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1 A rapid molecular diagnosis of cutaneous leishmaniasis by colorimetric malachite green-
2 loop-mediated isothermal amplification (LAMP) combined with an FTA card as a direct
3 sampling tool

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39 **ABSTRACT**

40 Leishmaniasis remains one of the world's most neglected diseases, and early detection of the
41 infectious agent, especially in developing countries, will require a simple and rapid test. In this
42 study, we established a quick, one-step, single-tube, highly sensitive loop-mediated isothermal
43 amplification (LAMP) assay for rapid detection of *Leishmania* DNA from tissue materials spotted
44 on an FTA card. An FTA-LAMP with pre-added malachite green was performed at 64 °C for 60
45 min using a heating block and/or water bath and DNA amplification was detected immediately
46 after incubation. The LAMP assay had high detection sensitivity down to a level of 0.01 parasites
47 per µl. The field- and clinic-applicability of the colorimetric FTA-LAMP assay was demonstrated
48 with 122 clinical samples collected from patients suspected of having cutaneous leishmaniasis in
49 Peru, from which 71 positives were detected. The LAMP assay in combination with an FTA card
50 described here is rapid and sensitive, as well as simple to perform, and has great potential
51 usefulness for diagnosis and surveillance of leishmaniasis in endemic areas.

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56 *Keywords: Leishmania, LAMP, FTA, malachite green, Peru*

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1. Introduction

Leishmaniasis is a wide spectrum of diseases caused by an intracellular protozoan parasite of the genus *Leishmania*, transmitted by the bite of an infected female sand fly. *Leishmania* infection can result in three main clinically distinct manifestations in the human host; cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL). A critical component towards an understanding of the epidemiology and proper control/treatment of leishmaniasis is early and accurate diagnosis.

Conventionally, leishmaniasis is diagnosed by microscopic examination of skin smear/biopsy samples or aspirates from lesions for CL and MCL, and splenic or bone marrow aspirates for VL (Reithinger and Dujardin, 2007; de Vries et al., 2015). Despite high specificity, these methods are insensitive, invasive, and also require technical expertise (Reithinger and Dujardin, 2007; de Vries et al., 2015). Molecular approaches such as polymerase chain reaction (PCR) assays have been employed in the diagnosis of leishmaniasis (Reithinger and Dujardin, 2007; de Ruiter et al., 2014; de Vries et al., 2015); however, the need for expensive specialized equipment, the long time to result and lack of field applicability have greatly hindered the integration of these techniques into the diagnostic algorithm in endemic areas.

Recently, a rapid and simplified molecular technique, the loop-mediated isothermal amplification (LAMP) has been shown to be an effective tool in detection of human pathogenic infectious agents (Notomi et al., 2000; Mori et al., 2012; Dhama et al., 2014). The technique has been applied in the detection of *Leishmania* using purified DNA extracted from patient's materials (Takagi et al., 2009; Adams et al., 2010; Khan et al., 2012) or swab boiled samples from CL model mice (Direct Boil-LAMP method; Mikita et al., 2014). However, the efficiency of the reported Direct Boil-LAMP method as a rapid diagnostic tool for CL remains to be

demonstrated with clinical samples. Furthermore, the Foundation for Innovative New
Diagnostics (FIND) has also devoted its effort towards reducing the burden of visceral
leishmaniasis through innovative LAMP technique (<http://www.finddiagnostics.org/>). Despite
the progress made with LAMP in diagnosis of leishmaniasis, a simple and efficient procedure for
field and clinic sample collection and storage without the need for liquid handling and
refrigerant/cold storage, is necessary. Flinders Technology Associates (FTA) cards (Whatman)
lyse spotted cells and pathogens, and protect the nucleic acids from oxidation, nucleases and UV
damage at room temperature for long storage. Studies have shown the utility of FTA cards for
nested PCR analyses in epidemiological studies of leishmaniasis (Kato et al., 2010, 2011).
However, the potential usefulness of FTA cards as a direct sampling tool for diagnosis of
leishmaniasis by LAMP assay has not yet been well-explored. Therefore, this study reports the
establishment of a quick, one-step, and single-tube, sensitive colorimetric malachite green (MG)
based LAMP in combination with an FTA card for the detection of *Leishmania* DNA from
patients' cutaneous lesion-materials.

104 2. Materials and methods

105 2.1. Parasites and template preparation

106 A WHO reference strain of *Leishmania* (*Leishmania*) *major* (MHOM/SU/1973/5ASKH) was
107 cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10%
108 fetal calf serum (Cansera International, Etobicoke Ontario, Canada), 2 mM L-glutamine, 100
109 units/ml of penicillin, and 100 µg/ml of streptomycin at 25°C. Parasites were harvested in the log
110 phase and suspended in phosphate-buffered saline (PBS), counted using a Neubauer counting
111 chamber (Hirschmann, Germany), and then 10^6 to 1 parasites were prepared from the cultures.
112 Each set of 10^6 to 1 parasites was applied to FTA cards (Whatman, Newton Center, MA, USA),
113 and the coded cards were allowed to air dry and stored at room temperature. Ten to eleven months
114 later, 2.0-mm-diameter discs were punched from each FTA card using a Harris micro-punch tool
115 (Whatman) and washed twice with an FTA purification reagent (Whatman) and once with distilled
116 water. The discs were air-dried and used directly as the DNA template for the LAMP assay. For
117 the 1 parasite level, LAMP assay was repeated with different single punch in order to achieve
118 amplification since parasite DNA is localized on the FTA card matrix. In addition to the analytical
119 sensitivity of the FTA-LAMP using live parasites, 10-fold serial dilutions of purified *L. (L.)*
120 *mexicana* (MNYC/BZ/1962/M379) DNA (equivalent to 10^4 to 0.01 parasites) were individually
121 applied to 2.0-mm-diameter pre-punched FTA cards and washed, air-dried, and used as templates
122 to verify the detection sensitivity of the LAMP. The specificity of the LAMP assay was assessed
123 against *Leishmania*-related human pathogenic *Trypanosoma* parasites using DNA prepared from
124 *Trypanosoma cruzi* (both Tulahuen and Y strains) and *Trypanosoma brucei gambiense* (both
125 IL2343 and Wellcome strains), as well as human and dog genomic DNAs.

Furthermore, to test the reliability of the LAMP assay in the amplification of *Leishmania* DNA on an FTA card, tissue materials were aspirated from skin lesion of a mouse experimentally infected with *L. (L.) major* and spotted onto an FTA card. Discs of 2.0-mm-diameter were punched out from the sample areas, washed, air-dried, and directly used as a template for the LAMP assay. The experiment was conducted following the guidelines of the Ethics Committee on Animal Experimentation of Hokkaido University (approval number: 13-0139).

2.2. Clinical samples

A total of 122 samples previously collected from patients with CL and MCL who visited the rural health centers at 15 Departments: Piura, Amazonas, Loreto, Lambayeque, Cajamarca, La Libertad, San Martin, Ancash, Lima, Pasco, Junin, Ayacucho, Apurimac, Cusco and Madre de Dios in Peru, for the diagnosis and treatment of leishmaniasis, analyzed by nested PCR (Kato et al., 2010) were used for the developed FTA-LAMP evaluation under the approval of the research ethics committee of Hokkaido University (license number: vet26-4). Briefly, the tissue materials were taken by aspirating or scraping the active edge of the lesions of a patient by local physicians and well-trained laboratory technicians and spotted onto an FTA card, coded, air dried and enclosed in self-sealing bag and stored at room temperature (Kato et al., 2010).

2.3. FTA-loop-mediated isothermal amplification assay

The LAMP assay was carried out as previously described (Nzulu et al., 2014). The primer sequences based on *Leishmania* 18S rRNA gene were forward inner primer (FIP–Le.rRNA), 5′ -

TACTGCCAGTGAAGGCATTGGTGGCAACCATCGTCGTGAG-3'; backward inner primer
(BIP-Le.rRNA) 5'-TGCGAAAGCCGGCTTGTTCCCATCACCAGCTGATAGGGC-3';
forward outer primer (F3-Le.rRNA) 5'-GGGTGTTCTCCACTCCAGA-3'; backward outer
primer (B3-Le.rRNA), 5'-CCATGGCAGTCCACTACAC-3' (Nzulu et al., 2014). One punch of
an FTA card from sample areas was used as a template for the LAMP assay. The mixture was
incubated at 64 °C for 60 or 30 min in a heating block and then heated at 80 °C for 5 min to
terminate the reaction. A positive control (DNA from a reference strain: *L. (V.) braziliensis* -
MHOM/BR/1975/M2904) and a negative (water) sample were included in each LAMP run. The
LAMP assay using one punch of an FTA card template per sample was repeated not more than
twice for samples negative in the first LAMP assay in order to achieve gene amplification.

2.4. Sequencing

To confirm that the LAMP products had the target sequence, direct sequencing of the LAMP
amplicons was performed. The LAMP products were purified using a PCR purification kit
(NIPPON Genetics, Tokyo, Japan) and then sequenced with a FIP-Le.rRNA primer (Nzulu et al.,
2014) using a BigDye Terminator version 3.1 Cycle-Sequencing Kit (Applied Biosystems, Foster
City, CA, USA).

173 3. Results and discussion

174 The LAMP assay could detect all the *L. (L.) major* (10^6 to 1 parasites) levels from 2.0-mm-
175 diameter FTA cards templates. No amplification was detected in the negative controls.
176 Additionally, the FTA-LAMP assay had high detection sensitivity down to a level of 0.01
177 parasites/ μ l (Fig. 1A), which agreed with our previous report using purified DNA as a template
178 (Nzulu et al., 2014). All the positive reactions turned light blue while the negative reactions
179 became colorless without adding any reagent after incubation. The colorimetric results were
180 confirmed by gel electrophoresis (Fig. 1B), and there was agreement in the detection of the
181 amplification products by both systems. Furthermore, we observed that DNA amplification could
182 also be detected within 30 min of incubation, even down to the level of 0.01 parasites/ μ l (data not
183 shown). No cross-reactivity was recorded from *Trypanosoma cruzi* (both Tulahuen and Y strains),
184 *Trypanosoma brucei gambiense* (both IL2343 and Wellcome strains), human and dog genomic
185 DNAs. In addition, the LAMP assay reliably yielded positive reactions from 2.0-mm-diameter
186 punch of an FTA card spotted with cutaneous lesion-materials of the experimentally-infected
187 mouse (data not shown). These observations indicated the robustness and efficiency of the present
188 LAMP assay in amplification of the target DNA from FTA card templates. The structures of the
189 LAMP amplicons were confirmed by direct sequencing.

190 We further evaluated the field- and clinic- applicability of the developed FTA-LAMP using
191 122 clinical samples, and LAMP amplicons were detected in 71/122 (58.2%) samples. Overall, the
192 results indicated that the FTA-LAMP was as efficient as the nested PCR using an FTA card as a
193 template (Kato et al., 2010). The accuracy of the LAMP amplicons was confirmed by direct
194 sequencing.

195 Particularly important is the fact that in this study LAMP could reliably amplify *Leishmania*
196 DNA directly from FTA card templates. Studies have shown that PCR has lower sensitivity for

197 amplification of DNA on FTA cards compared to LAMP (Kuboki et al., 2003); it is suggested to
198 be due to PCR inhibitors in blood components, which does not affect the *Bst* DNA polymerase
199 used in the LAMP assay. Recently, we showed that LAMP can amplify *Leishmania* DNA from
200 crude sand fly templates (Nzulu et al., 2014), and previous reports have also shown superior
201 tolerance of LAMP for biological substances (Poon et al., 2006; Kaneko et al., 2007). To our
202 knowledge, this is the first time tissue materials spotted on FTA cards have been used for
203 diagnosis of leishmaniasis by LAMP assay. Generally, the use of FTA card as a DNA template is
204 critical, especially when parasite density is very low; the probability of a punch containing a
205 parasite DNA is very low since parasite DNA is not evenly spread across the FTA matrix, but
206 localized in areas where parasites in the tissue materials are fixed. In addition to assay sensitivity,
207 to obtain a successful detection, careful attention is necessary during sample collection to avoid
208 spotting of diluted or blood-containing materials onto FTA cards, which reduces the density of
209 parasites on the cards.

210 The closed colorimetric MG detection system is highly sensitive, which enables visual
211 discrimination of results by the naked eye without the aid of any specialized instrument and UV
212 illuminator or ordinary light (Nzulu et al., 2014). Pre-addition of MG eliminates the openings of
213 tubes and completely avoids contamination problems. Collectively, the use of FTA cards for
214 direct sample collection and storage without the need for a cold chain, while MG dye which does
215 not require freezing for storage (Nzulu et al., 2014) coupled with the LAMP reagents reported to
216 be stable at 25 and 37 °C for 30 days (Thekisoe et al., 2009), indicates the assay's potential as a
217 field diagnostic tool deployable in developing countries. Furthermore, since the described LAMP
218 assay is not species-specific, as the primers can amplify both Old and New World *Leishmania*

219 species (Nzulu et al., 2014), the developed universal-LAMP assay allows for the screening and
220 detection of multiple species of the genus *Leishmania* in endemic areas.

221 In conclusion, this study provided a rapid, simple and highly sensitive colorimetric LAMP
222 assay combined with an FTA card as a direct sampling tool for diagnosis of leishmaniasis. The
223 established LAMP assay is in line with the recent global trend in seeking rapid, point-of-care tests
224 for the control of infectious diseases. The simplicity, rapidity and sensitivity of the LAMP assay
225 make it an ideal routine diagnostic tool that will strongly support the cooperation of patients and
226 clinicians in endemic areas. With such advantages, the established FTA-LAMP assay will be a
227 good molecular tool for active screening and diagnosis of leishmaniasis in endemic areas.

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242 References

- 243 Adams, E.R., Schoone, G.J., Ageed, A.F., Safi, S.E., Schallig, H.D., 2010. Development of a
244 reverse transcriptase loop-mediated isothermal amplification (LAMP) assay for the sensitive
245 detection of *Leishmania* parasites in clinical samples. Am. J. Trop. Med. Hyg. 82, 591-596.
- 246 De Ruiter, C. M., der Veer, C., Leeflang, M.M.G., Deborggraeve, S., Lucas, C., Adams E. R.,
247 2014. Molecular tools for diagnosis of visceral leishmaniasis: systematic review and meta-
248 analysis of diagnostic test accuracy. J. Clin. Microbiol. 52, 3147-3155.
- 249 De Vries, H.J.C., Reedijk, S. H., Schallig, H.D.F.H., 2015. Cutaneous leishmaniasis: recent
250 developments in diagnosis and management. Am. J. Clin. Dermatol. 16, 99-109.
- 251 Dhama, K., Karthik, K., Chakraborty, S., Tiwari, R., Kapoor, S., Kumar A., Thomas, P., 2014.
252 Loop-mediated isothermal amplification of DNA (LAMP): A new diagnostic tool lights the
253 world of diagnosis of animal and human pathogens: a review. Pakistan J. Biol. Sci. 17, 151-
254 166.
- 255 Kaneko, H., Kawana, T., Fukushima, E., Suzutani, T., 2007. Tolerance of loop-mediated
256 isothermal amplification to a culture medium and biological substances. J. Biochem. Biophys.
257 Methods. 70, 499-501.
- 258 Kato, H., Caceres, A.G., Mimori, T., Ishimaru, Y., Sayed, A.S.M. Fujita, M., Iwata, H., Uezato,
259 H., Velez, L.N., Gomez, E.A.L., Hashiguchi, Y., 2010. Use of FTA cards for direct sampling
260 of patients' lesions in the ecological study of cutaneous leishmaniasis. J. Clin. Microbiol. 48,
261 3661-3665.
- 262 Kato, H., Watanabe, J., Nieto, I.M., Korenaga, M., Hashiguchi, Y., 2011. *Leishmania* species
263 identification using FTA card sampling directly from patients' cutaneous lesions in the state
264 of Lara, Venezuela. Trans. R. Soc. Med. Hyg. 105, 561-567.

265 Khan, M.G.M., Bhaskar, K.R.H., Salam, M.A., Akther, T., Pluschke, G., Mondai, D., 2012.
 266 Diagnostic accuracy of loop-mediated isothermal amplification (LAMP) for detection of
 267 *Leishmania* DNA in buffy coat from visceral leishmaniasis patients. *Parasit. Vectors.* 5, 280.
 268 Kuboki, N., Inoue, N., Sakurai, T., Di Cello, F., Grab, D.J., Suzuki, H., Sugimoto, C., Igarashi, I.,
 269 2003. Loop-mediated isothermal amplification for detection of African trypanosomes. *J. Clin.*
 270 *Microbiol.* 41, 5517-5524.
 271 Mikita, K., Maeda, T., Yoshikawa, S., Ono, T., Miyahira, Y., Kawana, A. 2014. The direct boil-
 272 LAMP method: a simple and rapid diagnostic method for cutaneous leishmaniasis. *Parasitol.*
 273 *Int.* 63, 785-789.
 274 Mori, Y., Tomita, N., Kanda, H., Notomi, T., 2012. Novel molecular diagnostic platform for
 275 tropical infectious diseases, current topics in tropical medicine, Dr. Alfonso Rodriguez-
 276 Morales (Ed.), ISBN: 978-953-51-0274-8, In Tech, DOI: 10.5772/29561. Available from:
 277 [http://www.intechopen.com/books/current-topics-in-tropical-medicine/novel-molecular-](http://www.intechopen.com/books/current-topics-in-tropical-medicine/novel-molecular-diagnostic-platform-for-tropical-infectious-diseases)
 278 [diagnostic-platform-for-tropical-infectious-diseases.](http://www.intechopen.com/books/current-topics-in-tropical-medicine/novel-molecular-diagnostic-platform-for-tropical-infectious-diseases)
 279 Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T.,
 280 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* 28, E63.
 281 Nzelu, C.O., Gomez, E.A., Caceres, A.G., Sakurai, T., Martini-Robles, L., Uezato, H., Mimori, T.,
 282 Katakura, K., Hashiguchi, Y., Kato, H., 2014. Development of a loop-mediated isothermal
 283 amplification method for rapid mass-screening of sand flies for *Leishmania* infection. *Acta*
 284 *Trop.* 132, 1-6.
 285 Poon, L.L., Wong, B.W., Ma, E.H., Chan, K.H., Chow, L.M., Abeyewickreme, W., Tangpukdee,
 286 N., Yuen, K.Y., Guan, Y., Looareesuwan, S., Peiris, J.S., 2006. Sensitive and inexpensive

287 molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from
288 heat-treated blood by loop-mediated isothermal amplification. Clin. Chem. 52, 303-306.

289 Reithinger, R., Dujardin, J.C., 2007. Molecular diagnosis of leishmaniasis: current status and
290 future applications. J. Clin. Microbiol. 45, 21-25.

291 Takagi, H., Itoh, M., Islam, M.Z., Razzaque, A., Ekram, A.R.M., Hashiguchi, Y., Noiri, E.,
292 Kimura, E., 2009. Sensitive, specific and rapid detection of *Leishmania donovani* DNA by
293 loop-mediated isothermal amplification. Am. J. Trop. Med. Hyg. 81, 578-582.

294 Thekisoe, OM, Bazie, R.S., Coronel-Servian, A.M., Sugimoto, C., Kawazu, S., Inoue, N., 2009.
295 Stability of loop-mediated isothermal amplification (LAMP) reagents and its amplification
296 efficiency on crude trypanosome DNA templates. J. Vet. Med. Sci. 71, 471-475.

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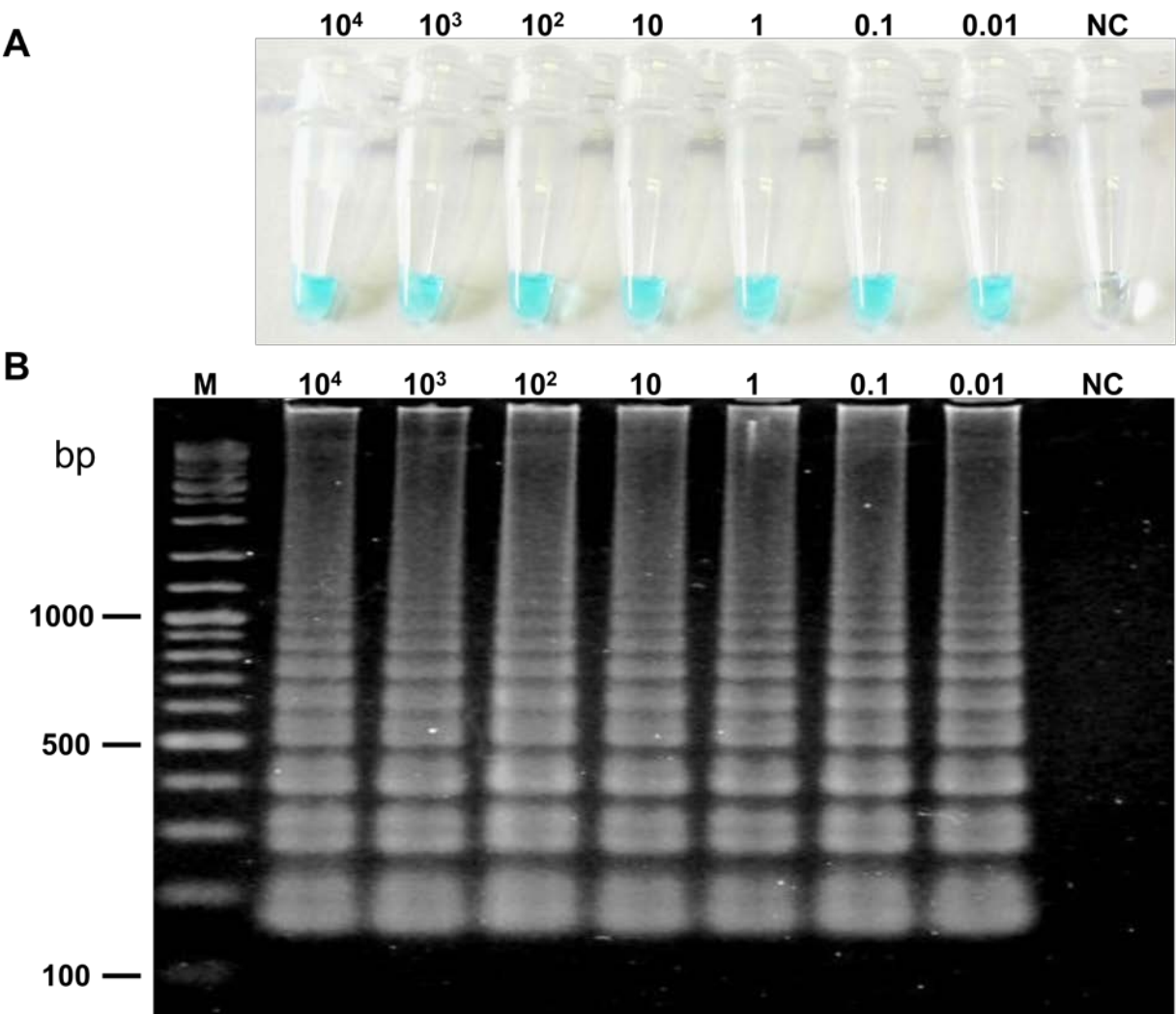
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299 **FIGURE LEGEND**

300 **FIG. 1.** Colorimetric FTA-LAMP detection sensitivity. Genome DNAs from 10-fold serially
301 diluted *Leishmania (Leishmania) mexicana* (MNYC/BZ/1962/M379) applied directly onto FTA
302 cards were used as templates. (A) Malachite green based FTA-LAMP sensitivity reaction products
303 inspected by the naked eye without a UV illuminator or ordinary light immediately after
304 incubation. Light blue: positive reactions; colorless: negative reaction. (B) Sensitivity of the FTA-
305 LAMP studied by agarose gel electrophoresis. bp: base pair; M: gene ruler; NC: negative control.

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Fig. 1