml of 96% ethanol per 1 ml of TRI reagent used for homogenization; thereafter, mix samples by inversion. Leave the samples at room temperature for 2–3 min and centrifuge at 2,000*g* for 5 min at 4 °C.

(v) Remove the supernatant.

(vi) Wash the pellet twice in 1 ml of DNA washing solution. At each wash, leave the DNA pellet in the DNA washing solution for 30 min at room temperature with periodic mixing by hand and centrifuge at 2,000*g* for 5 min at 4 °C; discard the supernatant.

(vii) Suspend the DNA pellet in 1 ml of 75% ethanol. Set aside for 10–20 min at room temperature with periodic mixing by hand and centrifuge at 2,000*g* for 5 min at 4 °C.

(viii) Remove ethanol and briefly air-dry DNA pellets by keeping tubes open for 5 min at room temperature.

(ix) Dissolve DNA pellets in 0.3 ml of 8 mM NaOH by slowly passing through the pipette tip. Leave DNA samples for about 1 h at room temperature to dissolve.

(x) Centrifuge at 12,000*g* for 10 min to remove insoluble material and transfer the resulting supernatant containing DNA to new tubes.

(xi) Measure DNA concentration using a NanoDrop spectrophotometer.
■ **PAUSE POINT** DNA can be left overnight at 4 °C or stored in a freezer at −20 or −70 °C for years.

(xii) Dilute the DNA samples from tissues to 10 ng μl^{-1}, and parasite DNA (standard) to 4 ng μl^{-1}.
■ **PAUSE POINT** DNA can be left overnight at 4 °C or stored in a freezer at −20 or −70 °C for several years.

(B) Proteinase procedure[21] ● TIMING ~2 d

(i) Add 750 µl of lysis buffer, containing 100 µg ml^{-1} of proteinase K, to the parasite solution (10^8 promastigotes in 500 ml of PBS) or to 50–100 mg of the tissue sample (fresh or frozen). Lyse the samples at 55 °C overnight.

(ii) Centrifuge the samples for 10 min at 3,220*g* at 4 °C to obtain a firm pellet.
▲ **CRITICAL STEP** For different tissues, the time of centrifugation may vary from 10 to 60 min. Nonlysed particles of tissue should be completely removed from the supernatant, because their presence can influence the quality of DNA.

(iii) Transmit the supernatant carefully to the microtube with isopropanol (1:1) for precipitation and centrifuge for 15 min at 3,220*g* at 4 °C.

(iv) Remove the supernatant carefully. Dry the precipitate for ~5 min at room temperature and resuspend in 250 µl of TE buffer. Leave DNA samples overnight at 4 °C to dissolve.

(v) Measure DNA concentration using the NanoDrop spectrophotometer.
■ **PAUSE POINT** DNA can be left overnight at 4 °C or stored in a freezer at −20 or −70 °C.

(vi) Dilute the DNA samples from tissues to 10 ng μl^{-1} and parasite DNA (standard) to 4 ng μl^{-1}.
■ **PAUSE POINT** DNA can be left overnight at 4 °C or stored in a freezer at −20 or −70 °C for a long period of time.

PCR ● TIMING ~2 h 30 min (procedure using 24 cycles)/~3 h 25 min (procedure using 40 cycles)

2| Prepare the reaction mixture. For each DNA sample, use 15 µl of the reaction mixture that contains 0.1 µl of each kinetoplast primer (50 pmol μl^{-1}); 2 µl of 10× PCR buffer without $MgCl_2$; 0.8 µl of 50 mM $MgCl_2$; 0.1 µl of each 100 mM dNTP; 0.4 µl of 5 U μl^{-1} Taq polymerase; and 11.2 µl of bidistilled water.

▲ **CRITICAL STEP** The mixture should be prepared under sterile conditions in a biological safety cabinet.

▲ **CRITICAL STEP** The final concentration of $MgCl_2$ in the reaction must be 2 mM. The primers used are highly sensitive to $MgCl_2$ concentration; subtle deviations from an optimal concentration (2 mM) resulted in either priming of nonspecific DNA synthesis (≥2.5 mM) or inhibition of DNA amplification and product absence (≤1.5 mM).

3| Add 15 µl of the reaction mixture and 5 µl of DNA sample diluted to 10 ng μl^{-1} (50 ng DNA per each reaction) to each well of a 96-well plate. As a standard, use 5 µl of parasite DNA diluted to 4 ng μl^{-1} (20 ng per reaction). As a negative control, add 5 µl of bidistilled water to the reaction instead of DNA.

4| Cover plates tightly with cap strips and run the PCR. The cycling conditions are as follows: 1 cycle at 94 °C for 90 s, followed by 24 cycles at 94 °C for 30 s, 53 °C for 45 s, 72 °C for 60 s and a final extension for 10 min at 72 °C.

ELISA ● TIMING ~20 h

5| Coat 96-well ELISA plates with streptavidin solution (0.5 µg ml^{-1}) in coating buffer at 100 µl per well and leave them overnight at 4 °C. It is possible to coat ELISA plates overnight at 4 °C or at room temperature for 3 h before Step 2 to speed up the procedure.

6| Wash four times with the ELISA washing solution (0.5% Tween 20 in PBS).

7| Dilute PCR samples to 2% concentration using 2% FCS/PBS (usually 2 µl of PCR product and 98 µl of 2% FCS/PBS in each well of the ELISA plate). Each sample should be tested in duplicate.

8| Dilute the standard (the PCR product of 20 ng of *Leishmania* parasite DNA per reaction—see Step 3). To prepare the highest concentration of the standard (2% PCR product in 2% FCS/PBS), dilute 4 µl of PCR reaction product in 196 µl of 2% FCS/PBS in a well of a 96-well ELISA plate. Prepare a series of 10 subsequent dilutions in which each of the subsequent standard samples is 2× diluted: take 100 µl from the first well (containing the most concentrated standard sample), add it to the next well with 100 µl of FCS/PBS, mix the solution by pumping it through the pipette tip, take 100 µl of the mixture from this (second) well and add to the next (third) well (also with 100 µl of FCS/PBS). Proceed until a complete row of 10 dilutions is prepared. Each standard dilution must be prepared in duplicate.

9| Incubate plates at room temperature for 2 h.

10| Wash six times with the ELISA washing solution.

11| Incubate at room temperature with the antidigoxigenin-detecting antibody Fab fragments (100 µl per well of 0.15 U ml^{-1} in 2% FCS/PBS) for 45 min.

12| Wash plates eight times with the ELISA washing solution.

13| Incubate in the dark with 100 µl per well of ABTS substrate solution with H_2O_2 (1 µl of 30% H_2O_2 per 1 ml of ABTS substrate solution) for 5–15 min to develop color reaction.
▲ **CRITICAL STEP** H_2O_2 should be mixed with the ABTS substrate solution immediately before addition.
? TROUBLESHOOTING

14| Stop the color reaction with a stop solution at a concentration of 50 µl per well.

15| Prepare a plate-reading form and a standard curve in the ELISA-reading software according to the manufacturer's instructions. Read the absorbance values of the plates at a wavelength of 405 nm with a reference filter of 620 nm using the ELISA reader. Estimate the concentration of *L. major* DNA in samples using the curve fitter program KIM-E for Windows (or a similar program) using least squares–based linear regression analysis.
? TROUBLESHOOTING

● TIMING

Step 1, DNA preparation
(A) DNA isolation using TRI reagent: ~4 h
(B) Proteinase procedure: ~2 d
Steps 2–4, PCR: ~2 h 30 min (procedure using 24 cycles)/~3 h 25 min (procedure using 40 cycles)
Steps 5–15, ELISA: ~20 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
13, 15	No color developed in standards, nor in the analyzed samples	Decayed H_2O_2	Use fresh H_2O_2
		Decayed ABTS substrate	ABTS substrate solution must be stored in the dark at −20 °C and must not be refrozen. Otherwise prepare a new solution
		Degraded detecting antibody	Ensure that detecting antibody has not expired and is stored at 4 °C. Otherwise use the fresh antibody
		No product in PCR. (It is possible to check the presence of product by running PCR samples on 2% agarose gel)	Ensure that all PCR conditions are proper and concentrations of all reagents are exact. Increase the number of PCR cycles. However, we do not recommend carrying out more than 40 cycles, because at very high cycle numbers the DNA might get degraded
	The color developed both in standards and analyzed samples, but the ELISA-reading software shows that some samples are out of the standard curve and their values are lower than the standard	Some experimental samples contain very low concentration of the parasite DNA. More detailed calibrating curve is necessary to quantitate them	Make more than 10 serial dilutions of the standard used in ELISA and include points of very low concentrations (**Fig. 3a**)
	The color has developed only in the standard samples but not in the analyzed samples	Number of PCR cycles is too small	Increase the number of PCR cycles. However, we do not recommend carrying out more than 40 cycles, because at very high cycle numbers the DNA might get degraded
		Concentration of DNA used in PCR is too low or too high	Try to increase or decrease the amount of total DNA used in PCR. In our experiments, the optimal concentration of the total DNA is 50 ng per reaction, but it can vary depending on the concentration of parasite DNA in total DNA. Using too much total DNA in PCR can inhibit amplification
	The color has developed only in the analyzed samples but not in the standards	Parasite DNA used as standard was degraded	Dilute fresh parasite DNA or isolate new DNA from parasites
	The color is very strong and there are no differences among the analyzed samples	The number of PCR cycles carried out is too high	Carry out a lower number of PCR cycles. However, we do not recommend carrying out less than 15 PCR cycle because at low cycle numbers, the amount of the PCR product is quite small even in samples with high parasite load. Carrying out too few cycles may lead to false negative results
		Concentration of total DNA in the PCR mixture is too high	Use smaller amount of total DNA in the PCR mixture
		The PCR is contaminated with parasite DNA	Prepare the PCR mixture under sterile conditions using pipette tips with filter. Store PCR reagents separated into small aliquots in microtubes. Find out which reagent is contaminated or take new microtubes of all PCR reagents
		Nonspecific products in PCR (check by running on 2% agarose gel)	Ensure that all PCR conditions are proper and concentrations of all reagents are exact. Use only recommended reagents

ANTICIPATED RESULTS

The improved PCR method is able to detect 1.5 fg of parasite DNA (40 cycles of amplification), which corresponds to 0.02 parasites (**Fig. 2**). By introduction of the signal amplification by ELISA, sensitivity was further increased to 0.3 fg of DNA, which approximately corresponds to 0.004 parasites (assuming an average diploid genome mass of 80 fg (ref. 15)). It is much higher

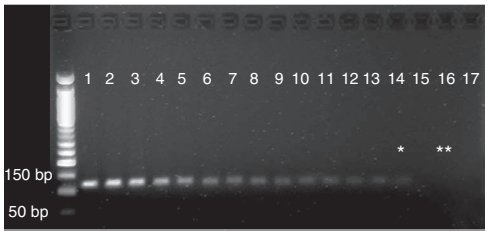

Figure 2 | Sensitivity of detection by PCR. DNA samples were diluted ((1) 5 ng per reaction, (2) 1 ng, (3) 0.5 ng, (4) 0.1 ng, (5) 50 pg, (6) 10 pg, (7) 5 pg, (8) 1 pg, (9) 0.5 pg, (10) 0.1 pg, (11) 20 fg, (12) 5 fg, (13) 3 fg, (14) 1.5 fg, (15) 0.7 fg, (16) 0.3 fg and (17) pure water) and amplified up to 40 cycles. A volume of 20 µl of PCR product was run on 2% agarose gel at 150 V. A 50 bp DNA ladder was used as a marker. *, 1.5 fg per reaction is the minimal DNA concentration at which the amplification product was visible on agarose gel. **, 0.3 fg per reaction is the lowest DNA concentration at which the amplification product was detectable by PCR-ELISA, and it is considered as the technique sensitivity.

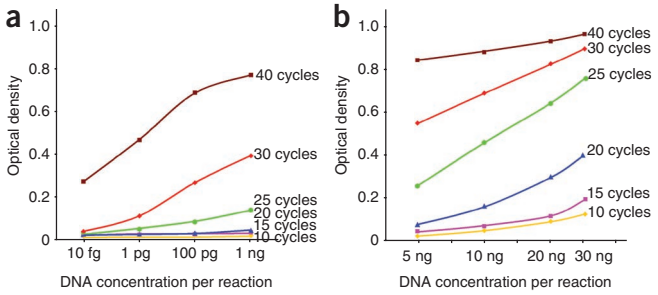

Figure 3 | Standard curves. Comparison of values of optical density obtained with lower (**a**) and higher (**b**) amounts of DNA using different numbers of cycles.

than the sensitivity of culture methods, which are generally lower in sensitivity than PCR methods[22]. It is also more sensitive than microscopy-based methods such as the tissue smear method, which uses a piece of the organ and might give misleading results when parasites are spread unevenly. It has higher or comparable sensitivity compared with other PCR-based techniques[8–17].

We tested the precision of the method by comparing OD values obtained with different amounts of *L. major* DNA using different numbers of cycles of PCR. The results shown in **Figure 3a,b** show a linear relationship between the amount of parasite DNA and the measured OD values over a wide range of DNA concentrations and cycle numbers. The described PCR-ELISA method can be used with 15–40 cycles of PCR. For most purposes, the intermediate number of cycles (around 25) would be optimal. However, the most suitable number of cycles should be selected by the user, taking into account the stage of the disease, susceptibility of the host organism, the organ tested and previous experience.

For analysis of strain differences in parasite loads, we used inguinal lymph nodes from infected male BALB/c and STS mouse strains, which are susceptible and resistant to *L. major*, respectively[23] (**Fig. 4**). Differences in parasite load between the strains were estimated by the Mann–Whitney *U*-test (**Fig. 5**).

The present PCR-ELISA method enabled for the first time the mapping of genetic loci that control parasite numbers in *L. major*-infected mice in a genome-wide search by individually testing 299 F2 hybrids between an intermediate and a susceptible strain[18].

As mentioned above, the primers used in this technique for the detection and quantification of *L. major* parasites are specific to the conserved region present in the kinetoplast minicircle. In addition to *L. major* detection, these primers were previously proven to be suitable for *L. mexicana*, *L. braziliensis*, *L. chagasi*, *L. donovani*, *L. aethiopica* and *L. enterietii*[8], and recently were also successfully tested in our laboratory for the detection and quantification of *L. tropica*. Needless to say, this method, using different primers, can be adapted to measure other parasites, bacteria or viruses.

In summary, the improved PCR-ELISA is an extremely sensitive method that allows simultaneous testing of a large number of samples (at least 150) by a single person and provides results in shorter time.

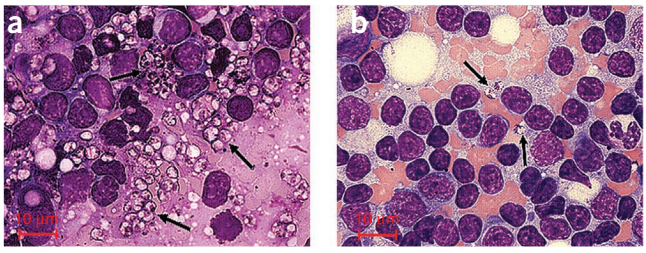

Figure 4 | Giemsa-stained lymph node smears. (**a**) BALB/c mice strain. Arrows show numerous *Leishmania* parasites with a dark nucleus, smaller kinetoplast and light cytoplasm. (**b**) STS mice strain. Arrows show single parasites among lymph node cells. Scale bar (10 µm) was calculated using LAS software (Leica).

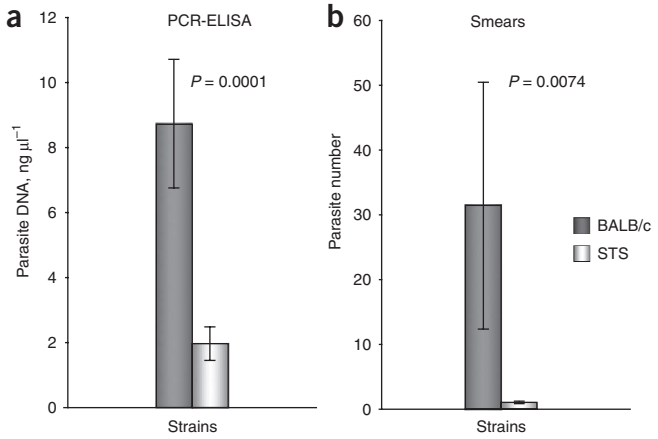

Figure 5 | Parasite load measured with two different techniques. Inguinal lymph nodes from BALB/c ($n=14$) and STS ($n=17$) male mice were used for analysis. (**a**) *Leishmania* DNA concentration measured with the PCR-ELISA technique. DNA was isolated from lymph node homogenates, 50 ng of DNA was amplified per reaction at 24 cycles and 2 µl of PCR product was used for ELISA. (**b**) Parasite count in Giemsa-stained lymph node smears. In each sample, the parasite number was counted as the mean amastigote number from 100 fields observed. Mean and standard error values (± s.e.m.) in BALB/c and STS groups tested by PCR-ELISA, as well as in tissue smears, were obtained by analysis of variance.

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