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(54) Title: RAPID MOLECULAR ASSAYS FOR SPECIFIC DETECTION AND QUANTITATION OF *LOA LOA* MICROFILAREMIA

(57) Abstract: A series of quantitative assays capable of quantifying *Loa loa* microfilaria burden from small-volume blood samples is provided. The present invention also provides methods of use of the quantitative assays to identify individuals at risk for encephalopathy due to antifilarial treatment, and also provides oligonucleotide primers used in the assay methods.



WO 2012/061283 A1

RAPID MOLECULAR ASSAYS FOR SPECIFIC DETECTION AND QUANTITATION
OF *LOA LOA* MICROFILAREMIA

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application No. 61/410,232, filed on November 4, 2010, the entire contents of which are incorporated by reference.

INCORPORATION-BY-REFERENCE OF MATERIAL ELECTRONICALLY FILED

[0002] Incorporated by reference in its entirety herein is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: One 12,656 Byte ASCII (Text) file named "709065.ST25," created on November 4, 2010.

BACKGROUND OF THE INVENTION

[0003] *Loa loa* (African eyeworm) is a filarial nematode estimated to infect 3-13 million people in central and western Africa, and is the causative agent for loiasis. Infected individuals may exhibit a range of relatively benign symptoms, however, those who are lifelong inhabitants of endemic areas are generally asymptomatic, even with the presence of large numbers of microfilariae circulating throughout their bloodstream. Loiasis is nonetheless an important public health concern due to the occurrence of greater than 1,000 severe adverse reactions (including fatal encephalopathies) in *Loa*-infected individuals receiving ivermectin, as a result of mass drug administration (MDA) programs aimed at the elimination of onchocerciasis and lymphatic filariasis. Consequently, disruption of further MDA has occurred in certain communities where these diseases are co-endemic.

[0004] The mechanism of *Loa*-related post-ivermectin encephalopathy is unclear, but risk appears to be greatest with patients having microfilaria (mf) counts above 8,000 organisms/ml in their blood. Reducing or preventing disruption of MDA therefore requires that patients with high-levels of circulating *L. loa* microfilaremia be identified and excluded from treatment. Microscopic evaluation of blood samples is currently the only diagnostic method used to detect *L. loa* microfilaremia in endemic areas, and is impractical for

widespread screening. As such, there remains a need for a diagnostic method that is quick, accurate, and sensitive, and can be performed in a clinical setting in the field, so that infected individuals can be identified and excluded from mass drug administration.

BRIEF SUMMARY OF THE INVENTION

[0005] Quantitative assays capable of quantifying *Loa loa* microfilarial burden from small-volume blood samples are provided.

[0006] In an embodiment, the present invention also provides a method of detecting the presence or absence of *L. loa* microfilaria in a sample comprising: a) subjecting a sample to a loop-mediated isothermal amplification (LAMP) procedure comprising 1) adding to the sample a LAMP primer mixture comprising (i) one or more forward inner primers (FIP) comprising a sequence having at least 90% identity to SEQ ID NOS: 31 and/or 37, (ii) one or more backward inner primers (BIP) comprising a sequence having at least 90% identity to SEQ ID NOS: 32 and/or 38, (iii) one or more forward outer primers (F3) comprising a sequence having at least 90% identity to SEQ ID NOS: 29 and/or 35, (iv) one or more backward outer primers (B3) comprising a sequence having at least 90% identity to SEQ ID NOS: 30 and/or 36, (v) one or more loop forward primers (LF) comprising a sequence having at least 90% identity to SEQ ID NOS: 33 and/or 39, and (vi) one or more loop backward primers (LB) comprising a sequence having at least 90% identity to SEQ ID NOS: 34 and/or 40; 2) incubating the mixture of 1) at a temperature of between 60 to 65 °C for 45 minutes to 90 minutes; 3) heating the mixture of 1) to 80 °C for between 2 to 5 minutes; b) detecting the presence or absence of amplified DNA resulting from (a); and c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *L. loa* microfilaria in the sample. In another embodiment, the present invention further comprises: d) correlating the amount of amplified DNA detected in b) with the quantity of *L. loa* microfilaria (mf) in the sample.

[0007] In a further embodiment, the present invention provides a method of detecting the presence or absence of *L. loa* microfilaria in a sample comprising: a) subjecting a sample to a real time quantitative PCR (qPCR) nucleic acid amplification procedure comprising 1) adding to the sample a mixture comprising (i) one or more forward primers comprising a sequence having at least 90% identity to SEQ ID NOS: 5 and/or 8, (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NOS: 6 and/or 9, and (iii) one or more probes comprising a sequence having at least 90% identity to SEQ ID

NOS: 7 and/or 10; 2) incubating the mixture of 1) for between 20 to 50 thermocycles; b) detecting the presence or absence of amplified DNA resulting from (a); and c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *L. loa* microfilaria in the sample.

[0008] In another embodiment, the present invention further comprises: d) correlating the amount of amplified DNA detected in b) with the quantity of *L. loa* microfilaria (mf) in the sample.

[0009] In an embodiment, the present invention provides a method of detecting the presence or absence of *L. loa* microfilaria in a sample comprising: a) subjecting a sample to a reverse transcriptase PCR (RT-PCR) nucleic acid amplification procedure comprising (i) one or more forward primers comprising a sequence having at least 90% identity to SEQ ID NOS: 1 and/or 3 and (ii) one or more reverse primers comprising a sequence having at least 90% identity to SEQ ID NOS: 2 and/or 4; b) detecting the presence or absence of amplified DNA resulting from (a); and c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *L. loa* microfilaria in the sample. In another embodiment, the present invention further comprises: d) correlating the amount of amplified DNA detected in b) with the quantity of *L. loa* microfilaria (mf) in the sample.

[0010] In an embodiment, the present invention provides polynucleotide sequences from *L. loa*, which are useful for developing the methods described herein. In particular, the present invention provides an isolated polynucleotide having the sequence of SEQ ID NO: 13, and an isolated polynucleotide which encodes the nucleotide sequence complementary to the polynucleotide of SEQ ID NO: 13.

[0011] In another embodiment, the present invention provides an isolated polynucleotide having the sequence of SEQ ID NO: 20, and an isolated polynucleotide which encodes the nucleotide sequence complementary to the polynucleotide of SEQ ID NO: 20.

[0012] In a further embodiment, the present invention provides an isolated polynucleotide having the sequence of SEQ ID NO: 23, and an isolated polynucleotide which encodes the nucleotide sequence complementary to the polynucleotide of SEQ ID NO: 23.

[0013] It is also contemplated that the present invention provides, in an embodiment, a method of identification of *L. loa* microfilaria (mf) in a subject comprising: a) obtaining a blood sample from the subject; b) subjecting the sample to a LAMP, qPCR, or RT-PCR procedure of the present invention using the primers identified above; and c) correlating the

presence or absence of amplified *L. loa* DNA with the presence or absence, respectively, of *L. loa* microfilaria (mf) in the subject.

[0014] In yet another embodiment, the present invention comprises: d) correlating the amount of amplified DNA detected in b) with the quantity of *L. loa* microfilaria (mf) in the subject.

[0015] In an embodiment, the present invention further comprises: e) comparing the quantity of *L. loa* microfilaria (mf) in the blood of the subject of d) with a predetermined quantity of *L. loa* microfilaria (mf) in the blood which is known to be positively correlated with the risk of the subject developing post antifilarial encephalopathy, and determining whether the subject should be excluded from treatment.

[0016] It is also contemplated that the present invention provides oligonucleotide primers for use in detection of *L. loa* by LAMP, qPCR or RT-PCR assay. In an embodiment, the present invention provides one or more oligonucleotide primers derived from the contig polynucleotide sequence of LLMF72 (SEQ ID NO: 13), LLMF269 (SEQ ID NO: 20) and LLMF342 (SEQ ID NO: 23). In particular, in an embodiment, the present invention provides the following oligonucleotide primers selected from the group consisting of SEQ ID NOS: 1-10 and SEQ ID NOS: 29-40.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

[0017] Figure 1 is a graph of the results of RT-PCR assay of an embodiment of the present invention with limiting dilutions of *L. loa* total RNA. Shown are concentrations of specific PCR product using primers for targets LLMF72 (circles, solid line) and LLMF269 (squares, dashed line). The lower X-axis indicates the number of *L. loa* microfilariae corresponding to the amount of RNA used as template (upper X-axis).

[0018] Figure 2 shows results of the qPCR assays of embodiments of the present invention using LLMF72 and LLMF269 primers with limiting dilutions of reverse-transcribed *L. loa* total RNA (2A), *L. loa* gDNA (2B) or 1% of total DNA extracted 200 μ l normal whole blood spiked with intact microfilariae (2C). Neither assay detected RNA from *Brugia malayi* (up to 10 ng) or gDNA from *B. malayi*, *Wuchereria bancrofti*, *Onchocerca volvulus*, or *Mansonella perstans* (up to 10 ng).

[0019] Figure 3 depicts amplification plots for the qPCR assay of an embodiment of the present invention (LLMF72 primers) with template DNA extracted from limiting dilutions of

intact microfilariae spiked into normal whole blood (1–10,000 organisms). The cutoff ΔR_n for a positive assay (0.2) is indicated by the horizontal line.

[0020] Figure 4 shows two graphs. Dried blood spots (50 μ l) were obtained from patients living in a *Loa*-endemic area in Cameroon. DNA was extracted from blood spots either manually by zinc BB disruption followed by Qiagen DNeasy® Blood & Tissue Kit (4A), using an automated EasyMAG™ process (4B). Microfilarial (MF) concentrations were predicted by LLMF72 qPCR using a standard curve based on limiting dilutions of intact *L. loa* MF spiked into whole blood and compared to MF concentrations determined microscopically from fresh 50 μ l blood samples.

[0021] Figures 5A and 5B are graphs showing predicted MF concentrations for boiling blood spot samples. Samples were boiled for 10 or 30 minutes. Using a standard curve derived from the previously spiked blood samples subjected to zinc BB/spin column extraction in Fig. 4A, the qPCR assay of an embodiment of the present invention (LLMF72 primers) was positive for all boiled blood samples except the sample spiked with a single organism (5A). There was a significant positive correlation between organism burden as assessed by microscopy (180 to 62,000 mf/ml) and organism burden as estimated by the qPCR method of the present invention (5B; Spearman $r = .71$; $P = .009$).

[0022] Figure 6 presents real-time turbidimetry data using the LAMP assay of an embodiment of the present invention (LLMF72 primers) with gDNA purified from *L. loa* (Ll), *B. malayi* (Bm), *W. bancrofti* (Wb), *O. volvulus* (Ov), or *M. perstans* (Mp), or 1% of DNA extracted from whole blood spiked with intact *L. loa* MF.

[0023] Figure 7 are three illustrations showing the sensitivity of an embodiment of the LAMP assay of the present invention. Multiplexing LLMF72 and LLMF342 LAMP primers increases analytic sensitivity compared to either primer set alone, with a lower limit of detection equal to about 0.01 pg gDNA.

[0024] Figure 8 depicts the colorimetric performance of an embodiment of the LAMP assay of the present invention. The assay shows three assays including LLMF72, LLMF342, and a LLMF72/LLMF342 multiplex colorimetric LAMP assay, each with 50 μ l dried blood spots subjected to 30 minutes of boiling. About 1% of each sample was used as template. The figure shows that the sensitivity of the multiplex assay is 100 fold more sensitive than either assay by itself.

DETAILED DESCRIPTION OF THE INVENTION

[0025] The assays of the present invention are highly sensitive, species specific, and may be used for rapid, high-throughput screening either with extracted DNA, or with boiled whole blood. The assays of the present invention are ready for immediate use in clinical laboratories where PCR equipment is available, and can also be adapted for use in resource-poor endemic areas in the field where minimal laboratory equipment is needed. The assays of the present invention represent an important step forward in the diagnosis of loiasis and may ultimately be of benefit in global health campaigns to eliminate filarial diseases.

[0026] In addition to the sensitivity, specificity, and ability to objectively quantify microfilaremia using embodiments of the methods of the present invention, the assays disclosed herein offer further advantages compared to conventional parasitology. First, the assays of the present invention eliminate the need for specialized training in filarial morphology, thereby making the assay accessible to anyone with general training in PCR techniques. Second, they enable rapid and high-throughput sample processing, which is of great benefit to centralized laboratories conducting population-based screening. Further, the *L. loa* assays of the present invention can be multiplexed with quantitative real-time PCR assays for other pathogens, using different fluorescent reporters. See, for example, Rao, R.U., et al., *Trans. R. Soc. Trop. Med. Hyg.*, 103: 365–70 (2009).

[0027] In an embodiment, the present invention also provides a method of detecting the presence or absence of *L. loa* microfilaria in a sample comprising: a) subjecting a sample to a loop-mediated isothermal amplification (LAMP) procedure comprising 1) adding to the sample a LAMP primer mixture comprising (i) one or more forward inner primers (FIP) comprising a sequence having at least 90% identity to SEQ ID NOS: 31 and/or 37, (ii) one or more backward inner primers (BIP) comprising a sequence having at least 90% identity to SEQ ID NOS: 32 and/or 38, (iii) one or more forward outer primers (F3) comprising a sequence having at least 90% identity to SEQ ID NOS: 29 and/or 35, (iv) one or more backward outer primers (B3) comprising a sequence having at least 90% identity to SEQ ID NOS: 30 and/or 36, (v) one or more loop forward primers (LF) comprising a sequence having at least 90% identity to SEQ ID NOS: 33 and/or 39, and (vi) one or more loop backward primers (LB) comprising a sequence having at least 90% identity to SEQ ID NOS: 34 and/or 40; 2) incubating the mixture of 1) at a temperature of between 60 to 65 °C for 45 minutes to 90 minutes; 3) heating the mixture of 1) to 80 °C for between 2 to 5 minutes; b) detecting the

presence or absence of amplified DNA resulting from (a); and c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *L. loa* microfilaria in the sample. In another embodiment, the present invention further comprises: d) correlating the amount of amplified DNA detected in b) with the quantity of *L. loa* microfilaria (mf) in the sample.

[0028] In a further embodiment, the present invention provides a method of detecting the presence or absence of *L. loa* microfilaria in a sample comprising: a) subjecting a sample to a real time quantitative PCR (qPCR) nucleic acid amplification procedure comprising 1) adding to the sample a mixture comprising (i) one or more forward primers comprising a sequence having at least 90% identity to SEQ ID NOS: 5 and/or 8, (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NOS: 6 and/or 9, and (iii) one or more probes comprising a sequence having at least 90% identity to SEQ ID NOS: 7 and/or 10; 2) incubating the mixture of 1) for between 20 to 50 thermocycles; b) detecting the presence or absence of amplified DNA resulting from (a); and c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *L. loa* microfilaria in the sample.

[0029] In another embodiment, the present invention further comprises: d) correlating the amount of amplified DNA detected in b) with the quantity of *L. loa* microfilaria (mf) in the sample.

[0030] In an embodiment, the present invention provides a method of detecting the presence or absence of *L. loa* microfilaria in a sample comprising: a) subjecting a sample to a reverse transcriptase PCR (RT-PCR) nucleic acid amplification procedure comprising (i) one or more forward primers comprising a sequence having at least 90% identity to SEQ ID NOS: 1 and/or 3 and (ii) one or more reverse primers comprising a sequence having at least 90% identity to SEQ ID NOS: 2 and/or 4; b) detecting the presence or absence of amplified DNA resulting from (a); and c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *L. loa* microfilaria in the sample. In another embodiment, the present invention further comprises: d) correlating the amount of amplified DNA detected in b) with the quantity of *L. loa* microfilaria (mf) in the sample.

[0031] In an embodiment, the present invention provides polynucleotide sequences from *L. loa*, which are useful for developing the methods described herein. In particular, the present invention provides an isolated polynucleotide having the sequence of SEQ ID NO:

13, and an isolated polynucleotide which encodes the nucleotide sequence complementary to the polynucleotide of SEQ ID NO: 13.

[0032] In another embodiment, the present invention provides an isolated polynucleotide having the sequence of SEQ ID NO: 20, and an isolated polynucleotide which encodes the nucleotide sequence complementary to the polynucleotide of SEQ ID NO: 20.

[0033] In a further embodiment, the present invention provides an isolated polynucleotide having the sequence of SEQ ID NO: 23, and an isolated polynucleotide which encodes the nucleotide sequence complementary to the polynucleotide of SEQ ID NO: 23.

[0034] It is also contemplated that the present invention provides, in an embodiment, a method of identification of *L. loa* microfilaria (mf) in a subject comprising: a) obtaining a blood sample from the subject; b) subjecting the sample to a LAMP, qPCR, or RT-PCR procedure of the present invention using the primers identified above; and c) correlating the presence or absence of amplified *L. loa* DNA with the presence or absence, respectively, of *L. loa* microfilaria (mf) in the subject.

[0035] In yet another embodiment, the present invention comprises: d) correlating the amount of amplified DNA detected in b) with the quantity of *L. loa* microfilaria (mf) in the subject.

[0036] In an embodiment, the present invention further comprises: e) comparing the quantity of *L. loa* microfilaria (mf) in the blood of the subject of d) with a predetermined quantity of *L. loa* microfilaria (mf) in the blood which is known to be positively correlated with the risk of the subject developing post antifilarial encephalopathy, and determining whether the subject should be excluded from treatment.

[0037] It is also contemplated that the present invention provides oligonucleotide primers for use in detection of *L. loa* by LAMP, qPCR or RT-PCR assay. In an embodiment, the present invention provides one or more oligonucleotide primers derived from the contig polynucleotide sequence of LLMF72 (SEQ ID NO: 13), LLMF269 (SEQ ID NO: 20) and LLMF342 (SEQ ID NO: 23). In particular, in an embodiment, the present invention provides the following oligonucleotide primers selected from the group consisting of SEQ ID NOS: 1-10 and SEQ ID NOS: 29-40.

[0038] The term "nucleic acid" as used herein, includes "polynucleotide," "oligonucleotide," and "nucleic acid molecule," and generally means an isolated or purified polymer of DNA or RNA, which can be single-stranded or double-stranded, synthesized or obtained (e.g., isolated and/or purified) from natural sources, which can contain natural, non-

natural or altered nucleotides, and which can contain a natural, non-natural or altered internucleotide linkage, such as a phosphoroamidate linkage or a phosphorothioate linkage, instead of the phosphodiester found between the nucleotides of an unmodified oligonucleotide. In some embodiments, the nucleic acid does not comprise any insertions, deletions, inversions, and/or substitutions. However, it may be suitable in some instances, as discussed herein, for the nucleic acid to comprise one or more insertions, deletions, inversions, and/or substitutions.

[0039] Preferably, the nucleic acids of the invention are recombinant. As used herein, the term "recombinant" refers to (i) molecules that are constructed outside living cells by joining natural or synthetic nucleic acid segments to nucleic acid molecules that can replicate in a living cell, or (ii) molecules that result from the replication of those described in (i) above. For purposes herein, the replication can be *in vitro* replication or *in vivo* replication.

[0040] The nucleic acids can be constructed based on chemical synthesis and/or enzymatic ligation reactions using procedures known in the art. See, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Press, Cold Spring Harbor, NY 2001; and Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and John Wiley & Sons, NY, 1994. For example, a nucleic acid can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed upon hybridization (e.g., phosphorothioate derivatives and acridine substituted nucleotides). Examples of modified nucleotides that can be used to generate the nucleic acids include, but are not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N⁶-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N⁶-substituted adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N⁶-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, 3-(3-amino-3-N-2-carboxypropyl) uracil, and 2,6-diaminopurine. Alternatively, one or more of the nucleic acids of the invention can be purchased from

companies, such as Macromolecular Resources (Fort Collins, CO) and SyntheGen (Houston, TX).

[0041] An embodiment of the invention also provides a nucleic acid comprising a nucleotide sequence that is at least about 70% or more, e.g., about 80%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identical to any of the nucleic acids described herein.

[0042] The nucleotide sequence which hybridizes under stringent conditions preferably hybridizes under high stringency conditions. By "high stringency conditions" is meant that the nucleotide sequence specifically hybridizes to a target sequence (the nucleotide sequence of any of the nucleic acids described herein) in an amount that is detectably stronger than non-specific hybridization. High stringency conditions include conditions which would distinguish a polynucleotide with an exact complementary sequence, or one containing only a few scattered mismatches from a random sequence that happened to have a few small regions (e.g., 3-10 bases) that matched the nucleotide sequence. Such small regions of complementarity are more easily melted than a full-length complement of 14-17 or more bases, and high stringency hybridization makes them easily distinguishable. Relatively high stringency conditions would include, for example, low salt and/or high temperature conditions, such as provided by about 0.02-0.1 M NaCl or the equivalent, at temperatures of about 50-70 °C. Such high stringency conditions tolerate little, if any, mismatch between the nucleotide sequence and the template or target strand, and are particularly suitable for detecting expression of any of the inventive nucleic acids. It is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide.

[0043] The invention also provides an isolated or purified nucleic acid comprising a nucleotide sequence which is complementary to the nucleotide sequence of any of the nucleic acids described herein or a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of any of the nucleic acids described herein.

[0044] Embodiments of the invention can comprise, consist essentially of, or consist of any of the nucleic acids, complementary nucleic acids, and SEQ ID NOs described herein.

[0045] The term "isolated" as used herein means having been removed from its natural environment. The term "purified" as used herein means having been increased in purity, wherein "purity" is a relative term, and not to be necessarily construed as absolute purity. For

example, the purity can be at least about 50%, can be greater than 60%, 70%, 80%, and 90%, or can be 100%.

[0046] The term “multiplex amplification reaction” means, in the context of this description, multiple target sequences of DNA or RNA in a test sample which are amplified by a PCR or LAMP procedure.

[0047] The term “primer” refers to an oligonucleotide, whether natural or synthetic, capable of acting as a point of initiation of DNA synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced, i.e., in the presence of four different nucleoside triphosphates and an agent for polymerization (i.e., DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. A primer is preferably a single-stranded oligodeoxyribonucleotide. The appropriate length of a primer depends on the intended use of the primer but typically ranges from about 10 to about 30 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to specifically hybridize with a template. When primer pairs are referred to herein, the pair is meant to include one forward primer which is capable of hybridizing to the sense strand of a double-stranded target nucleic acid (the “sense primer”) and one reverse primer which is capable of hybridizing to the antisense strand of a double-stranded target nucleic acid (the “antisense primer”).

[0048] “Probe” refers to an oligonucleotide which binds through complementary base pairing to a sub-sequence of a target nucleic acid. A primer may be a probe. It will be understood by one of skill in the art that probes will typically substantially bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are typically directly labeled (e.g., with isotopes or fluorescent moieties) or indirectly labeled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the target, by Southern blot for example.

EXAMPLES

[0049] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope. Unless otherwise specified, the examples

disclosed herein which identify a particular manufacturer in the example, follow the manufacturer's protocols for the particular method disclosed.

EXAMPLE 1

[0050] Patient samples. All samples were acquired under registered protocols approved by the Institutional Review Board of NIAID (NCT00001230), the Cameroon Ethical Committee, and the Cameroon Ministry of Health, with written informed consent obtained from all subjects.

EXAMPLE 2

[0051] Filarial organism collection. *L. loa*, *W. bancrofti*, and *Mansonella perstans* mf were purified from the blood of patients seen by the NIH/NIAID Clinical Parasitology Unit. *O. volvulus* adult worms were obtained from excised onchocercomas of Guatemalan patients. *B. malayi* mf and adult worms were obtained from the Filariasis Research Reagent Repository Center (Athens, GA).

EXAMPLE 3

[0052] Extraction of genomic DNA. *L. loa* (5 million mf) and *O. volvulus* (50 adult female worms) were digested overnight at 56 °C in buffer G2 (Qiagen, Valencia, CA) with 20 mg/ml proteinase K. Genomic DNA was extracted using Genomic tip-100/G and Genomic DNA buffer set (Qiagen). The remaining filarial organisms were digested as described above, and genomic DNA was extracted with phenol/chloroform.

EXAMPLE 4

[0053] Extraction of mf RNA. One million *L. loa* mf and 500,000 *B. malayi* mf were frozen under liquid nitrogen and disrupted by a stainless steel piston/mortar apparatus. Total RNA was extracted using the RNeasy Kit (Qiagen), and poly-A RNA isolation with the Oligotex mRNA Mini Kit (Qiagen).

EXAMPLE 5

[0054] Synthesis of mf cDNA. cDNA was synthesized from 1 µg of *L. loa* or *B. malayi* total RNA in 50-µl reactions containing 160 units MultiScribe™ reverse transcriptase, 5.5

mM MgCl₂, 2 mM dNTP mix, 2.5 mM random hexamers, 20 units RNase inhibitor, and 1X RT buffer (Applied Biosystems, Foster City, CA).

EXAMPLE 6

[0055] Construction and screening of a *L. loa* mf cDNA library. A cDNA library was created in the vector pTriplEx2 using the SMART cDNA Library Construction Kit (Clontech, Mountain View, CA). The library was screened by PCR amplification of individual plaques using primers specific to the pTriplEx2 phagemid insertion site (Table 1) and sequencing at the NIAID Rocky Mountain Laboratories Genomics Unit (Hamilton, MT).

[0056] Table 1. Primer and probe sequences for PCR assays.

Assay	Sequence	Product size (bp)
Reverse-transcriptase PCR		
LLMF72 5' primer	5'-GAGGGATCCATGCGGCATTCCTCTATAAAC-3' (SEQ ID NO: 1)	252
LLMF72 3' primer	5'-GAGCTCGAGTCAACTCCCTTTGAAACGTTT-3' (SEQ ID NO: 2)	
LLMF269 5' primer	5'-GAGGGATCCATGAAAGCTGTTGGTGCCATT-3' (SEQ ID NO: 3)	398
LLMF269 3' primer	5'-GAGCTCGAGTCACAACCTTTCGTTCTTCAT-3' (SEQ ID NO: 4)	
Real-time PCR		
LLMF72 5' primer	5'-CGGAAGACTCAACGTCAGAAATCA-3' (SEQ ID NO: 5)	
LLMF72 3' primer	5'-AGGAACGCTTGATGGTGATGT-3' (SEQ ID NO: 6)	62
LLMF72 probe	5'-FAM-CCAACAGCCTGCTTTT-NFQ-3' (SEQ ID NO: 7)	

LLMF269 5' primer	5'-GCAAGAGTCTTTACAACACTATATTTTGCAGAAA-3'	
	(SEQ ID NO: 8)	
LLMF269 3' primer	5'-GGCATCTTCATCCGGGTAACACTATAC-3'	78
	(SEQ ID NO: 9)	
LLMF269 probe	5'-FAM-TCGAGACGAGACTTTC-NFQ-3'	
	(SEQ ID NO: 10)	

EXAMPLE 7

[0057] Contig construction and bioinformatics analysis. *L. loa* mf ESTs were assembled into contigs using the Desktop cDNA Annotation System (dCAS 1.4.3) software package of Guo Y, et al., *Bioinformatics*, 25:1195–6 (2009). Contigs were selected for further evaluation as candidate assay targets based on the number of ESTs comprising the contig (abundance), length of at least 200 bp with a predicted open reading frame (ORF), and lack of sequence homology to *i)* the non-redundant protein database (nr), *ii)* ESTs from related filarial pathogens, and *iii)* *L. loa* L3 larval stage ESTs (D. L. Fink, et al., unpublished).

[0058] Table 2. Candidate PCR assay targets based on dCAS bioinformatics analysis of *Loa loa* microfilaria EST library screen. Contigs selected for further investigation by real-time PCR are shown in bold.

Contig name	GenBank accession number	ORF length (bp)
LLMF3 (SEQ ID NO: 11)	HM753544	378
LLMF49 (SEQ ID NO: 12)	HM753550	252
LLMF72 (SEQ ID NO: 13)	HM753552	237
LLMF99 (SEQ ID NO: 14)	HM753553	186
LLMF103 (SEQ ID NO: 15)	HM753536	195
LLMF178 (SEQ ID NO: 16)	HM753537	219
LLMF188 (SEQ ID NO: 17)	HM753538	204

LLMF199 (SEQ ID NO: 18)	HM753539	267
LLMF212 (SEQ ID NO: 19)	HM753540	201
LLMF269 (SEQ ID NO: 20)	HM753541	387
LLMF274 (SEQ ID NO: 21)	HM753542	267
LLMF288 (SEQ ID NO: 22)	HM753543	219
LLMF342 (SEQ ID NO: 23)	HM753545	288
LLMF357 (SEQ ID NO: 24)	HM753546	645
LLMF401 (SEQ ID NO: 25)	HM753547	615
LLMF415 (SEQ ID NO: 26)	HM753548	240
LLMF475 (SEQ ID NO: 27)	HM753549	874
LLMF505 (SEQ ID NO: 28)	HM753551	392

EXAMPLE 8

[0059] Reverse transcriptase PCR of candidate target transcripts. RT-PCR was performed on *L. loa* total RNA using the OneStep® RT-PCR kit (Qiagen) and primers specific to the 5' and 3' ends of each target transcript (Table 1). Quantification of specific PCR product was accomplished using a 2100 Bioanalyzer instrument and 2100 Expert software (Agilent Technologies, Waldbronn, Germany).

EXAMPLE 9

[0060] DNA extraction from whole blood spiked with *L. loa* mf. *L. loa* mf were spiked into 200 µl aliquots of whole blood obtained from a healthy volunteer with no history of exposure to filaria-endemic regions. Following the zinc BB disruption protocol of Laney S.J., et al., *PLoS Negl. Trop. Dis.*, 2:e251 (2008) (electronic publication), DNA was extracted using the QiaAmp DNA blood and tissue kit (Qiagen). Duplicate sets of spiked whole blood samples were created by adding intact *L. loa* mf as described above to 50 µl aliquots of whole blood. After addition of 150 µl distilled water, the samples were vortexed briefly and then boiled for 10–30 minutes. Following removal of 2 µl aliquots, samples were spun in a bench-top centrifuge at maximum speed for 5 minutes and supernatants recovered.

EXAMPLE 10

[0061] Blood spot collection. Mid-day venous blood samples were obtained by prick from Cameroonian volunteers living a region endemic for *L. loa* as part of a study on *Loa*-associated ophthalmologic, cardiac, and renal impact. An aliquot of 50 μ l of each collected blood sample was examined microscopically, while an additional 50–100 μ l was spotted onto filter paper.

EXAMPLE 11

[0062] DNA extraction from blood spots. Blood spots were partitioned into 6-mm circular sections using disposable sterile biopsy punch tools (Acuderm, Inc., Ft. Lauderdale, FL). A set of 36 punched blood spots were submerged in 200 μ l PBS and subjected to DNA extraction by the zinc BB/Qiagen spin column method described above. A second set of 36 punched blood spots was transferred into sterile tubes containing 2.0 ml easyMAGTM lysis buffer (BioMerieux, Durham, NC), pulse vortexed for 15 seconds, and then incubated for 10 minutes at room temperature. Samples were extracted into 50 μ l easyMAGTM elution buffer according to manufacturer's recommendations for off-board lysis. A third set of 12 punched blood spots was immersed in 200 μ l distilled water and boiled for 10–30 minutes at 99 °C while shaking.

EXAMPLE 12

[0063] Real-time PCR assays. qPCR was performed in an ABI 7900 sequence detection system using Taqman fast chemistry reagents (Applied Biosystems) using the primer/probe sets described in Table 1. Amplification conditions were 20 seconds at 95 °C, followed by 40 cycles of 1 second at 95 °C, and 20 seconds at 60 °C. Quality of template was confirmed for all samples using a control primer/probe set targeting a conserved region of the eukaryotic 18S ribosomal RNA gene (Applied Biosystems).

EXAMPLE 13

[0064] Loop-mediated Isothermal Amplification (LAMP) Assay. The LAMP assays used in the present invention are based on the method of Goto et al., *Biotechniques*, 46:167-172 (2009) and U.S. Patent 6,410,278. Briefly, LAMP is an autocycling and strand displacement DNA synthesis method involving the use of the large fragment of *Bst* DNA polymerase and a

set of six specially designed primers, a forward inner primer (FIP), a backward inner primer (BIP), a forward outer primer (F3), a backward outer primer (B3) a loop forward primer (LF) and a loop-backward primer (LB). Gene amplification by the LAMP method can be superior to that by PCR in general, because LAMP does not require an expensive thermocycler, as all reactions can be performed at a constant temperature ranging from 60 °C to 65 °C.

[0065] The use of expensive equipment decreases the versatility of LAMP and greatly limits the wide use of this procedure, especially in developing countries. Detection of turbidity by the naked eye is the simplest and most cost-efficient method for judging a positive or negative LAMP reaction, although this method requires some skill for assessing the result. For better visibility of the reaction result, a DNA intercalating dye such as SYBR® green (Applied Biosystems, Inc., Carlsbad, CA), Picogreen® (Molecular Probes Inc., Eugene, OR), or propidium iodide is added to the solution after the reaction is completed. When the LAMP reaction is positive, a color change is observed under ambient light. However, as in the case of analysis in gel electrophoresis, the colorimetric assay using the intercalating dye is associated with an increased risk of contamination of other subsequent LAMP reaction solutions because the assay requires opening of the tubes. To avoid such contamination, separate rooms should be used for LAMP setup and analysis.

[0066] The LAMP reaction solution used for spectrophotometric analysis consisted of the following components: 0.88 ng target *L. loa* mf DNA sample, 1.6 µM each of FIP and BIP, 0.2 µM each of F3 and B3, 0.8 µM each of LF and LB, 1.4 mM of each dNTP, 120 µM HNB, and MgSO₄ at various concentrations in the LAMP buffer (10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.8 at 25°C), 0.1% Tween 20, and 0.8 M betaine). An absorption spectra analysis was performed in a 0.2-ml quartz cuvette with a 10-mm path length by using the spectrophotometer (VersaMax, Molecular Dynamics, Sunnyvale, CA) with Spectra Manager software (Jaescio UK, Essex). The instrument was first set to zero at 750 nm for distilled water, and absorbance in the range of 750 nm to 350 nm was recorded at 1-nm intervals.

[0067] The enzyme is inactivated by heating the tubes at 80-95 °C for 2 minutes. Presence of fluorescence under a UV light (254-366 nm) indicates a positive result and visualization of DNA products on gel electrophoresis is not required for assessing successful DNA amplification, because a positive LAMP reaction causes the solution to become cloudy due to the formation of the magnesium pyrophosphate byproduct. The resulting turbidity of the solution has a high correlation with the amount of DNA synthesized, and a real-time

turbidimeter (LA-200™, Teramecs Ltd., Japan) is used for quantifying initial template DNA (Figure 6). LAMP can also be adapted to RNA amplification by simply adding a reverse transcriptase in the reaction solution. Thus, the adaption of this procedure in the present invention, for use in the field, has great advantages over other conventional methods.

[0068] TABLE 3. LAMP Primers used in the assay protocol.

Primer Name	Primer Sequence
LLMF72	
LLMF72-F3	AGATTTGACGGCAACGGAAG (SEQ ID NO: 29)
LLMF72-B3	GCGTCAGTTTCGTGTTGTGA (SEQ ID NO: 30)
LLMF72-FIP	CCGGAATCAGAGGAACGCTTGATCAACGTCAGAAATCAGCCA (SEQ ID NO: 31)
LLMF72-BIP	GCACAGCAGAGTCTTCTAGTGGCGTTGATGACGCTCCCAA (SEQ ID NO: 32)
LLMF72-LF	GGTGATGTA AAAAGCAGGCTGT (SEQ ID NO: 33)
LLMF72-LB	TAAGTTTTCCAGGAACTGCACC (SEQ ID NO: 34)
LLMF342	
LLMF342-F3	CAGCAGAGTGACTTACGT (SEQ ID NO: 35)
LLMF342-B3	AAAGCGATCGATTTGTCAAT (SEQ ID NO: 36)
LLMF342-FIP	CCGAGAATGTTGAGAGCATAGTAATGACGGTGAACAGTTACACA (SEQ ID NO: 37)
LLMF342-BIP	AGCTCATCAGGAATTATCCTTCTGAGAAAAATGACGGTTACGAAAT (SEQ ID NO: 38)
LLMF342-LF	TGCTTAATTGGTGCCTTGAAGC (SEQ ID NO: 39)
LLMF342-LB	TAGTTATCTCGTATACGGCGGTCA (SEQ ID NO: 40)

[0069] Primer mix. For each LAMP primer, the sample is reconstituted to 100 μ M in water (multiply nmol produced x 10 to get μ l of water to add to tube). For each target, a stock primer mix is made consisting of: 5 μ l F3; 5 μ l B3; 40 μ l FIP; 40 μ l BIP; 20 μ l LF; 20

μl LB; and 20 μl water to make a total volume of 150 μl, which is enough for 100 assays. This protocol can be scaled up as needed.

[0070] The reagents used are found in the LoopAmp® reagent kit (SA Scientific, San Antonio, TX), including the 192-assay kit (# E-2060), and reaction tubes (96) (# E-9030), following the manufacturer's protocol.

[0071] Analysis of template using whole blood or blood spots was performed as follows. For blood spots, 8 x 3 mm circles are punched out and immersed in 100 μl water, vortexed briefly, and then boiled for 30 minutes. The samples are then vortexed briefly again after boiling. The assay can be performed at this point using crude boiled samples, or the samples can be spun (5 minutes max speed) in a microfuge and supernatant collected for the assay.

[0072] To begin the assay set-up, a master mix needs to be prepared, consisting of (for each 25 μl assay): 12.5 μl 2X buffer; 1.5 μl each primer mix; 1 μl Bst polymerase; 1 μl 3 mM hydroxynaphthol blue; and adding water as needed, to bring final reaction volume to 25 μl (including template). The master mix is then dispensed into PCR plate wells or LoopAmp tubes. The volume dispensed should be 25 μl minus the template volume. The template is then added to each well, and the plate or tubes are spun down.

[0073] The samples are then heated using a heating block or thermocycler for 65 °C for 1 hour, followed by 80 °C for 10 minutes to deactivate the enzyme, followed by chilling the samples to 4 °C.

[0074] The samples are then visually inspected to determine if the reaction was completed and provides a qualitative result. The presence of a purple color in the tube indicates a negative reaction and the presence of a blue color in the tube indicates a positive reaction. The samples are then transferred to 96-well U-bottom clear plate and read in a plate reader at 650 nm, using negative control as blank (Figure 7).

EXAMPLE 14

[0075] Statistical analysis and calculations. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). To estimate concentration of mf in blood spot samples, a standard curve derived from spiked blood samples (SDS 2.2.2 software, Applied Biosystems) was used to calculate the number of organisms in each blood spot, normalizing for the percentage of extracted DNA used as template. This number was then divided by the estimated volume of blood (10 μl for each 6-mm punch, 2–5 punches per sample).

EXAMPLE 15

[0076] A screen of the *L. loa* mf-stage cDNA library produced sequence information for 1,882 ESTs, which were assembled into 518 unique contigs by dCAS analysis. From these, 18 potential PCR targets were identified based on their having limited sequence homology: a) to all publicly available nematode ESTs; b) to the non-redundant protein database (nr); and c) to clustered ESTs derived from *L. loa* L3 larvae. Each candidate transcript included a start codon and stop codon separated by at least 200 base pairs, indicating a potential ORF (Table 2).

[0077] Amplification of the 18 candidate transcripts was confirmed by RT-PCR using primers corresponding to the 5' and 3' ends of predicted ORFs. All but one of the RT-PCR reactions produced specific PCR products (data not shown). Two targets (predicted ORFs from contigs LLMF72 (SEQ ID NO: 13) and LLMF269 (SEQ ID NO: 20) were chosen for further evaluation based on abundance of specific RT-PCR product and lack of other nonspecific products. Using limiting dilutions of *L. loa* total RNA as template, RT-PCR detected as little as about 3.2 pg (LLMF72) or about 0.64 pg (LLMF269) total RNA, corresponding to the RNA present in a fraction of one mf (Figure 1). For both targets, the final concentration of specific PCR product was directly proportional to the log amount of RNA template used.

[0078] To shorten the running time of the assay and gain sensitivity, Taqman real-time PCR (qPCR) primers and probes were designed for the LLMF72 (SEQ ID NO: 13) and LLMF269 (SEQ ID NO: 20) targets. Following reverse transcription, qPCR detected limiting dilutions of *L. loa* total RNA from about 20 ng to about 2 pg, with a linear relationship between the log amount of RNA used and the number of reaction cycles needed to detect a signal above baseline (Figure 2A). Neither qPCR assay detected cDNA prepared from *B. malayi* mf RNA (10-fold dilutions from about 20 ng to about 2 pg), although from these same *B. malayi* samples, a conserved region of the 18S ribosomal RNA gene could be detected (data not shown) using a primer/probe set targeting this sequence.

[0079] Similar to the situation with total RNA, both qPCR assays detected limiting dilutions of *L. loa* genomic DNA from about 10 ng to about 0.1 pg. A linear relationship was again observed between the amount of genomic DNA used as template and the number of reaction cycles needed to detect signal above baseline (Figure 2B). Neither qPCR assay detected genomic DNA from *B. malayi*, *O. volvulus*, *W. bancrofti*, or *M. perstans* (10-fold

dilutions from about 10 ng to about 0.1 pg), although all samples were detected in a linear fashion using the 18S rRNA primer/probe set (data not shown). When both LLMF72 and LLMF269 primer/probe sets were used together in a single assay, there was no reduction compared with the assay performed using the LLMF72 primers alone, in the number of reaction cycles at which any amount of DNA was detected (data not shown).

EXAMPLE 16

[0080] The following example illustrates the successful identification of mf in whole blood samples using the methods described above.

[0081] Embodiments of the qPCR assays of the present invention were then evaluated with DNA extracted from whole blood samples spiked with limiting dilutions of intact *L. loa* mf (1 to 10,000 organisms). Using about 1% of the total extracted DNA from each sample as template, there was once more a linear relationship between the log number of mf spiked and the number of reaction cycles needed to detect signal above baseline (Figure 2C and Figure 3). The lower limit of detection for both assays was about 1% of DNA extracted from a single *L. loa* mf. Combining both LLMF72 and LLMF269 primer/probe sets into a single assay did not increase the sensitivity of the assay beyond that seen with the assay using the LLMF72 primers alone (data not shown).

[0082] Recognizing the advantage of fewer reaction cycles to positive (i.e., higher sensitivity), the assay using LLMF72 primers was selected for further evaluation with blood samples from a cohort of Cameroonian study subjects with well defined *L. loa* microfilaremia. DNA was extracted from a portion of the dried blood spots using the manual DNA extraction method described above, prior to performing the qPCR method of the present invention using the LLMF72 primers. Using the spiked blood samples as a standard curve to estimate the concentration of *L. loa* organisms present in each blood spot, there was a significant positive correlation between the extent of microfilaremia predicted by qPCR and the level confirmed previously by microscopy (Figure 4A; Spearman $r = .74$; $P < .0001$). Among 36 blood spots evaluated, only one (40 mf/ml by microscopy) was negative by qPCR.

[0083] A second set of 36 blood spots was subjected to automated DNA extraction by easyMAGTM, and concentration of mf was again estimated by the qPCR method of the present invention using the LLMF72 primers. Among this set, there was a strong linear correlation between predicted and observed microfilaremia (Figure 4B; $r^2 = .88$; $P < .0001$). There were two samples positive by microscopy (20 mf/ml and 200 mf/ml) but negative by

qPCR. Fifteen of the blood spots in this set were collected from individuals who were apparently amicrofilaremic by microscopy. Five of these samples, however, contained detectable *L. loa* genomic DNA by the qPCR of the present invention (predicted organism burden 1–7 mf/ml).

[0084] To investigate whether time and effort of DNA extraction could be reduced, DNA was extracted from another set of spiked whole blood samples by boiling for about 10–30 minutes. Using a standard curve derived from the previously spiked blood samples subjected to zinc BB/spin column extraction, the qPCR assay of the present invention (LLMF72 primers) was positive for all boiled blood samples except the sample spiked with a single organism (Figure 5A). There was a linear relationship between number of organisms spiked and number estimated by qPCR, with no increase in DNA extraction efficiency observed with boiling samples for 30 minutes compared with 10 minutes. Centrifugation of boiled samples and use of the supernatants as template did not have any effect on qPCR assay results (data not shown).

[0085] Efficiency of DNA extraction by boiling was also evaluated with a set of 12 dried blood spots. Boiling resulted in detection of *L. loa* DNA by the qPCR assay of the present invention (LLMF72 primers) in all 12 samples. Furthermore, there was a significant positive correlation between organism burden as assessed by microscopy (180 to 62,000 mf/ml) and organism burden as estimated by the qPCR method of the present invention (Figure 5B; Spearman $r = .71$; $P = .009$).

[0086] Recently, the Broad Institute at Harvard in conjunction with MIT, published the sequence assembly and initial annotation of the genomes of *L. loa*, *O. volvulus*, and *W. bancrofti* (http://www.broadinstitute.org/annotation/genome/filarial_worms/MultiHome.html). A preliminary analysis of the LLMF72 (SEQ ID NO: 13) and LLMF269 (SEQ ID NO: 20) target sequences indicate that they reside within single-copy genes encoding hypothetical proteins. Both target sequences have similarity to single regions of the *B. malayi*, *W. bancrofti*, *Schistosoma mansoni*, and *Caenorhabditis elegans* genomes, although there is no evidence of gene expression among ESTs of these other organisms. Species specificity of the targets is conferred by a lack of sequence similarity at the primer/probe binding sites. Consequently, the assays of the present invention are negative with as much as about 10 ng of purified genomic DNA (equivalent to about 10^4 – 10^5 mf) from *B. malayi*, *W. bancrofti*, *O. volvulus*, or *M. perstans*. This level of specificity is extremely

important, as *L. loa* may be co-endemic with both *W. bancrofti* and *M. perstans*, whose life cycles also include bloodstream mf.

[0087] The qPCR assays of embodiments of the present invention are highly sensitive. A lower limit of detection equivalent to about 2 pg reverse-transcribed RNA, about 0.1 pg genomic DNA, or about 1% of DNA extracted from 200 μ l of whole blood spiked with a single *L. loa* mf. Using DNA extracted from clinical samples as template, the qPCR assay (LLMF72 primers) was able to detect a single mf in a 20- μ l dried blood spot (equivalent to a burden of about 50 mf/ml). Overall, the assay described herein was positive for 65 of 68 samples with parasitologically proven *L. loa* microfilaremia and therefore has a sensitivity of about 96% with these specimens. Three false-negative results were obtained with samples where organism burden was very low (20–200 mf/ml, or 4 organisms in a 20- μ l blood spot). In light of the observed lower limits of detection for purified genomic DNA and spiked whole blood samples, these false negatives were most likely due either to sampling error (no organisms present in the processed blood spot) or to issues with the DNA extraction process.

[0088] Mf burdens predicted by the qPCR assay of an embodiment of the present invention using the LLMF72 primers were typically about 2- to about 10-fold lower than observed microscopically, no matter which DNA extraction method was used. These predictions were based on a standard curve derived from spiked whole blood samples, further indicating that extraction from dried blood spots is likely less efficient than extraction from fresh whole blood. Microfilaremia estimation was most accurate and most consistent with samples subjected to other extraction methods, for example, the easyMAG™ extraction. Preferably, this automated extraction process can be performed in laboratory settings where such equipment is available. Nevertheless, the methods disclosed herein, including the method comprising boiling blood spots for about 10 minutes enabled detection of as few as about 180 mf/ml (approximately 7 organisms in a 40- μ l blood spot) or greater. These numbers favor successful performance of the assay in endemic areas where conditions may necessitate small sample volumes, delay between sample collection and processing, and simplified extraction of template; however, the qPCR assay of an embodiment of the present invention will likely be even more sensitive, consistent, and accurate with larger volume (100 μ l and above) fresh blood samples.

[0089] The qPCR assay of an embodiment of the present invention (LLMF72 primers) also detected low-level mf in 5 of 15 samples thought to be amicrofilaremic by microscopy. These discordances were unlikely to be due to false-positive assay results, because the DNA

extraction and assay protocols were conducted under rigorous conditions designed to protect against cross-sample contamination, and all qPCR runs included internal “no template” controls with verified negative results. Rather, an embodiment of the qPCR assay of the present invention is likely detecting small numbers of microorganisms that were missed by microscopy due to sampling error with the 50- μ l aliquots that were examined. All five samples tested positive by serology for IgG against *L. loa* SXP-1 using the method of Burbelo, P.D., et al., *J. Clin. Microbiol.* 46:2298–304 (2008), supporting that these samples were indeed infected with *L. loa*.

[0090] Optimum PCR performance requires proper timing of blood collection at mid-day when microfilaremia is greatest; however, it is thought that smaller numbers of organisms can be detectable outside the window of peak microfilaremia, and a nomogram may be used to extrapolate qPCR results to predict true parasite burden using the methods disclosed herein.

[0091] In another embodiment of the present invention, the addition of hydroxynaphthol blue (1 mM) to the LAMP assay, while not quantitative, provides ready point-of care feasibility with single heat source requirement and visual colorimetric reporting system. The sensitivity of the LAMP assay for either LLMF72 or LLMF342 primers was in the range of approximately 1 pg DNA/ml.

[0092] In a further embodiment of the LAMP assay of the present invention, when the assay was multiplexed and both primer targets were assayed at the same time, the sensitivity of the assay of the present invention was as low as 0.01 pg DNA/ml (Figure 8).

[0093] The qPCR and LAMP assay embodiments of the present invention have analytic sensitivity of ≤ 0.1 pg gDNA (< 1 mf/ml, for qPCR), or clinical detection limits of about 200–400 mf/ml using boiled 50 μ l dried blood samples (for LAMP).

[0094] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0095] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely

intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0096] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIM(S):

1. A method of detecting the presence or absence of *Loa loa* (*L. loa*) microfilaria in a sample comprising:

a) subjecting the sample to a loop-mediated isothermal amplification (LAMP) procedure comprising:

1) adding to the sample a LAMP primer mixture comprising (i) one or more forward inner primers (FIP) comprising a sequence having at least 90% identity to SEQ ID NOS: 31 and/or 37; (ii) one or more backward inner primers (BIP) comprising a sequence having at least 90% identity to SEQ ID NOS: 32 and/or 38; (iii) one or more forward outer primers (F3) comprising a sequence having at least 90% identity to SEQ ID NOS: 29 and/or 35; (iv) one or more backward outer primers (B3) comprising a sequence having at least 90% identity to SEQ ID NOS: 30 and/or 36; (v) one or more loop forward primers (LF) comprising a sequence having at least 90% identity to SEQ ID NOS: 33 and/or 39; and (vi) one or more loop backward primers (LB) comprising a sequence having at least 90% identity to SEQ ID NOS: 34 and/or 40;

2) incubating the mixture of 1) at a temperature of between 60 to 65 °C for 45 minutes to 90 minutes;

3) heating the mixture of 1) to 80 °C for between 2 to 5 minutes;

b) detecting the presence or absence of amplified DNA resulting from (a); and

c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *L. loa* microfilaria in the sample.

2. The method of claim 1, further comprising:

d) correlating the amount of amplified DNA detected in b) with the quantity of *L. loa* microfilaria in the sample.

3. A method of detecting the presence or absence of *Loa loa* (*L. loa*) microfilaria in a sample comprising:

a) subjecting the sample to a real time quantitative PCR (qPCR) nucleic acid amplification procedure comprising:

1) adding to the sample a mixture comprising (i) one or more forward primers comprising a sequence having at least 90% identity to SEQ ID NOS: 5 and/or 8; (ii) one or more reverse primers comprising a sequence having at least 90% identity to SEQ ID NOS: 6 and/or 9; and (iii) one or more probes comprising a sequence having at least 90% identity to SEQ ID NOS: 7 and/or 10;

2) incubating the mixture of 1) for between 20 to 50 thermocycles;

b) detecting the presence or absence of amplified DNA resulting from (a); and

c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *L. loa* microfilaria in the sample.

4. The method of claim 3, further comprising:

d) correlating the amount of amplified DNA detected in b) with the quantity of *L. loa* microfilaria (mf) in the sample.

5. A method of detecting the presence or absence of *Loa loa* (*L. loa*) microfilaria in a sample comprising:

a) subjecting the sample to a reverse transcriptase PCR (RT-PCR) nucleic acid amplification procedure comprising (i) one or more forward primers comprising a sequence having at least 90% identity to SEQ ID NOS: 1 and/or 3 and (ii) one or more reverse primers comprising a sequence having at least 90% identity to SEQ ID NOS: 2 and/or 4;

b) detecting the presence or absence of amplified DNA resulting from (a); and

c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *L. loa* microfilaria in the sample.

6. The method of claim 5, further comprising:

d) correlating the amount of amplified DNA detected in b) with the quantity of *L. loa* microfilaria (mf) in the sample.

6. The method of any of claims 1-5, wherein the sample is obtained from a mammal.
7. The method of any of claims 1-5, wherein the sample is obtained from a human.
8. The method of any of claims 1-7, wherein the sample is a blood sample.
9. An isolated polynucleotide having the sequence of SEQ ID NO: 13.
10. An isolated polynucleotide which encodes the nucleotide sequence complementary to the polynucleotide of SEQ ID NO: 13.
11. An isolated polynucleotide having the sequence of SEQ ID NO: 20.
12. An isolated polynucleotide which encodes the nucleotide sequence complementary to the polynucleotide of SEQ ID NO: 20.
13. An isolated polynucleotide having the sequence of SEQ ID NO: 23.
14. An isolated polynucleotide which encodes the nucleotide sequence complementary to the polynucleotide of SEQ ID NO: 23.
15. A method of detection of *Loa loa* (*L. loa*) microfilaria in a subject comprising:
 - a) obtaining a blood sample from the subject;
 - b) subjecting the sample to the method of any of claims 1-5; and
 - c) correlating the presence or absence of amplified *Loa loa* (*L. loa*) DNA in the sample with the presence or absence, respectively, of *L. loa* microfilaria in the subject.
16. The method of claim 15, further comprising:
 - d) correlating the amount of amplified DNA detected in c) with the quantity of *L. loa* microfilaria (mf) in the blood of the subject.
17. The method of claim 16, further comprising:
 - e) comparing the quantity of *L. loa* microfilaria (mf) in the blood of the subject of d) with a predetermined quantity of *L. loa* microfilaria (mf) in the blood which is known to be

positively correlated with the risk of the subject developing post antifilarial encephalopathy, and determining whether the subject should be excluded from treatment.

FIGURE 1

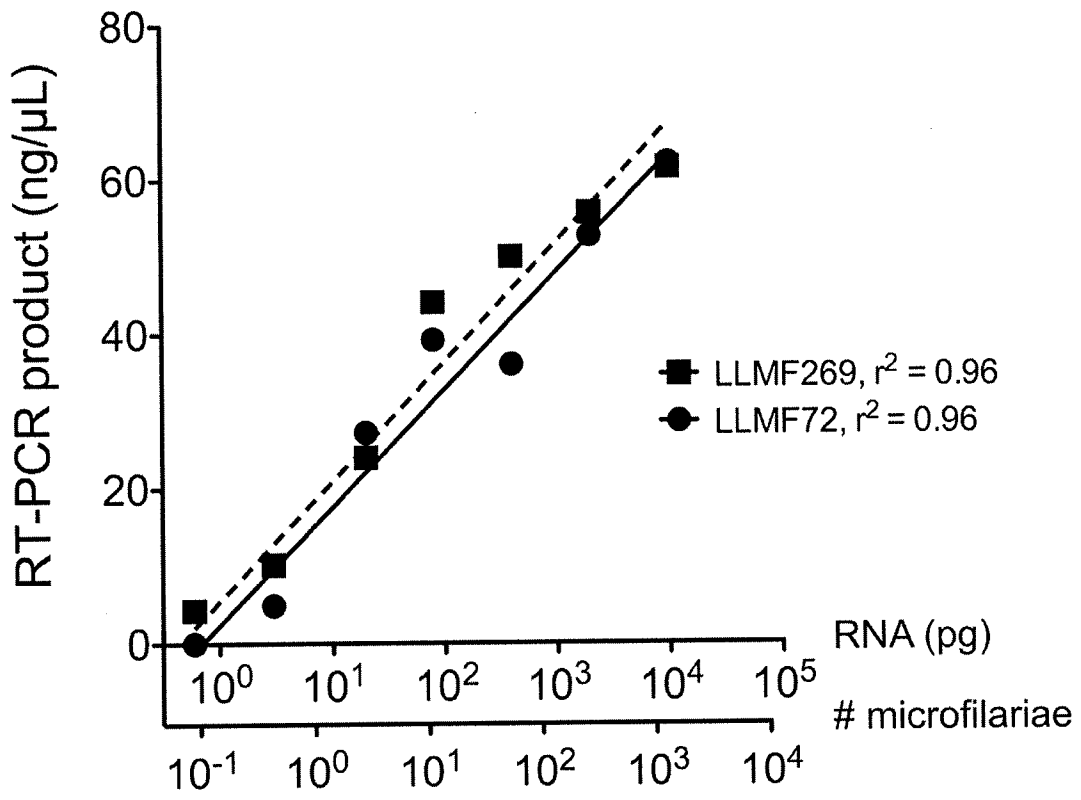


FIGURE 2

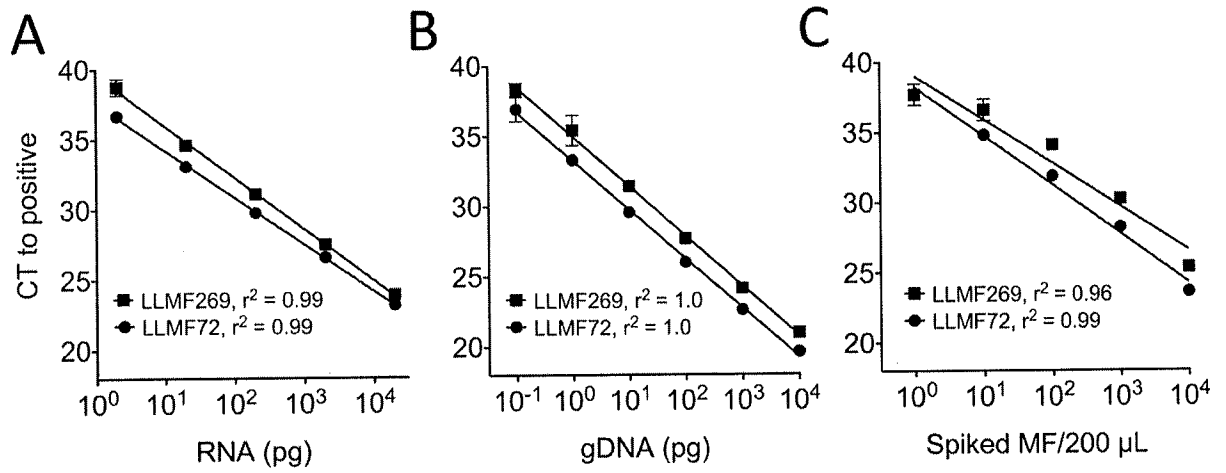


FIGURE 3

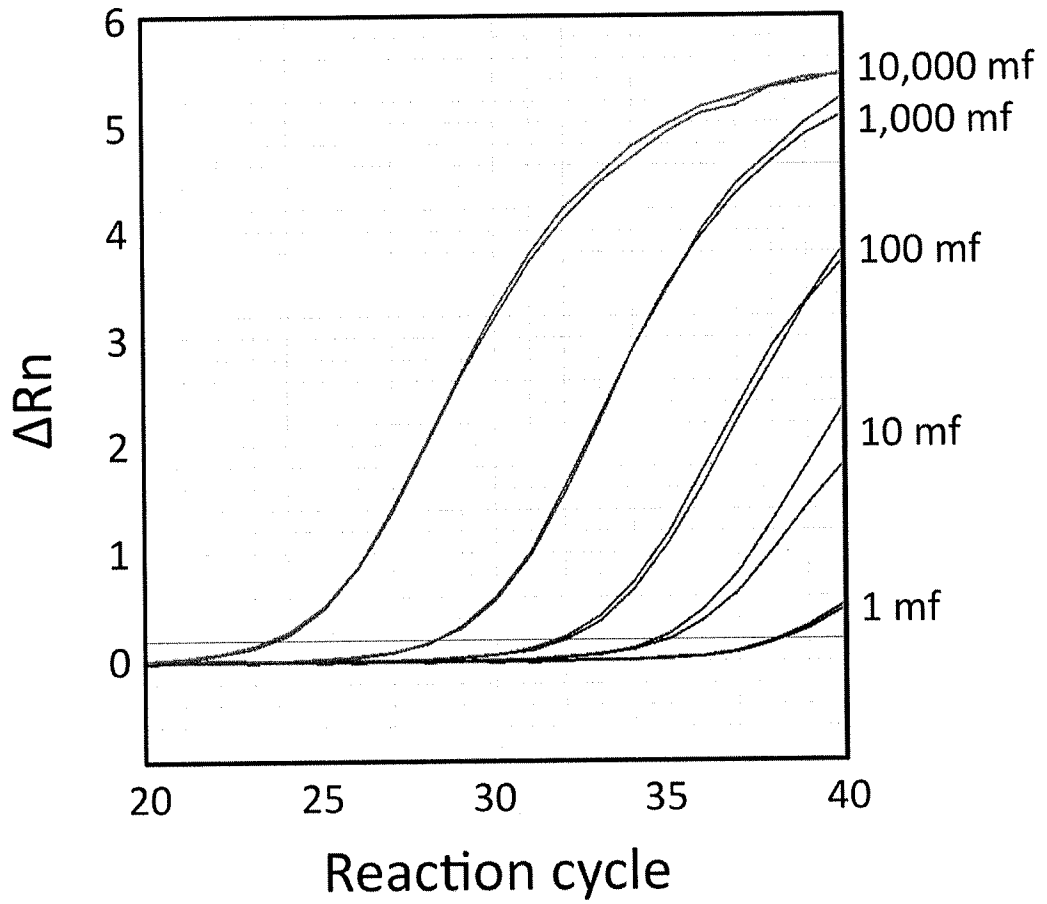


FIGURE 4

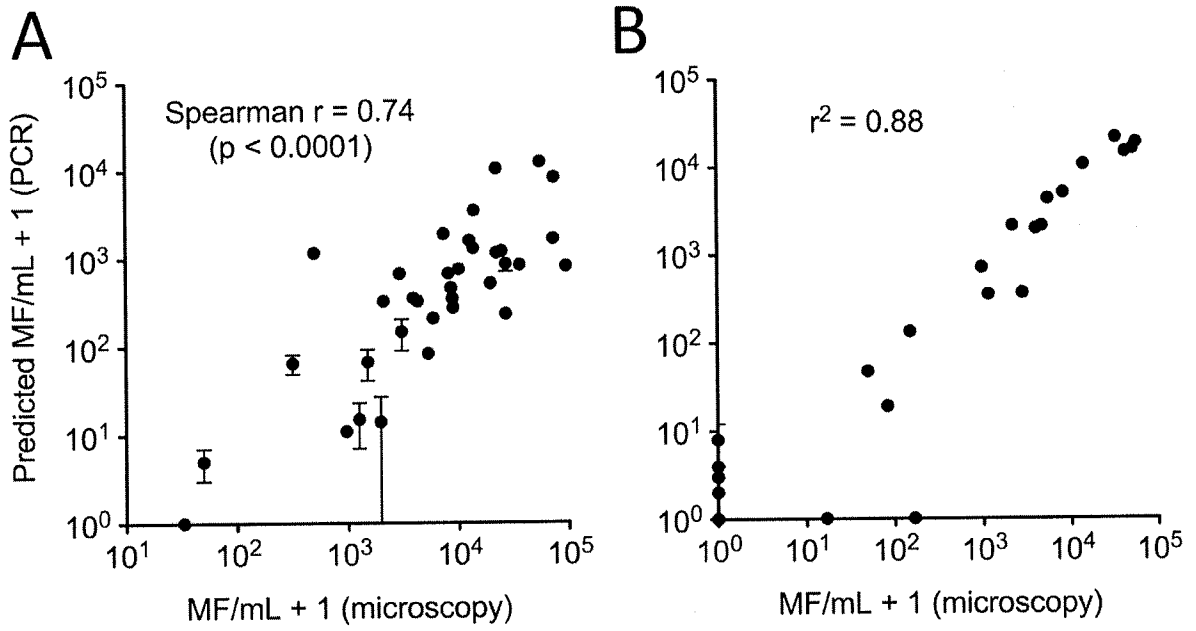


FIGURE 5

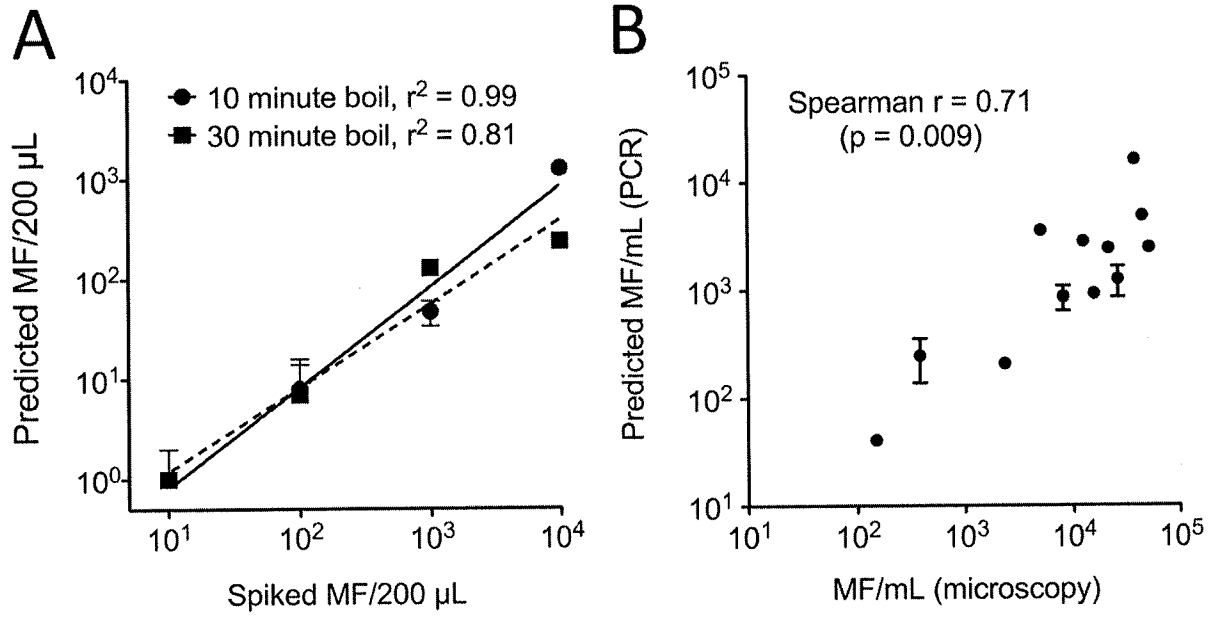


FIGURE 6

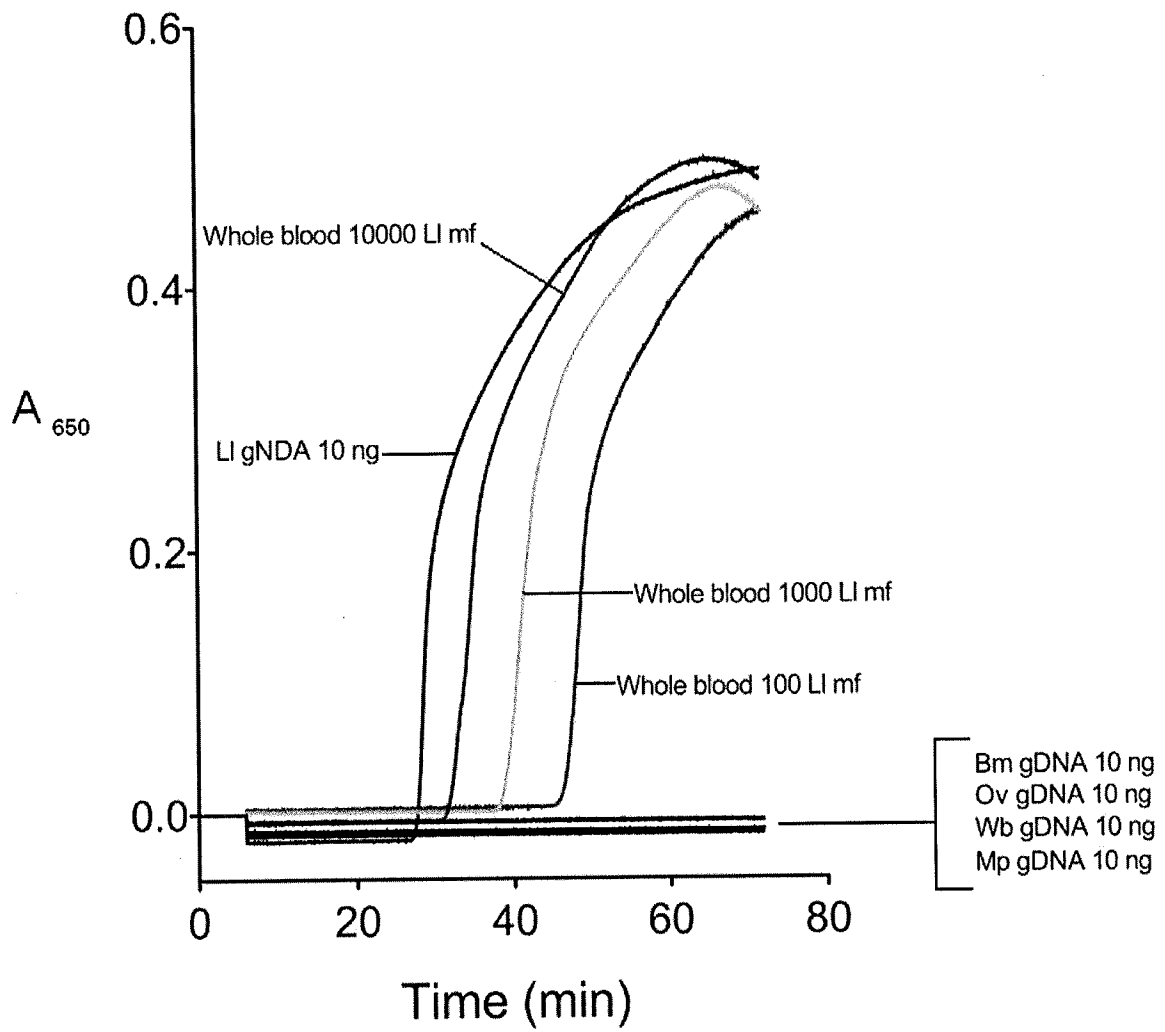


FIGURE 7

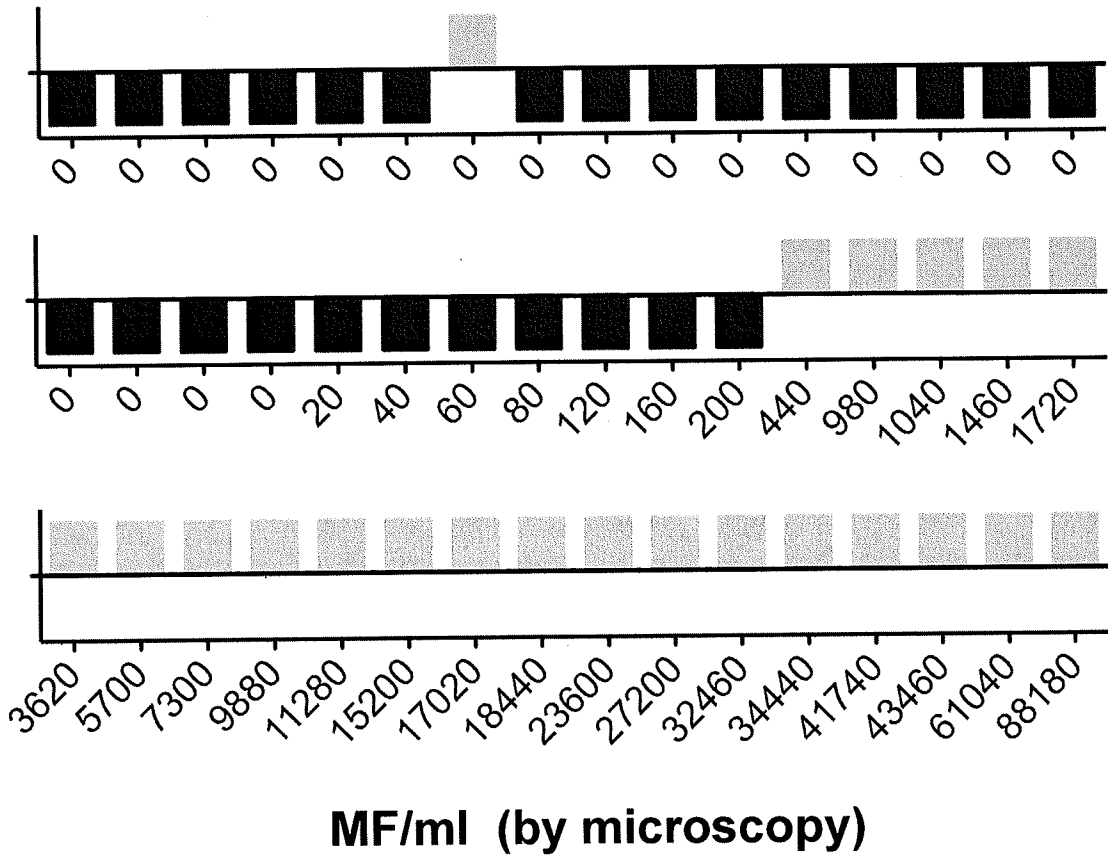
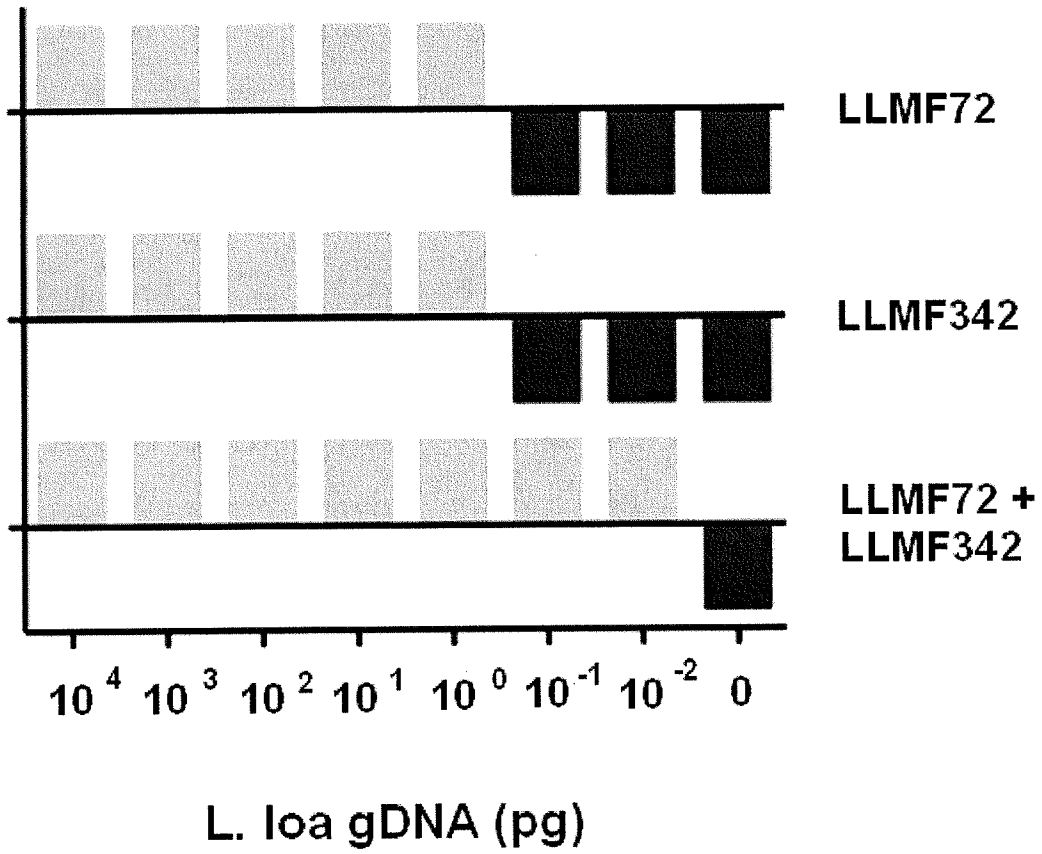


FIGURE 8



PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 709065	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/US2011/058565	International filing date (day/month/year) 31/10/2011	(Earliest) Priority Date (day/month/year) 04/11/2010
Applicant THE UNITED STATES OF AMERICA, AS REPRESENTED BY...		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of:

the international application in the language in which it was filed

a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

b. This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6**bis**(a)).

c. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. **Certain claims were found unsearchable** (See Box No. II)

3. **Unity of invention is lacking** (see Box No III)

4. With regard to the **title**,

the text is approved as submitted by the applicant

the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

the text is approved as submitted by the applicant

the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings**,

a. the figure of the **drawings** to be published with the abstract is Figure No. _____

as suggested by the applicant

as selected by this Authority, because the applicant failed to suggest a figure

as selected by this Authority, because this figure better characterizes the invention

b. none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2011/058565

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)
 - on paper
 - in electronic form
 - b. (time)
 - in the international application as filed
 - together with the international application in electronic form
 - subsequently to this Authority for the purpose of search
2. In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/058565

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12Q
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBASE, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE EMBL [Online]</p> <p>14 November 2009 (2009-11-14), "Label=LL-MF2-11-C01 DF09-LLMF Loa loa cDNA, mRNA sequence.", XP002665458, retrieved from EBI accession no. EM_EST:GT571296 Database accession no. GT571296 abstract; sequence</p> <p style="text-align: center;">----- -/--</p>	13,14

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search 13 December 2011	Date of mailing of the international search report 23/12/2011
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Reuter, Uwe
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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/058565

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE EMBL [Online]</p> <p>14 November 2009 (2009-11-14), "Label=LL-MF2-18-C06 DF09-LLMF Loa loa cDNA, mRNA sequence.", XP002665459, retrieved from EBI accession no. EM_EST:GT571424 Database accession no. GT571424 abstract; sequence</p>	9,10
X	<p>-----</p> <p>DATABASE EMBL [Online]</p> <p>14 November 2009 (2009-11-14), "Label=LL-MF2-20-B05 DF09-LLMF Loa loa cDNA, mRNA sequence.", XP002665460, retrieved from EBI accession no. EM_EST:GT571414 Database accession no. GT571414 abstract; sequence</p>	11,12
A	<p>-----</p> <p>FINK D L ET AL: "Toward molecular parasitologic diagnosis: Enhanced diagnostic sensitivity for filarial infections in mobile populations", JOURNAL OF CLINICAL MICROBIOLOGY 2011 AMERICAN SOCIETY FOR MICROBIOLOGY USA LNKD- DOI:10.1128/JCM.01697-10, vol. 49, no. 1, 27 October 2010 (2010-10-27), pages 42-47, XP002665457, ISSN: 0095-1137 abstract; table 1</p>	1-17
A	<p>-----</p> <p>JIMENEZ M ET AL: "Detection and discrimination of Loa loa, Mansonella perstans and Wuchereria bancrofti by PCR-RFLP and nested-PCR of ribosomal DNA ITS1 region", EXPERIMENTAL PARASITOLOGY, NEW YORK, NY, US, vol. 127, no. 1, 19 June 2010 (2010-06-19) , pages 282-286, XP027577246, ISSN: 0014-4894 [retrieved on 2010-06-19] page 284</p> <p>-----</p> <p style="text-align: center;">-/--</p>	1-17

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/058565

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>FOUSSEYNI S. TOURE ET AL: "Human occult loiasis: field evaluation of a nested polymerase chain reaction assay for the detection of occult infection", TROPICAL MEDICINE AND INTERNATIONAL HEALTH, vol. 3, no. 6, 1 January 1998 (1998-01-01) , pages 505-511, XP55014336, ISSN: 1360-2276, DOI: 10.1046/j.1365-3156.1998.00260.x abstract page 506</p> <p>-----</p>	1-17