A Novel Colorimetric Assay for Early Differentiation of Mucocutaneous and Cutaneous Leishmaniasis via Species-Specific Identification

Nancy Villegas Villao[a,b,c,d,e¥], Mavys Tabraue-Chavez[f,¥], Cristina Megino-Luque[f‡], Araceli Aguilar-Gonzalez [a,b,c,d], Juan J. Guardia-Monteagudo[f], F. Javier Lopez-Delgado[f], Agustin Robles-Remacho[a,b,c,d‡‡], Victoria Cano-Cortés[a,b,c,d], Juan J. Diaz-Mochon[a,b,c,d], Rosario M. Sanchez-Martin[a,b,c,d,*], Salvatore Pernagallo[f,*],

[a] GENYO Centre for Genomics and Oncological Research, Pfizer/University of Granada/Andalusian Regional Government. PTS Granada - Avenida de la Ilustración, 114– 18016, Granada, Spain.

[b] Department of Medicinal & Organic Chemistry, Faculty of Pharmacy, University of Granada, Campus de Cartuja s/n, Granada, Spain.

[c] Unit of Excellence in Chemistry Applied to Biomedicine and the Environment of the University of Granada, Granada, Spain.

[d] Instituto de Investigación Biosanitaria ibs.GRANADA, Granada, Spain.

[e] Departamento de Parasitología y Medicina Tropical. Carrera de Medicina, Facultad de Ciencias de la Salud, Universidad Católica de Santiago de Guayaquil, Guayaquil, Ecuador.

[f] DESTINA Genomica S.L. Parque Tecnológico Ciencias de la Salud (PTS), Avenida de la Innovación 1, Edificio BIC, 18016, Armilla, Granada, Spain.

¥ These two authors have contributed equally to this work.

‡Current affiliation: Department of Medicine, Division of Hematology and Oncology, The Tisch Cancer Institute. Icahn School of Medicine at Mount Sinai, 10029, New York, USA.

‡‡ Current affiliation: Department of Biochemistry and Biophysics, Tomtebodavägen 23B 171 65, Karolinska Campus Solna | Sweden

*Corresponding author. GENYO Centre for Genomics and Oncological Research, Pfizer/University of Granada/Andalusian Regional Government, PTS Granada - Avenida de la Ilustracion, ´ 114– 18016, Granada, Spain

*Corresponding author. DESTINA Genomica S.L. Parque Tecnológico Ciencias de la Salud (PTS), Avenida de la Innovación 1, Edificio BIC, 18016, Armilla, Granada, Spain.

E-mail addresses: salvatore.pernagallo@destinagenomics.com (S. Pernagallo), rmsanchez@go.ugr.es (R.M. Sanchez-Martin).

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

Abstract

Mucocutaneous leishmaniasis (MCL) is a severe and debilitating progression of cutaneous leishmaniasis (CL) that occurs when the disease spreads to involve mucosal tissues. Contrasting CL, which can often be treated with local therapies, MCL requires aggressive systemic treatment, strict adherence to a 30-day regimen and regular monitoring to prevent recurrence. These requirements highlight the critical need for accurate and rapid early diagnosis to guide effective treatment strategies. However, differentiating between the Leishmania species responsible for MCL and CL remains a significant challenge, particularly in resource-limited settings.

To address this gap, this study introduces a novel colorimetric assay that integrates the Spin-Tube platform with Dynamic Chemical Labelling (DCL) technology for species-specific identification of Leishmania parasites. This approach targets single nucleotide fingerprints (SNFs) within the conserved hsp70 gene, allowing precise differentiation between species associated with MCL and CL. The assay employs single-plex PCR followed by DCL-based detection of SNFs, providing rapid and visually interpretable results to facilitate species differentiation.

The assay demonstrated remarkable sensitivity, with a detection limit of 1 copy of parasite DNA per µL and performed effectively even under resource-limited conditions. It was used to identify ten MCL patients, with the results confirmed through DNA sequencing. Its simplicity, affordability (approximately €1 per test) and rapid turnaround make it an ideal diagnostic solution for endemic regions. By providing accurate early differentiation between CL and MCL, this assay enables the implementation of personalised treatment plans, minimising unnecessary exposure to toxic therapies and reducing the risk of irreversible mucosal damage for affected patients

Keywords: Mucocutaneous leishmaniasis (MCL), Dynamic Chemical Labeling (DCL), Spin-Tube device, hsp70 gene, Infectious disease diagnostics.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

1. Introduction

Leishmaniasis is a vector-borne disease caused by protozoa of the genus Leishmania transmitted to humans by the bite of infected female sandflies (Mathison and Bradley, 2023; Mann et al., 2021; Parkash et al. 2024; Yadav et al., 2023). In the Old World, transmission is mainly by sandflies of the genus Phlebotomus, while in the New World the genus Lutzomyia is the main vector (Kevric et al., 2015). After transmission, the parasites are phagocytosed by host macrophages, where they evade the immune response by transforming into amastigotes, an intracellular form that replicates within the macrophages (Conde et al., 2022; Costa-da-Silva et al., 2022). This ability to survive and multiply within immune cells is central to the pathogenesis of leishmaniasis, leading to a spectrum of clinical outcomes that depend on the infecting Leishmania species and the immune status of the host (Mathison and Bradley, 2023; Mann et al., 2021; Conde et al., 2022).

The World Health Organization (WHO) continues to classify leishmaniasis as one of the most significant parasitic diseases worldwide, owing to its broad distribution, high disease burden, and considerable socioeconomic impact. As of 2023, the disease is endemic in over 98 countries, with an estimated 600,000 to 1 million new cases reported annually (Kaye et al., 2023). Most of these cases occur in tropical and subtropical regions, where factors such as poor housing, malnutrition, and inadequate access to healthcare exacerbate the disease's effects (Mathison and Bradley, 2023; Mann et al., 2021; Kevric et al., 2015). Moreover, environmental factors, including climate change and deforestation, are contributing to the spread of leishmaniasis by disrupting ecosystems and forcing disease-carrying animals into new areas, thereby increasing the risk of transmission (Valero et al., 2021).

Leishmaniasis manifests in two primaries clinical forms: visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL). Among the two, CL is the most common form and is characterised by ulcerative skin lesions that typically appear on exposed areas of the body, such as the face, arms, and legs (Mathison and Bradley, 2023; Parkash et al., 20241). These lesions often take months to heal, and even after healing, they frequently leave permanent scars, that can lead to social stigma and psychological distress for affected individuals. In some cases, CL can progress to a more severe secondary form known as mucocutaneous leishmaniasis (MCL), which is considered a complication of CL. MCL, a more aggressive form, typically arises from untreated CL or CL patients who have not adhered to the treatment properly. It involves the destruction of mucosal tissues in the nose, mouth, and throat, causing ulcerations that can lead to severe disfigurement and functional impairments. If left untreated, MCL may result in significant tissue damage and permanent disfigurement, particularly affecting breathing, eating, and speaking (Mathison and Bradley, 2023; Mann et al., 2021; Kevric et al., 2015; Costa-da-Silva et al., 2022; Kaye et al., 2023).

The clinical form of leishmaniasis is largely species-dependent (Figure 1). For example, *Leishmania braziliensis* is commonly associated with MCL, while *Leishmania mexicana* and *Leishmania amazonensis* is more commonly linked to CL. Understanding the species involved is critical not only for determining the appropriate treatment but also for controlling disease spread. Early differentiation between CL and MCL is particularly important due to the severity of MCL, which can cause extensive mucosal tissue damage, leading to permanent disfigurement and functional impairments if left untreated.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

MCL requires more aggressive treatment than CL, often involving higher concerted potentially toxic therapies such as pentavalent antimonials (e.g., meglumine antimoniate or sodium stibogluconate) or amphotericin B, especially in cases where standard therapies fail (Goto and Lindoso, 2010). These treatments carry significant risks, including cardiac, renal, and hepatic toxicity (Haldar et al., 2011). Therefore, accurate species identification is essential to ensure appropriate therapy. Misidentification can result in suboptimal treatment or unnecessary exposure to toxic drugs, further complicating the patient's condition (Pinheiro and de Souza, 2022). Additionally, delayed treatment can lead to irreversible damage to mucosal tissues, resulting in long-term disability and disfigurement (Goto and Lindoso, 2010; Pinheiro and de Souza, 2022). Early and accurate diagnosis is therefore crucial to improve clinical outcomes by reducing the risk of complications and ensuring timely intervention (Poloni et al., 2023).

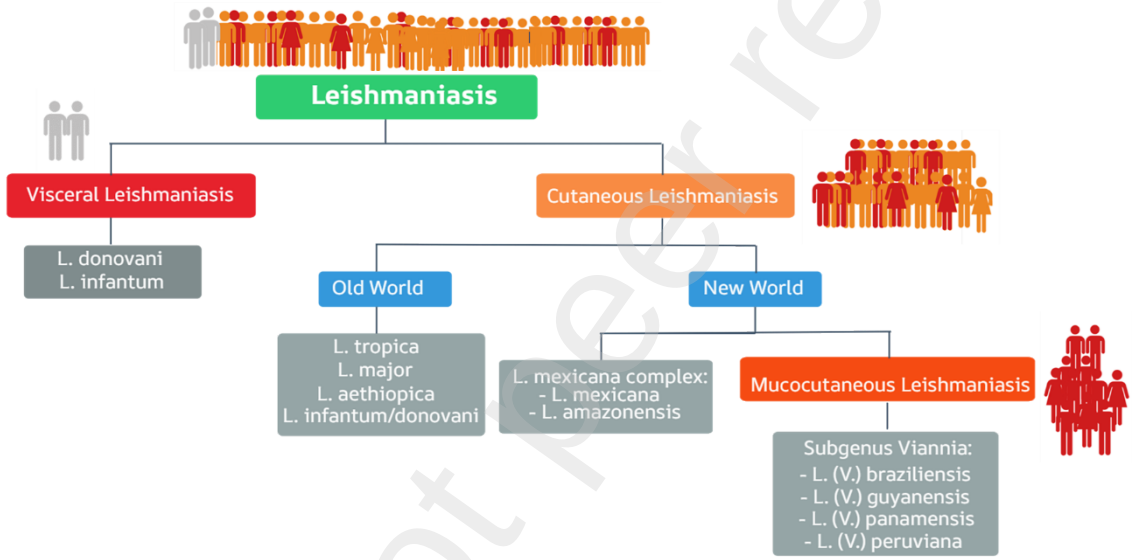


Figure 1. This figure shows the clinical forms of leishmaniasis alongside the primary *Leishmania* species responsible, classified by their geographical distribution. The diagram distinguishes between Old World and New World species (Kevric et al., 2015), highlighting species-specific associations with visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL). This Figure 1 reports only the principal species, although the authors acknowledge that other species may also be present.

Traditional diagnostic methods, such as microscopy and serological tests, have limited sensitivity and cannot reliably differentiate between *Leishmania* species, particularly in early-stage infections or asymptomatic patients (Mann et al., 2021; Thakur et al., 2020; Aronson et al., 2016). Molecular techniques, such as real-time quantitative PCR (qPCR), are now considered the gold standard for diagnosing leishmaniasis due to their high sensitivity (Tawfeeq and Ali, 2022; Wu et al., 2020; Bell and Ranford-Cartwright, 2002). Commonly used qPCR assays, such as those using TaqMan, Molecular Beacons, or Scorpion probes, are effective but require sophisticated equipment that is often unavailable in resource-limited settings (Mimori et al., 1998; Jariyapan et al., 2021; Chicharro et al., 2023; Soares et al., 2024). Moreover, qPCR and similar methods struggle to resolve the high genomic sequence homology among *Leishmania* species, limiting their ability to differentiate closely related species. Capture-based molecular assays provide a promising alternative for low-resource environments. However, they

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

lack the triple binding capability offered by the other technologies —forward primer, reverse primer, and internal probe— that is key to distinguish highly homologous sequences, thus reducing their utility for species-specific *Leishmania* diagnosis (Jani and Peter, 2022; Yin et al., 2024). These limitations underscore the need for specialized molecular assays capable of addressing genomic homology while remaining accessible in low-resource settings.

To overcome these challenges, we developed a novel diagnostic platform that combine high specificity through a triple binding capabilities provided by the Dynamic Chemical Labeling (DCL) technology (Delgado-Gonzalez et al., 2019; Detassis et al., 2019a; Detassis et al., 2024; Garcia-Fernandez et al., 2019; López-Longarela et al., 2020; Marín-Romero et al., 2018; Marín-Romero et al., 2023; Padial-Jaudenes et al., 2024; Pernagallo et al., 2012; Rissin et al., 2017; Smirnova et al., 2020; Tabraue-Chávez et al., 2019; Venkateswaran et al., 2016) that is compatible with formats suitable for low-resource settings, the Spin-Tube colorimetric device. This platform is specifically designed for low-resource environments and interrogates Single Nucleotide Fingerprint (SNF) markers—single-nucleotide variations in conserved genomic regions that enable precise differentiation of *Leishmania* species. Our group first introduced the term "Single Nucleotide Fingerprint (SNF)" to describe specific single-nucleotide variations in highly conserved nucleic acid sequences that allow differentiation of closely related pathogenic species (Luque-González et al., 2018).

In this study, SNF markers were selected from the conserved region of the *hsp70* gene (Jariyapan et al., 2021; Soares et al., 2024), which is particularly effective for distinguishing species responsible for CL and MCL. Our group previously applied the DCL reaction with the Spin-Tube platform to detect SNF markers in trypanosomatid parasites (Tabraue-Chávez et al., 2019). This innovative combination was later adapted for the ARREST-TB project, which focused on tuberculosis diagnostics and drug resistance detection (Smirnova et al., 2020). Additionally, DCL technology has been widely applied to RNA detection, including its use in SARS-CoV-2 diagnostics (Martín-Sierra et al., 2023). By integrating these technologies, this novel platform offers a reliable, low-cost solution for species-specific *Leishmania* diagnosis, addressing the key limitations of existing methods.

As shown in **Figure 2**, this novel approach utilizes DCL with biotinylated aldehyde-modified nucleobases (SMART-Biotin) in combination with abasic peptide nucleic acid (PNA) capture probes (DGL-Probes). DGL-Probes are immobilized on the membrane of the Spin-Tube in a precise spotting pattern to analyse selected SNFs (**Figure 3**). The DGL-Probes hybridize with complementary target DNA sequences amplified from the hsp70 gene of samples, forming a "Chemical Pocket" between the Abasic site on the DGL-Probe and the SNF nucleotide of interest within the hsp70 amplicon. Selected SNFs in the hsp70 target DNA sequences, involving single-nucleotide variations of Cytosine (C), Adenine (A), or Guanine (G), are analysed using a SMART-Biotin, specifically the biotinylated aldehyde-modified cytosine nucleobase (C-SMART-Biotin), as shown in **Figure 3**. When a "G" nucleotide is present at the SNF site, the C-SMART-Biotin covalently binds to the Abasic site position of the DGL-Probe via a thermodynamically controlled reductive amination reaction, which is templated by the SNF nucleotide according to Watson-Crick base pairing rules (Bowler et al., 2010, Bowler et al., 2011).

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

If a nucleotide other than "G" is present at the SNF site (“C” or “A”), no reaction occurs, thereby preventing the incorporation of C-SMART-Biotin (**Figure 2**). The assay's final step uses a reporter molecule, streptavidin-alkaline phosphatase, to detect the biotin tag. This interaction generates a visible blue chromogenic precipitate through the enzymatic hydrolysis of the chromogenic substrate BCIP®/NBT (5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride), confirming the presence or absence of guanine at the SNF site.

The DGL-Probes are spotted in specific patterns on the membrane, enabling species differentiation through C-SMART-Biotin incorporation. As illustrated in **Figure 4**, the results of the assay demonstrate that the generated pattern allows for the identification of *Leishmaniasis* and the differentiation between CL and MCL in a single reaction. Moreover, this differentiation is achieved through a visual test with the naked eye.

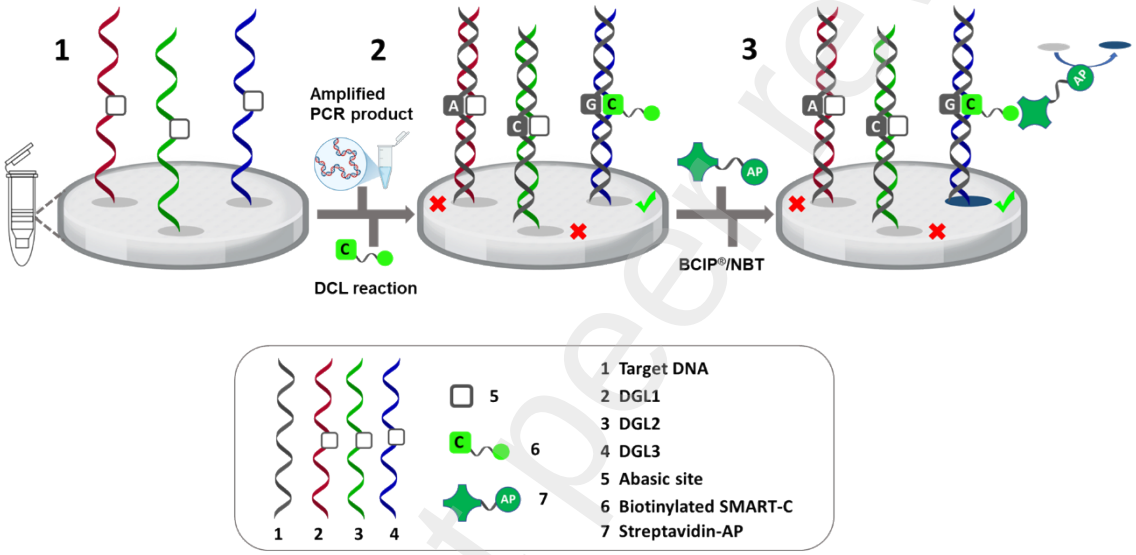


Figure 2. Schematic representation of the assay and its mechanism for interrogating SNFs. (1) Three DGL-Probes, designed to capture complementary target DNA sequences and interrogate the three SNFs (**Figure 3**), are spotted onto the membrane of the Spin-Tube. **(2)** Amplification of the hsp70 target gene (target DNA) is added to the Spin-Tube. These DNA targets, containing the three specific SNFs, hybridize with the DGL-Probes. C-SMART-Biotin is incorporated exclusively when a guanine (G) is present opposite the “abasic site” within the “chemical pocket.” **(3)** Detection is carried out using a colorimetric reaction with BCIP®/NBT, producing a blue precipitate on specific spots, which can be visualized with the naked eye (as a blue spot on the membrane). In cases where cytosine (C) or adenine (A) is present, the incorporation of C-SMART-Biotin does not occur, and therefore no blue spot is produced (appearing as a gray spot on the membrane).

2. Material and methods

2.1. Materials and Reagents

All chemicals were provided from Sigma Aldrich and used as received. The DCL buffer was prepared from 0.3M citrate buffer and 0.1% sodium dodecyl sulphate (SDS), with the pH adjusted to 6.0 using HCl. Synthetic double strand DNAs (gBlocks™ Gene Fragments) that mimics hsp70 fragment of 249bp for *L. braziliensis*, *L. mexicana* and *L. peruviana* were purchased from Integrated DNA Technologies, BV (Leuven, Belgium). Parasitic gDNA from *L. major* - ATCC ID 30012D was purchased from the American

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

Type Culture Collection (ATCC). Human gDNA was purchased from Jena Bioscience. Abasic PNA probes (DGL-Probes) were provided by DESTINA Genomica SL (Granada, Spain) and were HPLC-purified. The concentration of DGL-Probes was measured using a FLUOstar Omega multidetector microplate reader (BMG LABTECH, Offenburg, Germany) based on extinction coefficients for C, T, A, and G (Ɛ260 = 6.6, 8.8, 13.7, and 11.7 mM−1 cm−1, respectively). The biotinylated aldehyde-modified cytosine (C-SMART-Biotin) and other necessary buffers (I-III) were also provided by DESTINA Genomica SL, while buffers IV to VI were purchased from Vitro SA (Granada, Spain). Biodyne C membranes were purchased from Pall Corporation (US), and pre-activation of the membranes was carried out by DESTINA Genomica SL using proprietary chemistry. The Spin-Tube plastic components were purchased from Ciro Manufacturing Corporation (FL, US), and the devices were manually assembled by DESTINA Genomica SL.

2.2. In Silico Design and Identification of Single Nucleotide Fingerprints Target Regions

A set of sequences covering a partial coding sequence (CDS) of the hsp70 gene, containing SNFs within conserved regions across Leishmania species of interest, was selected. These sequences were obtained from the European Nucleotide Archive and the GenBank database. Alignment of the sequences was performed using the NCBI BLAST tool, confirming 100% homology with reference genomes and no significant alignment with the human genome (**Table S1**). Multiple sequence alignment was further conducted using the EMBL-EBI Clustal Omega tool, identifying three conserved regions with specific SNFs (**Figure S1**). Based on these regions and SNFs, three DGL-Probes (DGL1, DGL2 and DGL3) and a single set of PCR primers were designed as reported in **Tables S2** and **S3**.

2.3. DGL-Probe Spotting and Spin-Tube Fabrication

The DGL-Probes were modified with amino-PEGylated groups at the N-terminal end, incorporating glutamic acid residues as a side chain at specific gamma positions (Rissin, et al., 2017; Venkateswaran, et al. 2016). These probes were designed to target regions and interrogated SNFs identified in section 2.2 (**Figure S1**). As a positive control, a biotinylated 5'-amino-modified ssDNA oligo was also spotted. DGL-Probe concentrations were determined using a Thermo Fisher Scientific Nanodrop® ND-2000C spectrophotometer as reported in section 2.1.

The DGL-Probes were covalently immobilized onto preactivated Biodyne C nylon membranes, as previously described by our group (Martín-Sierra et al., 2023; Smirnova et al., 2020; Tabraue-Chávez et al., 2019). DGL-Probes were solubilized in $NaHCO_3$ buffer (pH 8.3, 0.125 M) with amaranth dye (0.2025 mg/mL) and DMSO (30%), and manually spotted onto membranes using a P2 Gilson Pipette. The Spin-Tube devices were assembled by integrating these spotted membranes with plastic components, following the procedure outlined in our previous publications (Tabraue-Chávez et al., 2019).

2.4. Dynamic Chemical Labeling (DCL) with C-SMART-Biotin and Colorimetric Workflow

Protocol was carried out as follows: a) blocking the membranes with 400 µL of pre-heated reagent I; b) washing with 600 µL of pre-heated Reagent II; c) 2 min incubation

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

with 200 µL pre-heated DCL buffer; d) DCL reaction: 20 µL PCR product or 20 µL 5nM ssDNA, 10uL 200 µM C-SMART-Biotin, 10 µL 20mM $NaBH_3CN$ and 160 µL DCL buffer at 45 ºC for 1 hour. Before use, PCR products were denatured at 95 ºC for 10 minutes and kept on ice per 2 minutes just before the addition. Negative controls were prepared by substituting nucleic acids with water or PCR negative controls; e) incubation with 200µL of pre-heated reagent III at 45 ºC for 5 minutes; f) washing with 600 µL reagent II; g) blocking of membranes with 200 µL reagent IV at room temperature for 5 minutes to avoid non-specific binding of streptavidin; h) Incubation with 200 µL AP-Streptavidin (Reagent V) at room temperature for 5 minutes to recognize the biotin incorporated during the DCL reaction; i) washing with 600 µL reagent VI to remove streptavidin excess; j) Chromogenic reaction using 200 µL of reagent VII (BCIP®/NBT) during 10 min at 45 ºC, to generate a dark blue colour with each streptavidin recognition; k) optional washing with 200 µL reagent II. In all steps, the Spin-Tubes were centrifuged at 6000 rpm for 15 seconds to discard the solutions between each step. The protocol required only low-cost equipment, including a Nahita digital thermostatic bath and a VWR MiniSpin centrifuge.

2.5. Single-plex PCR Sensitivity and Specificity Testing

Three pairs of forward and reverse primers were designed to amplify segments of varying lengths from the conserved hsp70 gene in the target *Leishmania* species (**Table S3**). Primer design was performed using the Primer-BLAST 2.0 server (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and cross-reactivity with the human genome was assessed using the Genome Browser tool (http://genome.ucsc.edu/). Primer self-dimer and heterodimer formations were evaluated using Oligo Analyzer 3.1 (IDT, http://www.idtdna.com/calc/analyzer). Primers were purchased by Integrated DNA Technologies (IDT).

PCR amplification was conducted using a Techne TC-5000 PCR thermal cycler. After selecting the optimal primer pair (Forward 2 and Reverse 2), PCR conditions were optimized by testing different annealing temperatures (55 ºC, 57 ºC, and 60 ºC) (**Table S4**) and bioanalyzer results in **Figure S2**. The final PCR reaction was carried out in a 50 µL solution containing 1X GoTaq® G2 Hot Start Colourless Master Mix (Promega) and 0.2 µM of each primer. 10 ng of gBlocks™ were used as templates (**Figure S2**) and various genomic DNA concentrations were tested. Nuclease-free water was used as a negative control. Additionally, 1 ng of human gDNA was included for specificity testing. The final thermal cycling conditions consisted of an initial denaturation at 95 ºC for 5 minutes, followed by 35 cycles of 95 ºC for 30 seconds, annealing at 57 ºC for 30 seconds, and extension at 72 ºC for 30 seconds. A final extension at 72 ºC for 10 minutes was followed by a hold at 4 ºC. PCR products were analysed using an Agilent 2100 Bioanalyzer and DNA 1000 Kit (Agilent Technologies Inc.) and directly used for genotyping by DCL on the Spin-Tube colorimetric device without further purification.

2.6 Patient enrollment and sample collection

For this study, patients were recruited from the province of Santo Domingo de los Tsáchilas, an endemic region for CL in Ecuador (see **Section S1**). All participants were fully informed about the objectives of the research and the diagnostic procedures to be performed on their samples. Upon agreeing to participate, they signed an informed consent form in accordance with HCK-CEISH regulations (see **Section S1**).

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

The study received ethical approval from the Human Research Ethics Committee of HOSPITAL CLÍNICA KENNEDY (HCK-CEISH) in Guayaquil, Ecuador (approval code: HCK-CEISH-2022-013). Patient sample collection adhered to all ethical, methodological, and legal requirements stipulated by current regulations (see **Section S2**).
Biopsies were collected following the procedure described in **Section S2**, and the diagnosis of CL was confirmed through smear analysis (see **Section S3**).

2.7 DNA sequencing

DNA samples from patients were extracted from FTA cards sent from Ecuador to Spain for processing (Whatman, catalogue number WHAWB120210, QIAcard™ Non-Indicating FTA™ Cards). Sample collection was carried out as described in Section S4. Upon arrival in Spain, the FTA cards were stored at room temperature. To ensure traceability and standardized collection for subsequent analysis, relevant patient and sample information—including sample ID, collection date, and observations—was thoroughly documented.

For DNA extraction, the protocol provided by Whatman (catalogue number WB120210) was followed. A 2 mm disc was cut from the FTA card sample and transferred to an Eppendorf tube containing 500 µL of Milli-Q water. The sample was vortexed three times for 5 seconds each. The water was then removed using a sterile pipette, centrifuged for 5 seconds, and any excess liquid was pipetted off. Subsequently, 50 µL of sterile water was added, and the sample was heated at 95°C for 30 minutes. DNA extraction was completed using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

PCR amplification was performed using primers and the PCR protocol described in Section 2.5, with 25 ng of total patient DNA as the template. Amplification products (5 µL) were separated on a 2% agarose gel stained with Gel Red (Biotium, Fremont, CA, USA) and visualized using a UV transilluminator. Results of the gel electrophoresis are presented in Figure S3.

For sequencing, PCR products were purified using the QIAquick PCR Product Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Purified products were sequenced using Sanger sequencing (Stab Vida, Caparica, Portugal) with both Forward 2 and Reverse 2 primers. Sequencing was performed at Genyo (Granada, Spain). Sequencing results are provided in **Figure S6**.

3. Results

3.1. Design of the Assay

PCR approaches targeting the hsp70 gene offer significant advantages for diagnosing and identifying medically relevant *Leishmania* species from both the New and Old Worlds. These methods have been described in the past and allow for the direct detection of parasites from lesion samples, eliminating the need for parasite culture (Mimori et al., 1998; Poloni et al., 2023). The hsp70 gene is highly conserved across *Leishmania* species globally, with minimal size variation, making it an ideal target for species comparison. Additionally, it effectively discriminates between relevant species in

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

both *Leishmania* subgenera, and its multiple copies in the genome facilitate direct amplification from most clinical samples (Mimori et al., 1998).

In this study, we conducted an in-silico analysis of the hsp70 gene to identify SNFs capable of differentiating between CL and MCL in New World *Leishmania* species. The target species included *L. braziliensis, L. panamensis, L. guyanensis*, *L. peruviana*, *L. Mexicana* and *L. amazonensis* (**Figure 1**). Sequences covering a conserved partial region of the hsp70 gene were selected for analysis. Multiple sequence alignments using the Clustal Omega program identified diagnostic regions containing three SNFs that enable species differentiation (**Figure S1**). These regions were chosen based on their proximity, allowing amplification using a single pair of primers (Single-plex PCR). The selected SNFs contain either “Guanine (G), Cytosine (C) or Adenine (A)” nucleobases, enabling species differentiation via a single C-SMART-Biotin incorporation (**Figure 3**).

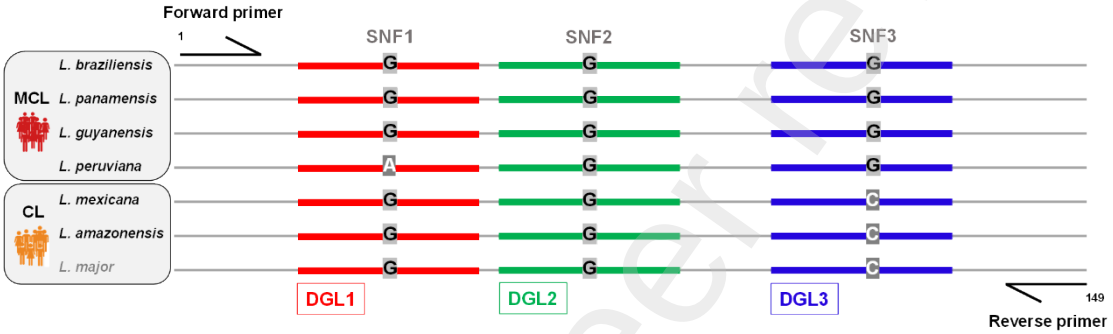


Figure 3. Alignment of Variant Regions in Leishmania hsp70 Target DNA Sequences. This alignment, generated by Single-plex PCR using a single pair of primers, highlights the differentiating SNF targets for DGL-Probe 1 (DGL1, shown in red), DGL-Probe 2 (DGL2, shown in green), and DGL-Probe 3 (DGL3, shown in blue). The nucleobases in the three SNFs are indicated in bold, capital letters, either in black or white for contrast. The complete sequences are provided in **Figure S3**.

This design discriminates species based on two SNFs, serving as target sequences for DGL1 and DGL3 (**Figure 3**). The DGL2 sequence acts as a control for the hsp70 amplicon, confirming the presence of *Leishmania* across all species. As shown in **Table 1**, combining the results from the three DGL-Probes allows differentiation between CL and MCL. This discrimination is based on the incorporation of C-SMART-Biotin, which only occurs when Guanine are opposite the abasic site positions of the complementary DGL-Probes as described in **Figure 2**. For instance, *L. peruviana* is distinguished by the lack of C-SMART-Biotin incorporation at DGL1, as there is an Adenine rather than a Guanine at this, while *L. mexicana* and *L. amazonensis* are discriminate by the absence of C-SMART-Biotin incorporation at DGL3, as their DNA has a Cytosine in front of the Abasic site position of DGL3 (**Figure 3**). The DGL2, targeting a Guanine, consistently shows a positive result across all species, confirming the presence of *Leishmania* and serving as a control.

Table 1. Expected C-SMART-Biotin Incorporation for the Selected DGL-Probes Based on Parasitic Infection and Clinical Manifestation. Legend: (+) Indicates C-SMART-Biotin incorporation, resulting in a detectable colorimetric signal. (-) Indicates no C-SMART-Biotin incorporation, meaning no colorimetric signal is generated.

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

Clinical manifestation	Parasite	DGL1	DGL2	DGL3
Mucocutaneous Leishmaniasis	*L. braziliensis* *L. panamensis* *L. guyanensis*	+	+	+
	L. peruviana	-	+	+
Cutaneous Leishmaniasis	*L. mexicana* *L. amazonensis*	+	+	-

3.2. Spin-Tube Device Fabrication

The Spin-Tube device previously developed by our group (Tabraue-Chávez et al., 2019; -43) consists of the following components: (i) a centrifuge collection tube; (ii) an internal assay column; (iii) a frit; (iv) a nylon membrane with DGL-Probes covalently immobilised; and (v) a plastic pressure ring securing the nylon membrane to the bottom of the column (**Figure 4-(1)**). This innovative platform is designed to perform the DCL reaction and subsequent colourimetric detection. The device supports a reverse dot blot assay format (Tabraue-Chávez et al., 2019), which is processed using a simple benchtop centrifuge. The assay concludes with a visually detectable colourimetric signal that can be easily read with the naked eye, making the Spin-Tube an efficient and accessible diagnostic tool. The three DGL-Probes designed in Section 2.1 were synthesised to complement the selected hsp70 amplicon sequence (shown in **Figure 3**) and to bind in an antiparallel alignment (with the N-terminal of the DGL-Probes facing the 3' end of the target DNA and the C-terminal aligning with the 5' end). These probes were tested on nylon membranes using DCL with C-SMART-Biotin incorporation, with gBlocks as templates and colourimetric readouts for detection (**Figure S3**).

3.3 SNF Identification of Leishmania Species

The Spin-Tube devices were used to validate the differentiation between CL and MCL by interrogating three SNFs through DCL, as shown in **Figure 2**, with a colorimetric readout (see Materials and Methods for details). The three DGL-Probes were manually spotted onto the nylon membrane in specific patterns, enabling species differentiation based on the incorporation of C-SMART-Biotin. A biotinylated 5'-amino-modified ssDNA oligo was also spotted at the top of the membrane as a positive control for the colorimetric reaction (**Figure 4-(2)**).

The DCL-Flow colorimetric assays, conducted on the Spin-Tube platform, utilized synthetic gBlocks™ Gene Fragments of 249 base pairs, representing sequences from *L. braziliensis*, *L. mexicana*, and *L. peruviana* (**Figure S3**). The results are as follows: (a) **Figure 4** (**3-I**): The gBlocks™ sequence for *L. braziliensis* mimicked infections with *L. braziliensis*, *L. panamensis*, and *L. guyanensis*, all species associated with MCL, producing the characteristic pattern shown in **Figure 4** (**4-I**). (b) **Figure 4** (**3-II**): The gBlocks™ sequence for *L. peruviana* simulated infections with *L. peruviana* and generated the expected pattern depicted in **Figure 4** (**4-II**). (c) **Figure 4** (**3-III**): The gBlocks™ sequence for *L. mexicana* represented infections with *L. mexicana* and *L. amazonensis*, both associated with CL, producing the corresponding pattern shown in **Figure 4** (**4-III**). (d) **Figure 4** (**3-IV**): A negative control was performed using water instead of synthetic gBlocks™ Gene Fragments to simulate the absence of parasitic infection. As expected, no colorimetric signal was observed except for the assay control (**CTRL**)

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

spot, which produced a precipitate, as shown in **Figure 4** (**4-IV**). The results confirmed that C-SMART Biotin incorporation occurred only when the target contained a guanine nucleotide within the "abasic site," as indicated in **Table 1**. The negative control (PCR with water) validated the absence of a colorimetric signal, confirming the assay's specificity (**Figure 4** (**4-IV**)).

The assay successfully distinguished species based on the expected DCL reaction patterns, with all results aligning with predictions. This demonstrates the robustness and accuracy of the Spin-Tube platform for species-specific identification and differentiation between CL and MCL.

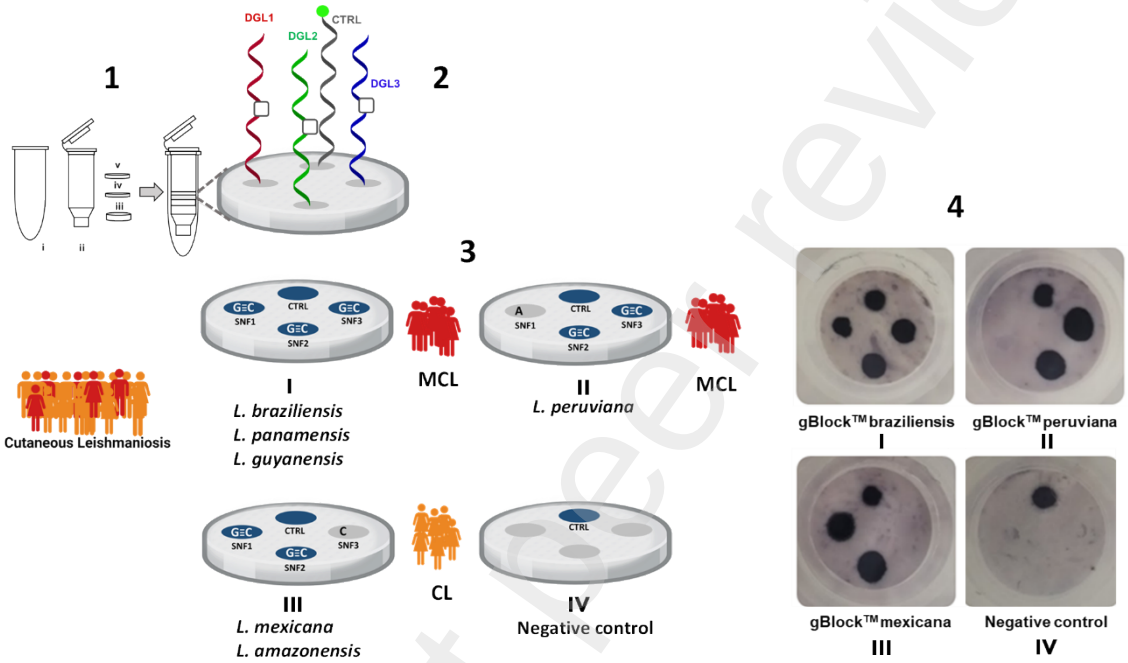


Figure 4. (1) Structure of the Spin-Tube Device: **i**) A centrifuge collection tube; **ii**) An internal column for the assay; **iii**) A frit; **iv**) A nylon membrane (pre-spotted with DGL-Probes and a biotinylated DNA control) immobilized at the bottom of the column with a plastic pressure ring (**v**). **(2) Schematic representation**: The nylon membrane includes three multi-capture DGL-Probes (DGL1, DGL2, and DGL3) and biotinylated oligo DNA spots, immobilized to enable species-specific detection through SNF interrogation. **(3) Illustration of the four test scenarios**: **I**) *L. braziliensis*: detection pattern for gBlocks™ simulating infections with *L. braziliensis*, *L. panamensis*, and *L. guyanensis*; **II**) *L. peruviana*: detection pattern for *L. peruviana*; **III**) *L. mexicana*: detection pattern for *L. mexicana* and *L. amazonensis*; **IV**) Negative control: PCR with no template, simulating no parasitic infection. **(4) Detection via colorimetric reaction**: The assay detects blue precipitate spots after incubation with streptavidin-alkaline phosphatase. Pictures visualization of the four cases described in **(3)** is provided, demonstrating results visible to the naked eye

3.4 Sensitivity and Specificity of the Assay

Single-plex PCR was implemented to amplify a segment of the conserved hsp70 region in the selected *Leishmania* species (**Figure S1**). The sensitivity of this colorimetric assay depends largely on the quality of the single-plex PCR. The detection limit was determined using single-plex PCR with decreasing amounts of *L. major* gDNA, chosen for its similarity to *L. mexicana* at the SNF under investigation with DGL2 (**Figure 3**). Eight concentration points and a negative control were tested (10 ng, 1 ng, 0.1 ng, 10 pg, 5 pg, 2.5 pg and 1.25 pg, of total parasite genomic DNA). PCR amplicons were analysed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

by capillary electrophoresis to assess the quantity and size of the PCR products (**Figure S4**). The expected bands were observed at all concentrations, although a very low yield was obtained with 1.25 pg. DCL colorimetric detection using the Spin-Tube showed clear positive results for samples starting from 10 ng down to 0.1 ng of *L. major* gDNA, while samples with 10 pg to 1.25 pg showed lower but still distinguishable signals from the negative control. Thus, the assay's detection limit is tied to PCR sensitivity, with 1.25 pg of gDNA (equivalent to 0.025 pg/µL or 1 copy/µL) being the lower limit (**Figure 5**).

To assess specificity, the single-plex PCR was challenged using the same gDNA templates with 1 ng of human gDNA at various ratios (50%, 10%, 1%, 0.5%, 0.25%, 0.125%, and 0% parasite DNA). Capillary electrophoresis results showed no amplification with only human gDNA. The amplification was comparable, or slightly better, in parasite-human DNA mixtures than in parasite DNA alone. **Figure S6** shows an example of capillary electrophoresis results for 0.1 ng *L. major*, 0.1 ng *L. major* with 1 ng human gDNA, and 1 ng human gDNA, demonstrating that the selected primers and PCR protocol selectively amplify parasitic gDNA. All colorimetric assays-maintained specificity in the presence of human gDNA (**Figure 6**).

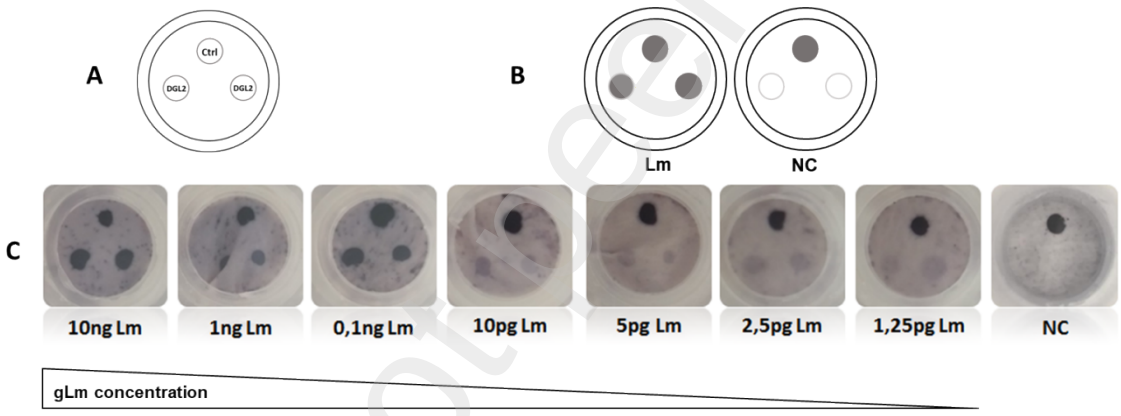
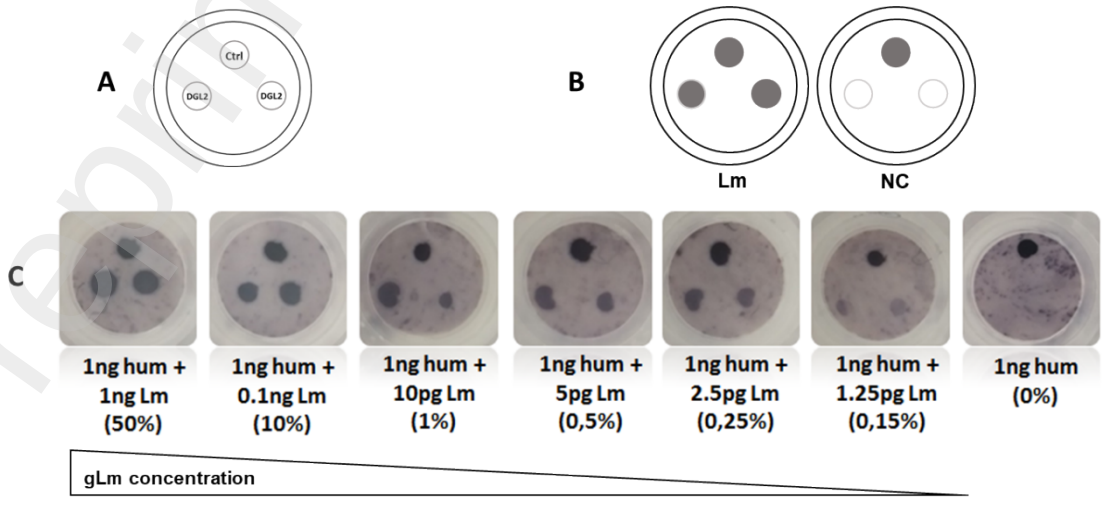


Figure 5. **Sensitivity of the assay**. (**A**) Spotting patterns of the DGL2 probe (left and right) and biotinylated oligo DNA (top) in the nylon membrane of the Spin-Tube. (**B**) Expected results with the incorporation of C-SMART-Biotin. (**C**) Test results with the PCR products at different initial amount of *L. major* gDNA.



This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

Figure 6. **Specificity of the assay in presence of human gDNA**. (**A**) Spotting patterns of the DGL2 probe (left and right) and biotinylated oligo DNA (top) in the nylon membrane of the Spin-Tube. (B) Expected results with the incorporation of C-SMART-Biotin. (C) Test results for PCR products in presence of 1 ng of human DNA and different amounts of starting parasite gDNA templates. **Lm**: *Leishmania* gDNA; **hum**: human gDNA.

3.5. Assay validation with clinical samples

The Spin-Tube devices were used to analyse clinical samples from ten patients diagnosed with CL through smear analysis, as described in **Section S3**. While smear analysis is effective for identifying pathogens, it cannot distinguish between species responsible for simple CL or the more severe MCL. To address this limitation, the Spin-Tube device and the newly developed assay were used following the workflow showed in **Figure 7A**. Tissue samples were collected from patients' ulcers, DNA was extracted and amplified using single-plex PCR, and the resulting amplicons were analysed in the Spin-Tube using the sDCL colorimetric reaction (DCL Rxn).

A novel Spin-Tube pattern was designed to differentiate CL from MCL by interrogating three SNFs with three DCL-Probes. The first row of the Spin-Tube contained a spot with a biotinylated DNA oligo serving as a control (CTRL), while the second row contained three spots corresponding to DGL1, DGL3, and DGL2. These spots were used to interrogate SNF1, SNF3, and SNF2, respectively (**Figure 7A**). Before to Spin-Tube incubation, PCR amplification products were verified using gel electrophoresis, as shown in Figure S6.

The Spin-Tubes were then used to process the PCR products from all ten clinical samples with the established colorimetric protocol. The results (**Figure 7B**) demonstrated successful hybridization of the DNA amplicons with the DGL-Probes, which were interrogated using C-SMART Biotin. Spin-Tubes from all ten patients showed a consistent positive pattern across all spots, indicating the presence of one of the following species: *L. braziliensis*, *L. panamensis*, or *L. guyanensis* (according to **Table 1**). This confirmed the diagnosis of MCL in all cases. Negative controls, prepared using water, displayed the expected negative control (NC) pattern, with only the CTRL spot showing a positive signal.

Further analysis of the PCR products via sequencing confirmed the presence of characteristic MCL sequences, including a “G” at all three SNF positions. These sequencing results validated the diagnoses made using the novel colorimetric assay, as shown in **Figure S7**.

Additionally, the signal-to-background ratio and the positive CTRL signal were quantified, as detailed in **Table S6**. A cut-off of 5% was established to classify a signal as positive. This analysis confirmed that all three spots in the pattern displayed clear positive signals when assessed through absolute quantification using a quantitative method, rather than relying solely on visual interpretation. These results underscore the robustness and reliability of the assay in diagnosing *leishmaniasis* and differentiating between CL and MCL, providing an effective tool for species-specific diagnosis.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

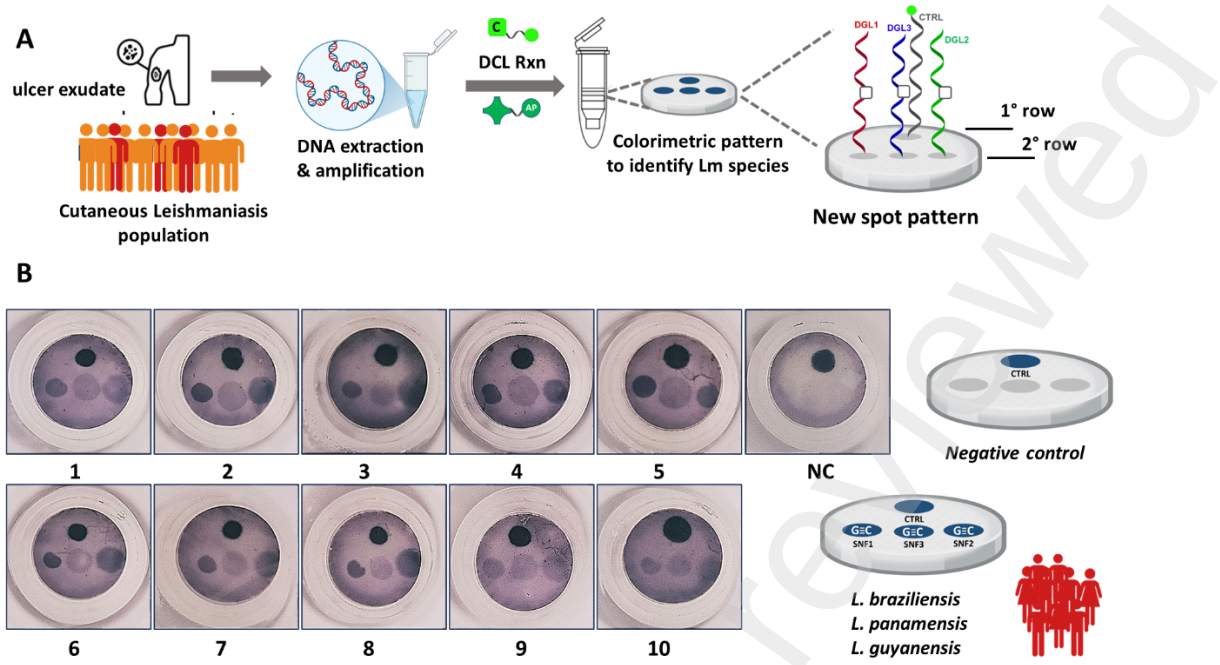


Figure 7. **Validation with clinical samples**. **(A)** The complete workflow of the assay, presented from left to right, illustrates the process from sample collection to interrogation using the novel spot pattern on the Spin-Tube Device. **(B)** Results obtained from the ten patient samples and the negative control (NC). The spot pattern indicates the incorporation of C-SMART-Biotin across the three spots in the second row.

4. Discussion

Molecular diagnostic methods, such as qPCR-based assays, have been pivotal in advancing the detection and identification of *Leishmania* parasites (Bell and Ranford-Cartwright, 2002; Tawfeeq and Ali, 2022; Wu et al., 2020). However, while these techniques provide high sensitivity, they remain costly and inaccessible in many endemic regions, and they struggle to differentiate between species that cause varying clinical manifestations. In the context of leishmaniasis, species identification is critical not only for effective treatment but also for disease control, especially in distinguishing between CL and the more severe MCL (Chicharro et al., 2023; Jariyapan et al., 2021; Mimori et al., 1998; Soares et al., 2024).

In this study, we addressed the need for early and accurate species differentiation by developing a novel colorimetric assay. This assay combines DCL technology with the Spin-Tube Device to identify *Leishmania* species responsible for CL and MCL in the New World (**Figure 1**). By targeting SNF markers within the highly conserved hsp70 gene, we provide a robust platform for rapid and specific diagnosis (**Figure 3**). The assay's ability to detect and differentiate between key species, such as *L. braziliensis*, *L. panamensis*, *L. guyanensis* and *L. peruviana* (associated with MCL) and *L. Mexicana* and *L. amazonensis* (primarily responsible for CL), is central for guiding appropriate treatment, especially considering the toxic side effects of aggressive treatments required for MCL (Goto and Lindoso, 2010; Pinheiro and de Souza, 2022)

Our approach takes advantage of the conserved nature of the hsp70 gene, which contains SNF markers specific to different Leishmania species. These three SNFs form a unique molecular signature, and their simultaneous interrogation allows for the precise discrimination between CL and MCL. By integrating DCL technology, we enable a

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

straightforward detection process. The presence of a guanine (G) nucleotide at the SNF site templates a dynamic covalent reaction between the C-SMART-Biotin and the DGL-Probe, generating a colorimetric signal. In contrast, other nucleobases, such as cytosine (C) and adenine (A), do not permit signal generation, as showed in **Figure 2**.

The method introduces several novel aspects: a unique design that combines SNFs for differentiating species associated with CL and MCL leishmaniasis, which has not been previously described, and a single-plex PCR specifically targeting these SNFs. Furthermore, it utilizes the innovative DCL approach for their interrogation, providing 100% specificity. Any mismatch during hybridization or the presence of a nucleobase other than "G" within the chemical pocket prevents the incorporation of C-SMART-Biotin, thereby eliminating the generation of a colorimetric signal.

This method significantly simplifies the detection process by eliminating the need for target labeling, a common requirement in other molecular diagnostics that rely solely on capture-based methods. Furthermore, the use of a single primer set for PCR amplification enhances the assay's efficiency and reduces complexity, making it particularly suitable for clinical samples in resource-limited settings.

One of the key advantages of this assay is its capacity to differentiate between species with clinical relevance to MCL and CL. The ability to detect species, which requires more aggressive treatment, due to their association with MCL, ensures that healthcare providers can implement timely and appropriate interventions. This early differentiation is crucial because delaying treatment in MCL cases can lead to irreversible mucosal tissue damage, severe disfigurement, and long-term functional impairments (Haldar et al., 2011; Pinheiro and de Souza, 2022). Furthermore, the simplicity of the Spin-Tube device and the colorimetric readout makes the assay accessible and affordable, potentially enabling widespread use in endemic regions where access to molecular diagnostics is limited.

The assay demonstrates a detection limit of 1 copy of parasite DNA per µL, which is highly advantageous for early diagnosis, especially in asymptomatic or low-parasite-load cases. This sensitivity, coupled with the assay's specificity in differentiating between clinically important *Leishmania* species, makes it a valuable tool for disease management in both endemic and non-endemic regions. While qPCR remains the gold standard for *leishmaniasis* diagnosis, our assay offers a cost-effective alternative that can be easily adapted for field use, particularly in high-risk populations where rapid diagnosis is critical.

Validation of the assay on clinical samples further demonstrates its reliability and robustness. Ten clinical samples from patients diagnosed with CL were analysed using the Spin-Tube device and subsequently validated by sequencing. The sequencing results confirmed the presence of the characteristic MCL-associated sequences, including the "G" nucleotide at all three SNF positions, which is perfectly consistent with the diagnosis of MCL (Figure 7). These results highlight the ability of the assay to provide accurate species differentiation and diagnostic accuracy.

Signal intensities were slightly lower for DGL2, probably due to manual spotting variability. However, automation of the spotting process and optimisation of spotting

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

concentrations are expected to normalise signal intensities and further improve assay performance and overall quality.

5. Conclusion

This study presents proof of concept for a novel molecular assay that facilitates rapid and accurate identification and differentiation of *Leishmania* species in the New World. The assay distinguishes species associated with CL from those causing MCL with 100% specificity. Using specific SNF markers in the hsp70 gene, it provides a robust alternative to traditional three-step binding technologies such as TaqMan, Molecular Beacons or Scorpion probes. The assay's simple colourimetric readout system makes it particularly suitable for resource-limited settings. With a detection limit of 1 copy of parasite gDNA per µL, it maintains high specificity even in the presence of human DNA. The total assay time is approximately 3 hours, including 90 minutes for PCR amplification, 60 minutes for the dynamic chemical reaction and 30 minutes for colour development.

This novel assay represents a unique solution for the early diagnosis of *Leishmania* infections, offering significant advantages in terms of cost (€1 per test manufacturing cost) and simplicity (no need for large equipment). The ability of the DCL technology to achieve triple binding specificity while maintaining a simple colorimetric output makes it particularly valuable for early species differentiation, allowing timely and appropriate treatment decisions for CL and MCL cases. Our group is actively investigating the integration of isothermal endpoint single-plex PCR with lyophilised reagents, which would allow the entire workflow, including PCR, to be performed as an on-site test suitable for resource-limited settings. In addition, this approach has potential applications for a wider range of diagnostic assays for other infectious diseases where species differentiation or single nucleotide variant detection is essential, such as malaria and tuberculosis.

The validation of the assay using clinical samples strongly supports its diagnostic potential. Ten patient samples diagnosed with CL were analysed using the Spin-Tube device, and the results were confirmed through sequencing. Sequencing verified the presence of MCL-specific sequences, including the “G” nucleotide at all three SNF positions, confirming the assay’s accuracy in differentiating species responsible for CL and MCL. These findings demonstrate the assay’s robustness and applicability in real-world diagnostic settings.

Further studies involving a larger cohort of patients are needed to validate the diagnostic accuracy of the assay across a broader range of clinical scenarios, including infected patients, healthy controls, and individuals with other conditions. We anticipate significant improvements in the performance of the assay with the proper manufacturing of the Spin-Tube device, incorporating automated spotting and high clinical-grade molecular diagnostic device manufacturing standards. Additionally, integrating a quantification system with the device would enable the interpretation of results in a numerical and quantitative manner, further enhancing the precision and usability of the assay.

We highly believe that a diagnostic tool capable of detecting low parasite levels in early symptomatic patients and reliably differentiating between *Leishmania* species would provide substantial benefits for disease management in both endemic and non-endemic regions. The approach presented here has the potential to address this critical need.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

Declaration of competing interest

The authors declare the following financial interests which may be considered as potential competing interests: JJDM is a founder, shareholder and Director of DESTINA Genomics Ltd. SP is a shareholder of DESTINA Genomics Ltd. DESTINA Genomica SL is a wholly owned subsidiary of DESTINA Genomics Ltd. DESTINA is interested in the exploitation of the technology developed here.

Data availability

Data will be made available on request.

Acknowledgements

This research work was funded by Instituto de Investigación e Innovación en Salud Integral, Universidad Católica de Santiago de Guayaquil, Guayaquil, Ecuador, Leishmaniasis Project (SINDE-SIU #592). This research was partially supported by the the European Union Next GenerationEU/ PRTR (grant number: PID2019.110987RB.I00 and PID2022-141065OB-I00) The authors are members of the NANOCARE 2.0 network (Grant RED2022-134560-T) funded by MCIN/AEI/10.13039/501100011033.

Appendix A. Supplementary data

Supplementary data to this article can be found online at

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used DeepL Write and ChatGPT in order to provide language refinement and proofreading purposes during the preparation of this manuscript. These tools were used to improve clarity and coherence without changing the scientific content or interpretations. All intellectual contributions and the final responsibility for the content remain solely with the authors. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

References

Aronson, N., Herwaldt, B.L., Libman, M., Pearson, R., Lopez-Velez, R., Weina, P., Carvalho, E.M., Ephros, M., Jeronimo, S., Magill, A., 2016. Diagnosis and Treatment of Leishmaniasis: Clinical Practice Guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH). Clin. Infect. Dis. 63, 1539–1557 https://doi.org/10.1093/cid/ciw742

Bell, A.S., Ranford-Cartwright, L.C., 2002. Real-time quantitative PCR in parasitology. Trends Parasitol. 18, 337-342 https://doi.org/10.1016/S1471-4922(02)02331-0

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

Bowler, F.R., Diaz-Mochon, J.J., Swift, M.D., Bradley, M., 2010. DNA analysis by dynamic chemistry. Angew. Chem. Int. Ed. Engl. 49, 1809–1812 https://doi.org/10.1002/anie.200905699

Bowler, F.R., Reid, P.A., Boyd, C.A., Diaz-Mochon, J.J., Bradley, M., 2011. Dynamic chemistry for enzyme-free allele discrimination in genotyping by MALDI-TOF mass spectrometry. Anal. Methods 3, 1656–1663 https://doi.org/10.1039/C1AY05176H

Chicharro, C., Nieto, J., Miguelañez, S., Garcia, E., Ortega, S., Peña, A., Rubio, J.M., Flores-Chavez, M., 2023. Molecular Diagnosis of Leishmaniasis in Spain: Development and Validation of Ready-To-Use Gel-Form Nested and Real-Time PCRs To Detect Leishmania spp. Microbiol. Spectr. 11, e0335422 https://doi.org/10.1128/spectrum.03354-22

Conde, L., Maciel, G., de Assis, G.M., Freire-de-Lima, L., Nico, D., Vale, A., Freire-de-Lima, C.G., Morrot, A., 2022 Humoral response in Leishmaniasis. Front. Cell. Infect. Microbiol. 12, 1063291 https://doi.org/10.3389/fcimb.2022.1063291

Costa-da-Silva, A.C., Nascimento, D.O., Ferreira, J.R.M., Guimarães-Pinto, K., Freire-de-Lima, L., Morrot, A., Decote-Ricardo, D., Filardy, A.A., Freire-de-Lima, C.G., 2022. Immune Responses in Leishmaniasis: An Overview. Trop. Med. Infect. Dis. 7, 54 https://doi.org/10.3390/tropicalmed7040054

Detassis, S., Grasso, M., Tabraue-Chávez, M., Marín-Romero, A., López-Longarela, B., Ilyine, H., Ress, C., Ceriani, S., Erspan, M., Maglione, A., Díaz-Mochón, J.J., Pernagallo, S., Denti, M.A., 2019. New Platform for the Direct Profiling of microRNAs in Biofluids. Anal. Chem. 91, 5874–5880 https://doi.org/10.1021/acs.analchem.9b00213

Detassis, S., Precazzini, F., Brentari, I., Ruffilli, R., Ress, C., Maglione, A., Pernagallo, S., Denti, M.A., 2024. SA-ODG platform: a semi-automated and PCR-free method to analyse microRNAs in solid tissues. Analyst, 149, 3891-3899. https://doi.org/10.1039/d4an00783b

Delgado-Gonzalez, A., Robles-Remacho, A., Marin-Romero, A., Detassis, S., Lopez-Longarela, B., Lopez-Delgado, F.J., de Miguel-Perez, D., Guardia-Monteagudo, J.J., Fara, M.A., Tabraue-Chavez, M., Pernagallo, S., Sanchez-Martin, R.M., Diaz-Mochon, J.J., 2019. PCR-free and chemistry-based technology for miR-21 rapid detection directly from tumour cells. Talanta, 200, 51–56. https://doi.org/10.1016/j.talanta.2019.03.039

Garcia-Fernandez, E., Gonzalez-Garcia, M.C., Pernagallo, S., Ruedas-Rama, M.J., Fara, M.A., López-Delgado, F.J., Dear, J.W., Ilyine, H., Ress, C., Díaz-Mochón, J.J., Orte, A., 2019. miR-122 direct detection in human serum by time-gated fluorescence imaging. Chem. Commun. 55, 14958–14961. https://doi.org/10.1039/c9cc08069d

Goto, H., Lindoso, J.A., 2010. Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. Expert Rev. Anti. Infect. Ther. 8, 419–33. https://doi.org/10.1586/eri.10.19

Haldar, A.K., Sen, P., Roy, S., 2011. Use of antimony in the treatment of leishmaniasis: current status and future directions. Mol. Biol. Int. 571242. https://pmc.ncbi.nlm.nih.gov/articles/PMC3196053/

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

Jani, I.V., Peter, T.F., 2022. Nucleic Acid Point-of-Care Testing to Improve Diagnostic Preparedness. Clin. Infect. Dis. 75, 723–728. https://doi.org/10.1093/cid/ciac013

Jariyapan, N., Bates, M.D., Bates, P.A., 2021. Molecular identification of two newly identified human pathogens causing leishmaniasis using PCR-based methods on the 3' untranslated region of the heat shock protein 70 (type I) gene. PLoS Negl. Trop. Dis. 15, e0009982. https://doi.org/10.1371/journal.pntd.0009982

Kevric, I., Cappel, M.A., Keeling, J.H., 2015. New World and Old World Leishmania Infections: A Practical Review. Dermatol. Clin. 33, 579–593. https://doi.org/10.1016/j.det.2015.03.018

Lopez-Longarela, B., Morrison, E.E., Tranter, J.D., Chahman-Vos, L., Léonard, J.F., Gautier, J.C., Laurent, S., Lartigau, A., Boitier, E., Sautier, L., Carmona-Saez, P., Martorell-Marugan, J., Mellanby, R.J., Pernagallo, S., Ilyine, H., Rissin, D.M., Duffy, D.C., Dear, J.W., Díaz-Mochón, J.J., 2020. Direct Detection of miR-122 in Hepatotoxicity Using Dynamic Chemical Labeling Overcomes Stability and isomiR Challenges. Anal. Chem. 92, 3388–3395. https://doi.org/10.1021/acs.analchem.9b05449

Luque-González, A.M., Tabraue-Chávez, M., López-Longarela, B., Sánchez-Martín. M.R., Ortiz-González, M., Soriano-Rodríguez, M., García-Salcedo, A.J., Pernagallo, S., Díaz-Mochón, J.J., 2018. Identification of Trypanosomatids by detecting Single Nucleotide Fingerprints using DNA analysis by dynamic chemistry with MALDI-ToF. Talanta, 176, 299–307. https://doi.org/10.1016/j.talanta.2017.07.059

Mann, S., Frasca, K., Scherrer, S., Henao-Martínez, A.F., Newman, S., Ramanan, P., Suarez, J.A., 2021. A Review of Leishmaniasis: Current Knowledge and Future Directions. Curr. Trop. Med. Rep. 8, 121–132. https://doi.org/10.1016/j.det.2015.03.018

Marín-Romero, A., Pernagallo, S., 2023. A comprehensive review of Dynamic Chemical Labelling on Luminex xMAP technology: a journey towards Drug-Induced Liver Injury testing. Anal. Methods 15, 6139–6149. https://doi.org/10.1039/d3ay01481a

Marín-Romero, A., Regele, V., Kolanovic, D., Hofner, M., Díaz-Mochón, J.J., Nöhammer, C., Pernagallo, S., 2023. MAGPIX and FLEXMAP 3D Luminex platforms for direct detection of miR-122-5p through dynamic chemical labelling. Analyst, 148, 5658–5666. https://doi.org/10.1039/d3an01250f

Marín-Romero, A., Tabraue-Chávez, M., Dear, J.W., Díaz-Mochón, J.J., Pernagallo, S., 2022. Open a New Window on the World of Circulating microRNAs by Merging ChemiRNA Tech with Luminex Platform. Sensors & Diagnostics, 1, 1243-1251. https://doi.org/10.1039/D2SD00111J

Marín-Romero, A., Tabraue-Chávez, M., López-Longarela, B., Fara, M.A., Sánchez-Martín, R.M., Dear, J.W., Ilyine, H., Díaz-Mochón, J.J., Pernagallo, S., 2021. Simultaneous Detection of Dott.ug-Induced Liver Injury Protein and microRNA Biomarkers Using Dynamic Chemical Labelling on a Luminex MAGPIX System. Analytica, 2, 130–139. https://doi.org/10.3390/analytica2040013

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

Marín-Romero, A., Tabraue-Chavez, M., Dear, J.W., Sanchez-Martin, R.M., Ilyine, H., Guardia-Monteagudo, J.J., Fara, M.A., Lopez-Delgado, F.J., Diaz-Mochon, J.J., Pernagallo, S., 2020. Amplification-free profiling of microRNA-122 biomarker in DILI patient serums, using the luminex MAGPIX system. Talanta, 219, 121265. https://doi.org/10.1016/j.talanta.2020.121265

Martín-Sierra, C., Chavez, M.T., Escobedo, P., García-Cabrera, V., López-Delgado, F.J., Guardia-Monteagudo, J.J., Ruiz-García, I., Erenas, M.M., Sanchez-Martin, R.M., Capitán-Vallvey, L.F., Palma, A.J., Pernagallo, S., Diaz-Mochon, J.J., 2023. SARS-CoV-2 viral RNA detection using the novel CoVradar device associated with the CoVreader smartphone app. Biosens. Bioelectron. 15, 115268. https://doi.org/10.1016/j.bios.2023.115268

Mathison, B.A., Bradley, B.T., 2023. Review of the Clinical Presentation, Pathology, Diagnosis, and Treatment of Leishmaniasis. Lab Med 54, 363–371. https://doi.org/10.1093/labmed/lmac134

Mimori, T., Sasaki, J., Nakata, M., Gomez, E.A., Uezato, H., Nonaka, S., Hashiguchi, Y., Furuya, M., Saya, H., 1998. Rapid identification of Leishmania species from formalin-fixed biopsy samples by polymorphism-specific polymerase chain reaction. Gene, 210, 179–86. https://doi.org/10.1016/s0378-1119(97)00663-x

Padial-Jaudenes, M., Tabraue-Chávez, M., Detassis, S., Ruedas-Rama, M.J., Gonzalez-Garcia, M.C., Fara, M.A., López-Delgado, F.J., González-Vera, J.A., Guardia-Monteagudo, J.J., Diaz-Mochon, J.J., Garcia-Fernandez, E., Pernagallo, S., Orte, A., 2024. Multiplexed MicroRNA Biomarker Detection by Bridging Lifetime Filtering Imaging and Dynamic Chemistry Labeling. Sensors and Actuators B: Chemical, 136136. https://doi.org/10.1016/J.SNB.2024.136136

Parkash, V., Ashwin, H., Dey, S., Sadlova, J., Vojtkova, B., Van Bocxlaer, K., Wiggins, R., Thompson, D., Dey, N.S., Jaffe, C.L., Schwartz, E., Volf, P., Lacey, C.J.N., Layton, A.M., Kaye, P.M., 2024. Safety and reactogenicity of a controlled human infection model of sand fly-transmitted cutaneous leishmaniasis. Nat. Med. 30, 3150–3162. https://doi.org/10.1038/s41591-024-03146-9

Pernagallo, S., Ventimiglia, G., Cavalluzzo, C., Alessi, E., Ilyine, H., Bradley, M., Diaz-Mochon, J.J., 2012. Novel biochip platform for nucleic acid analysis. Sensors (Basel) 12, 8100–8111. https://doi.org/10.3390/s120608100

Pinheiro, A.C., de Souza, M.V.N., 2022. Current leishmaniasis drug discovery. RSC Med. Chem. 13, 1029–1043. https://doi.org/10.1039/d1md00362c

Poloni, A., Giacomelli, A., Corbellino, M., Grande, R., Nebuloni, M., Rizzardini, G., Ridolfo, A.L., Antinori, S., 2023. Delayed diagnosis among patients with cutaneous and mucocutaneous leishmaniasis. Travel. Med. Infect. Dis. 2023, 55, 102637. https://doi.org/10.1016/j.tmaid.2023.102637

Rissin, D.M., López-Longarela, B., Pernagallo, S., Ilyine, H., Vliegenthart, A.D.B., Dear, J.W., Díaz-Mochón, J.J., Duffy, D.C., 2017. Polymerase-free measurement of microRNA-122 with single base specificity using single molecule arrays: Detection of drug-induced liver injury. PLoS One. 12, e0179669. https://doi.org/10.1371/journal.pone.0179669

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

Smirnova, T., Andreevskaya, S., Larionova, E., Andrievskaya, I., Kiseleva, E., Chernousova, L., Ergeshov, A., Diaz Mochon, J., Trabue, M., Pernagallo, S., Fara, M.A., Martínez-Murcia, A., Garcia Sierra, A., Navarro Garcia, A., Manganelli, M., Barzon, L., Sinigaglia, A., Bradley, M., Norman, D., Venkateswaran, S., 2020. Evaluation of DestiNA 'spin-tube' technology a novel test for the diagnosis of tuberculosis and mycobacteriosis. Eur. Respir. J. 56(suppl 64), 526. https://doi.org/10.1183/13993003.congress-2020.526

Soares, A.R.C., Faria, V.C.S., Avelar, D.M., 2024. Development and accuracy evaluation of a new loop-mediated isothermal amplification assay targeting the HSP70 gene for the diagnosis of cutaneous leishmaniasis. PLoS One. 19, e0306967. https://doi.org/10.1371/journal.pone.0306967

Tabraue-Chávez, M., Luque-González, M.A., Marín-Romero, A., Sánchez-Martín, R.M., Escobedo-Araque, P., Pernagallo, S., Díaz-Mochón, J.J., 2019. A colorimetric strategy based on dynamic chemistry for direct detection of Trypanosomatid species. Sci Rep. 9, 3696. https://doi.org/10.1038/s41598-019-39946-0

Tawfeeq, H.M., Ali, S.A., 2022. Molecular-based assay for genotyping Leishmania spp. from clinically suspected cutaneous leishmaniasis lesions in the Garmian area, Kurdistan Region of Iraq. Parasite Epidemiol. Control. 17, e00240. https://doi.org/10.1016/j.parepi.2022.e00240

Thakur, S., Joshi, J., Kaur, S., 2020. Leishmaniasis diagnosis: an update on the use of parasitological, immunological and molecular methods. J. Parasit. Dis. 44, 253–272. https://doi.org/10.1007/s12639-020-01212-w

Valero, N.N.H., Prist, P., Uriarte, M., 2021. Environmental and socioeconomic risk factors for visceral and cutaneous leishmaniasis in São Paulo, Brazil. Sci. Total Environ. 797, 148960. https://doi.org/10.1016/j.scitotenv.2021.148960

Venkateswaran, S., Luque-González, M.A., Tabraue-Chávez, M., Fara, M.A., López-Longarela, B., Cano-Cortes, V., López-Delgado, F.J., Sánchez-Martín, R.M., Ilyine, H., Bradley, M., Pernagallo, S., Díaz-Mochón, J.J., 2016. Novel bead-based platform for direct detection of unlabelled nucleic acids through Single Nucleobase Labelling. Talanta. 161, 489–496. https://doi.org/10.1016/j.talanta.2016.08.072

Wu, Y., Tian, X., Song, N., Huang, M., Wu, Z., Li, S., Waterfield, N.R., Zhan, B., Wang, L., Yang, G., 2020. Application of Quantitative PCR in the Diagnosis and Evaluating Treatment Efficacy of Leishmaniasis. Front. Cell. Infect. Microbiol. 10, 581639. https://doi.org/10.3389/fcimb.2020.581639

Yadav, P., Azam, M., Ramesh, V., Singh, R., 2023. Unusual Observations in Leishmaniasis-An Overview. Pathogens 12, 297. https://doi.org/10.3390/pathogens12020297

Yin, Y., Zhu, P., Guo, Y., Li, Y., Chen, H., Liu, J., Sun, L., Ma, S., Hu, C., Wang, H., 2024. Enhancing lower respiratory tract infection diagnosis: implementation and clinical assessment of multiplex PCR-based and hybrid capture-based targeted next-generation sequencing. EBioMedicine 107, 105307. https://doi.org/10.1016/j.ebiom.2024.105307

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006

Web references

European Nucleotide Archive. https://www.ebi.ac.uk/ena/ (16th December 2024).

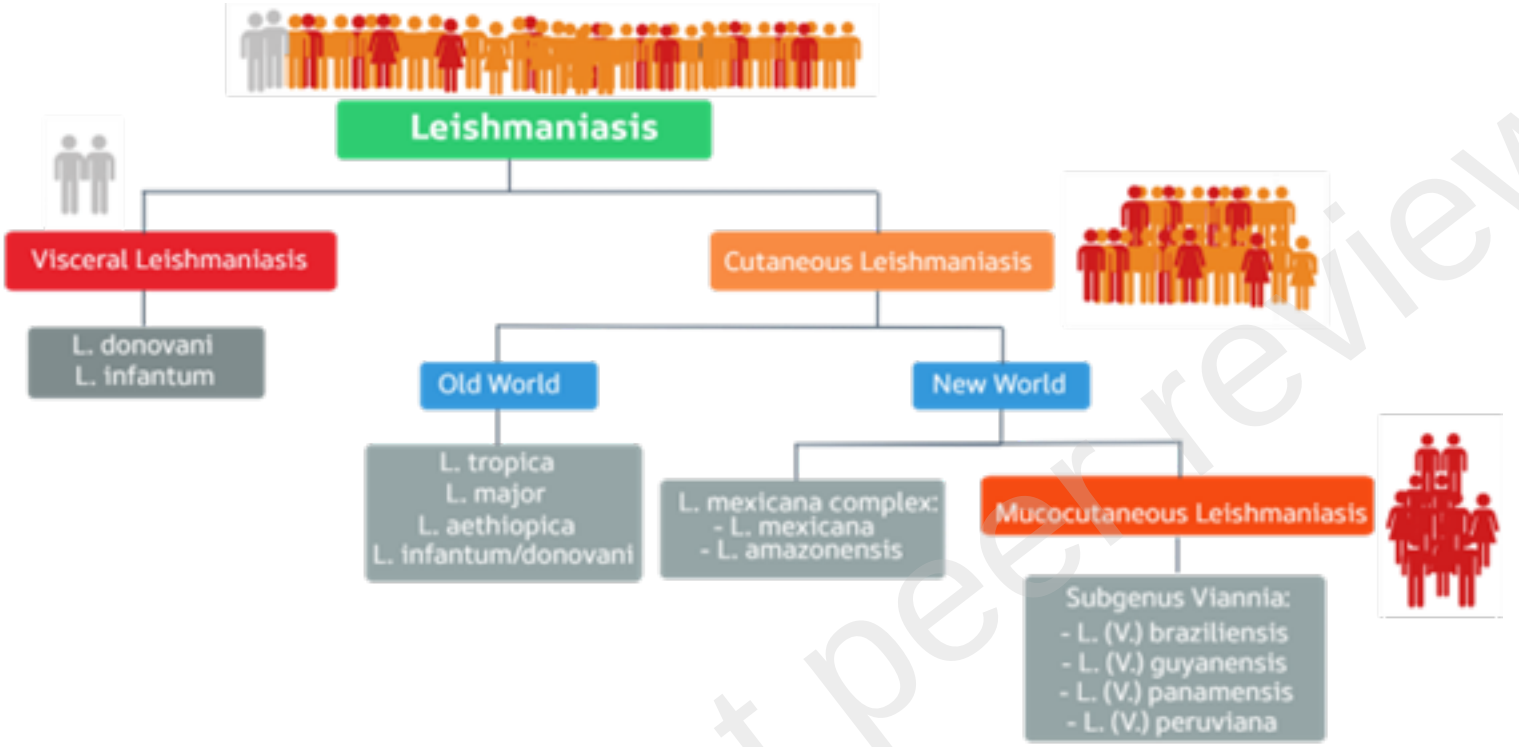
GenBank database. https://www.ncbi.nlm.nih.gov/genbank/ (16th December 2024).

NCBI BLAST tool. https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch (16th December 2024).

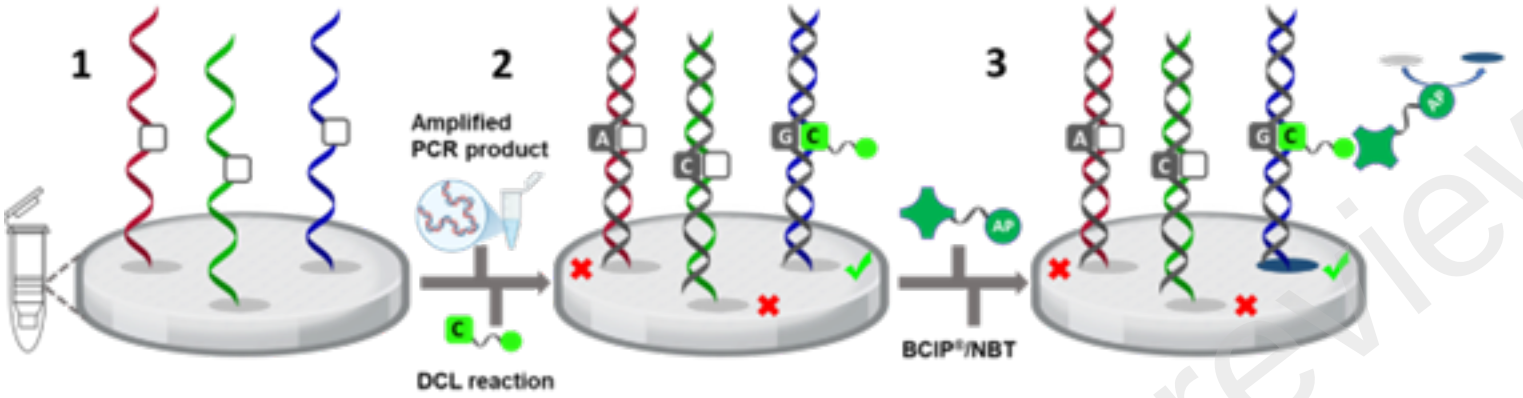
EMBL-EBI Clustal Omega tool. http://www.ebi.ac.uk/Tools/msa/clustalo/ (16th December 2024).

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=5131006



Preprint not peer reviewed

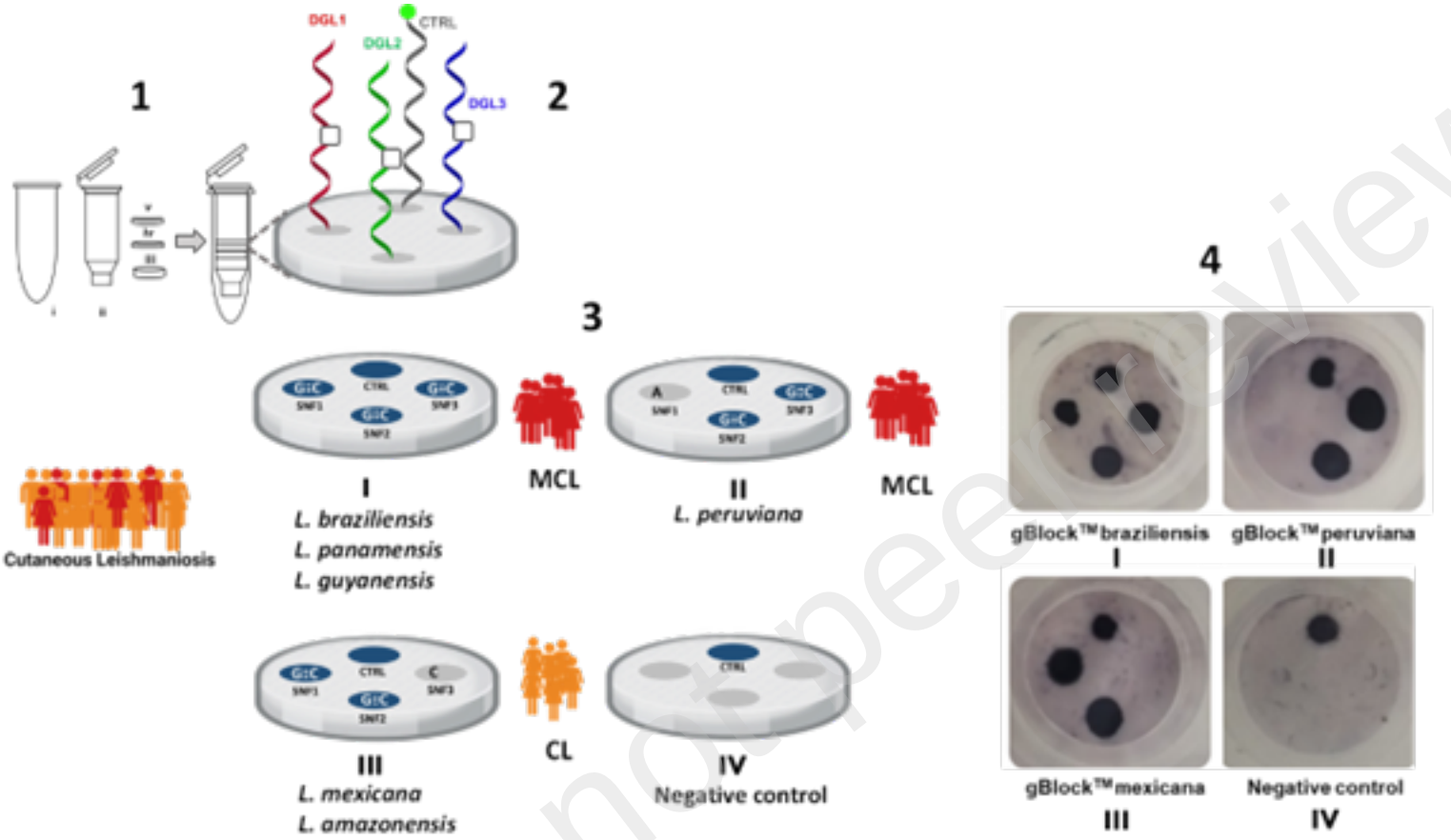




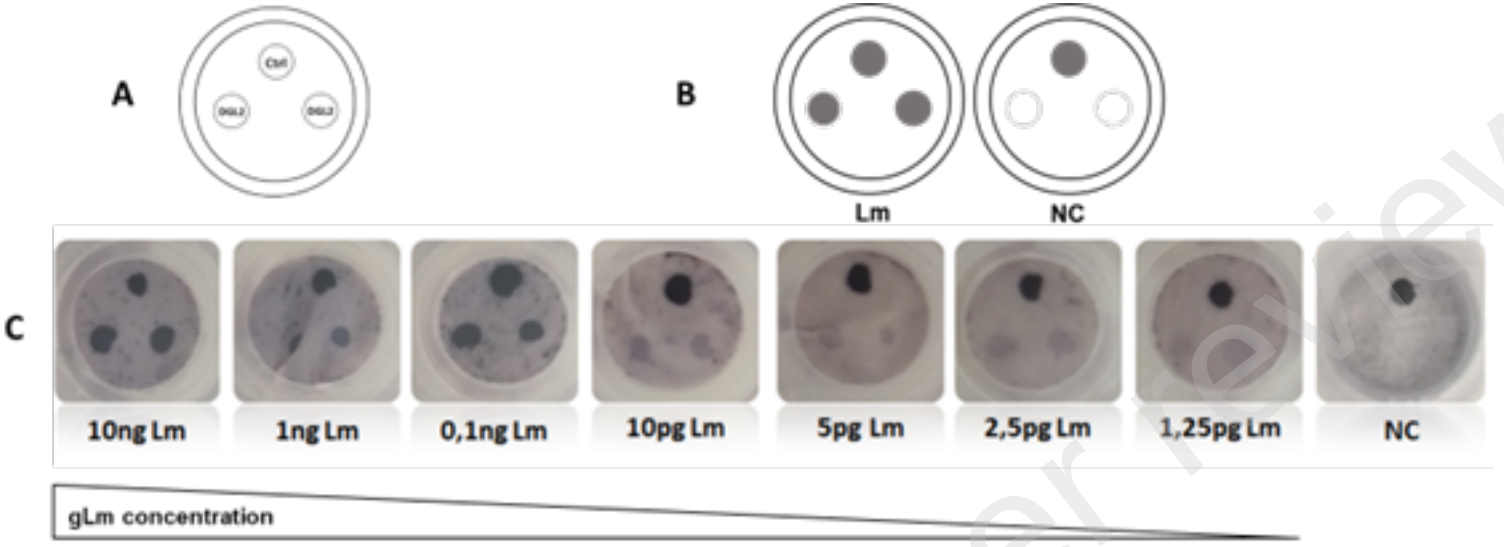
Preprint not peer reviewed



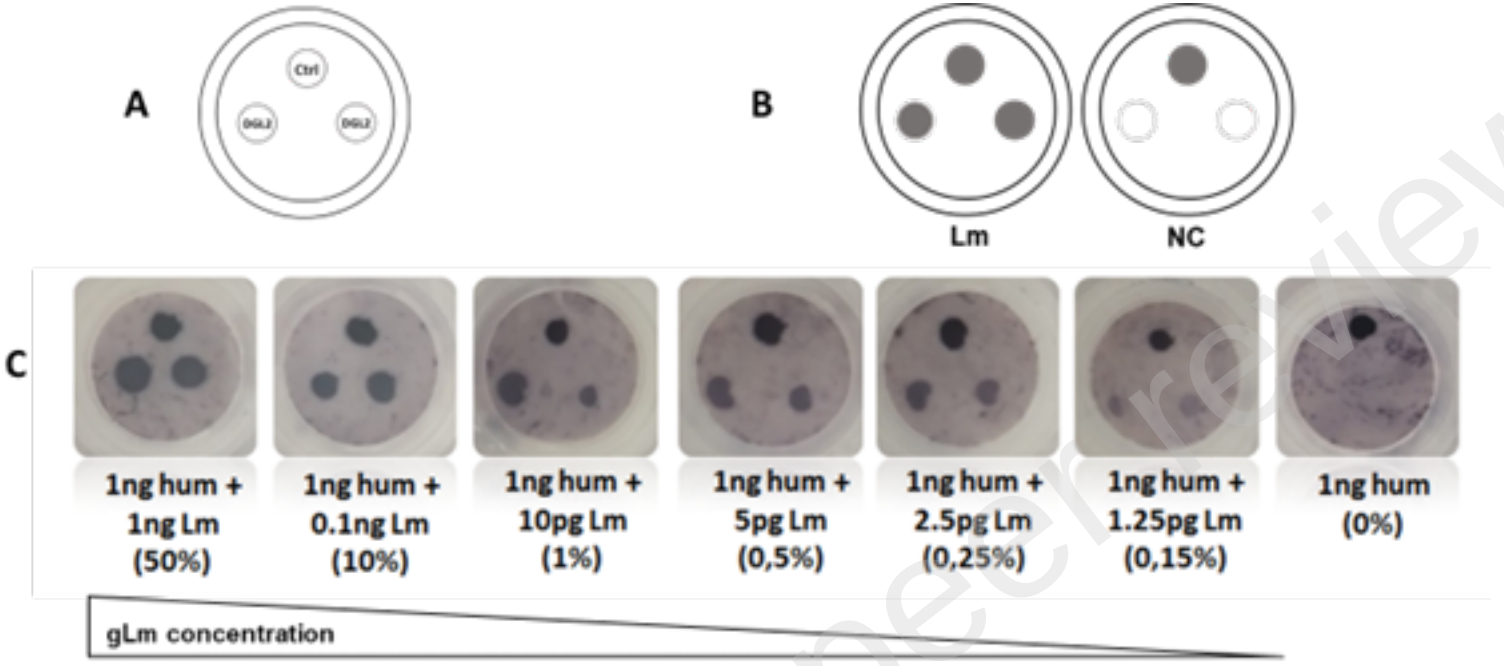
Preprint not peer reviewed



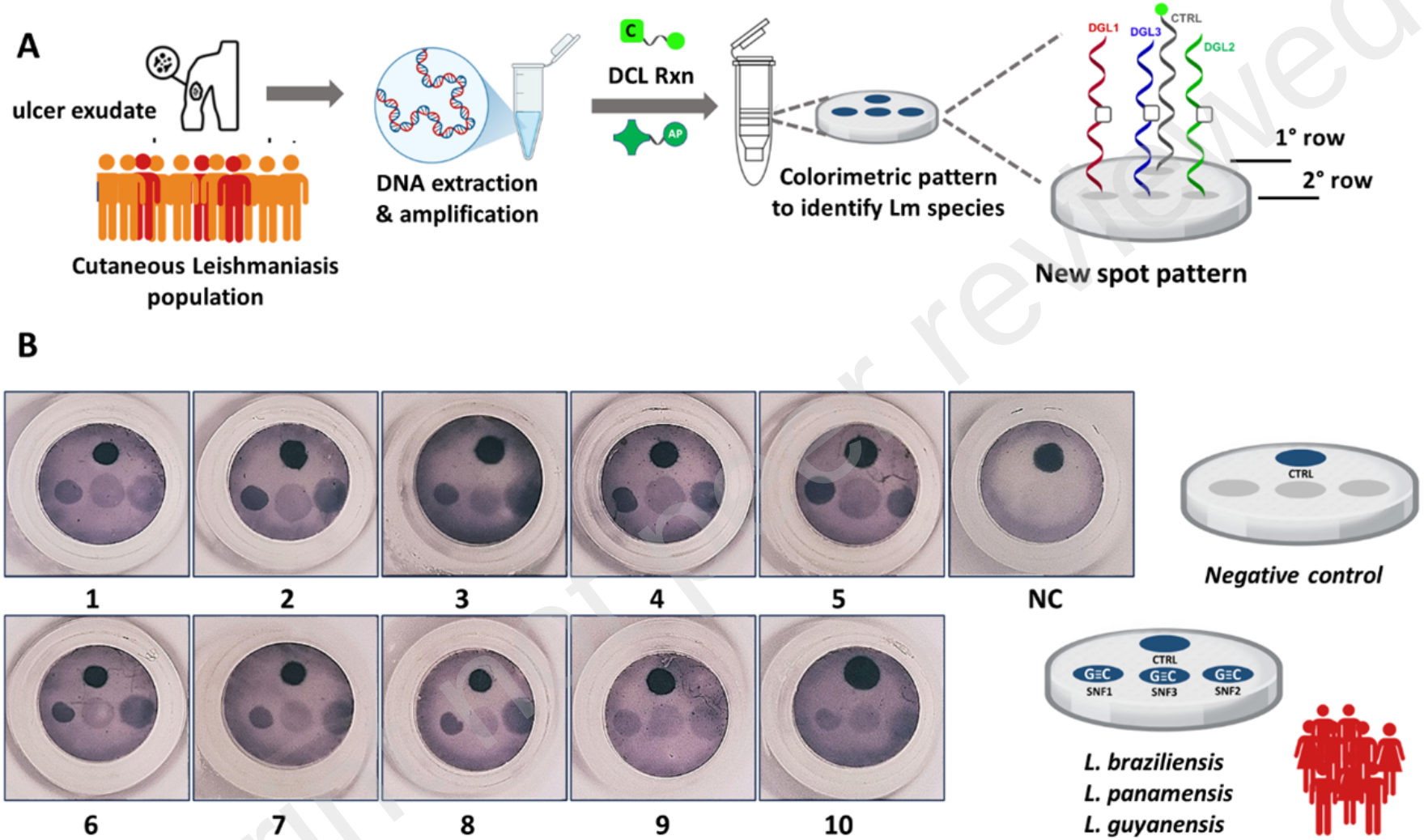
Preprint not peer reviewed



Preprint not peer reviewed



Preprint not peer reviewed



Preprint not peer reviewed