IDENTIFICATION OF *TOXOPLASMA GONDII* DNA IN LEAFY GREEN VEGETABLE BY LAMP

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1. Aim and field of application

To identify *Toxoplasma gondii* DNA in leafy green vegetables by LAMP (Loop-Mediated Isothermal Amplification). This method can be applied to fresh leafy green vegetables.

2. Principle of the method

LAMP is a molecular biology technique that allows the amplification of specific nucleic acid fragments, which initial and terminal nucleotide sequences are known (oligonucleotide pair). By selecting a specific and unique DNA portion in the genome of the organism of interest, it is possible to design 2 or 3 oligonucleotides (primers) pairs, which combined use allows the DNA amplification of the target organism, highly increasing the specificity of the method. The reaction is performed by a particular DNA polymerase, having both high strand displacement and replication activities. The reaction is carried out at a constant temperature (62-65°C) and the final products are stem–loop DNAs with cauliflower-like structures. Compared to conventional Polymerase Chain Reaction, LAMP has higher specificity, efficiency and rapidity, allowing amplification of small traces of target DNA in the matrix.

Toxoplasma gondii is a protozoan parasite able to infect all warm blood animal (mammals or birds), including humans. The sexual stage of the parasite life cycle occurs in felids, the definitive hosts, and the parasite environmental resistant stage, the oocysts, are shed with feces. Following oocysts ingestion by mammals or birds, the intermediate hosts, the parasite invades different host tissues (especially muscles and brain), where the asexual stage, the tachyzoite, undergoes multiple replication cycles and finally forms tissue cysts containing tents to thousands parasites, named bradizoytes. The cycle is completed when felids eat meat containing *T. gondii* tissue cyst (or directly oocysts). Humans can acquire toxoplasmosis by the ingestion of fruits, vegetables or water contaminated by oocysts or by the consumption of raw or undercooked meat containing tissue cysts. Usually, toxoplasmosis is asymptomatic, but in immunocompromised persons, it can cause encephalitis, myocarditis, pneumonia, retinochoroiditis and hepatitis. In case of infection acquired during pregnancy, the parasite can be transmitted from the mother to the fetus causing serious fetal diseases, eventually resulting in abortion or to permanent damages that can arise later at the adult age.

By LAMP approach, it is possible to detect *T. gondii* oocyst DNA from vegetables by the amplification of a specific nucleotide sequence of 529 bp, present only in the genome of *T. gondii* and repeated up to 300 times. A mix of 6 primers (see Table A) allows the specific amplification of the 529 bp sequence. After the LAMP reaction, the amplification products can be visualized by agarose gel electrophoresis, with positive reactions showing a typical ladder of bands.

Table A – LAMP Primer Mix c

Oligonucleotide sequence	Name
5'-TGGTTGGGAAGCGACGAGAGTTCCAGGAAAAGCAGCCAAG -3'	BIP
5'-TCCTCACCCTCGCCTTCATCTAGGACTACAGACGCGATGC -3'	FIP
5'-CCACAGAAGGGACAGAAGTC -3'	F3
5'-TCCGGTGTCTCTTTTTCCAC -3'	B3
5'-TCCAAGACGGCTGGAGGAG -3'	LF
5'-CGGAGAGGGAGAAGATGTTTCC -3'	LB

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4. Definitions

529 bp, it is a non-coding nucleotide sequence present up to 300 copies in a single genome of *T. gondii*.

Oligonucleotide (Primer), short sequence (15/30 nucleotide bases) used to amplify a specific DNA fragment.

LAMP Mix, mix of 6 oligonucleotides to specifically amplify the 529 bp sequence.

Reference DNA, purified DNA from *T. gondii* tachizoytes.

Positive control for the DNA extraction, baby lettuce pellet (900 µl ± 50 µl) spiked with 100 ± 10 *T. gondii* oocysts. It is analysed in the same working session of test samples, to verify the efficacy of the DNA extraction session.

Sample DNA, DNA extracted from a single sample.

Positive control for the amplification, reference DNA; this control is used in the amplification session to verify the efficacy of the LAMP.

Negative control for the amplification, reagent grade water; used in the amplification session to verify the absence of contamination.

The definitions and terminology used in the *EN ISO 22174:2005. Microbiology of food and animal feeding stuffs - Polymerase chain reaction (PCR) for the detection of food-borne pathogens - General requirements and definitions* are applied in the present method.

5. Devices/instruments

5.1 Reagen grade water system production

5.2 Analytical balance, readability 0.1 g

5.3 pH-meter, with ± 0.3 pH accuracy

5.4 Paddle blender

5.5 Refrigerated centrifuge for 50 mL tubes, minimum 7.500 x g

5.6 Freezer ≤-15°C

5.7 Bench top beat beater homogenizer for microtubes (like, FastPrep Instrument)

5.8 Bench top centrifuge for 1.5 mL tubes, minimum 10,000 xg

5.9 Thermoblock with vibration, temperature range 25÷100 °C

5.10 Rotary shaker for microtube

5.11 Refrigerator, temperature range 1÷8 °C

5.12 PCR thermocycler

5.13 Horizontal electrophoretic apparatus

5.14 UV transilluminator

5.15 Digital imaging system

5.16 Adjustable volume pipettes, volume range: 1-1000 µL

5.17 Vortex

6. Reagents and chemicals

6.1 **Glycine buffer**. Buffer of 1 M Glycine ($C_2H_5NO_2$) pH 5.5. The necessary amount of the buffer is freshly prepared using glycine powder commercially available.

Dissolve 75 g of Glycine in 800 ml of analytical grade water on a magnetic mixer and adjust the pH to 5.5 ± 0.2 with 1 M HCl or NaOH, then bring to 1 L volume. Store at 1÷ 8°C °C for up to 1 month. The glycine powder is stored according to manufacturer.

6.2 **Paddle-filtered bags**. Paddle bags with filters suitable for up to 400 ml volume. Commercially available material (such as, Bag BA6041/STR strainer for 400 Stomacher, Seward).

6.3 **Stand for Paddle-filtered bags**. Commercially available material.

6.4 **Resuspension buffer**. Commercial Sodium phosphate buffer (such as, Sodium Phosphate Buffer, FastDNA Spin kit for Soil, MP Biochemicals). The buffer is stored according to manufacturer.

6.5 **Homogenization buffer**. Commercial solution (such as, MT buffer, FastDNA Spin kit for Soil, MP Biochemicals). The buffer is stored according to manufacturer.

6.6 **Lysis tubes**. Commercially available screw cup tubes containing beads of different sizes and materials (such as, Lysis Matrix E tube, FastDNA Spin kit for Soil, MP Biochemicals).

6.7 **Lysis buffer**. Commercial solution (such as, PPS buffer, FastDNA Spin kit for Soil, MP Biochemicals). The buffer is stored according to manufacturer.

6.8 **Silica resin**. Commercial solution (such as, Binding Matrix, FastDNA Spin kit for Soil, MP Biochemicals). The buffer is stored according to manufacturer.

6.9 **Collection column**. Commercially available material (such as, FastDNA Spin kit for Soil, MP Biochemicals, and identified as “SPIN filter” by the manufacturer).

6.10 **Wash buffer**. Commercial solutions (such as, FastDNA Spin kit for Soil, MP Biochemicals). To be prepared and stored according to manufacturer and to be identified as 'SEWS-N'.

6.11 **Collecting tube**. Commercially available 2 mL tube (such as, FastDNA Spin kit for Soil, MP Biochemicals, and identified as "catch tube" by the manufacturer).

6.12 **Elution buffer**. Commercial solutions (such as, FastDNA Spin kit for Soil, MP Biochemicals, and identified as "DES" by the manufacturer). The buffer is stored according to manufacturer.

6.13 **Centrifuge tubes**. Commercially available molecular biology grade material, 0,2 mL, 1,5 mL, conical 50 mL.

6.14 **LAMP reaction mix 2x**. Buffer obtained by mixing commercial solutions, 10X ThermoPol DF (detergent-free) and 100 mM MgSO4; 5M Betaine; 100% Tween 20 (to prepare 1 ml of 2X reaction buffer mix: 320 µl of betaine 5M, 200 µl of 10X ThermoPol DF; 120 µl of MgSO4 100mM, 2 µl of Tween 20 100% and 358 µl reagent grade H2O). Do not add Tween 20 detergent if it is in the 10x ThermoPol. Aliquote the mix and store at -20 °C (5.4) up to 24 months

6.15 **Deoxynucleotide (dNTP) Solution Mix**. Commercial solution (100 mM) of deoxynucleotide (dNTP) mix. Each dNTP is used at 25 µmol for each amplification reaction; Store at -20 °C (5.6).

6.16 **Bst DNA polymerase (Large Fragment).** Bst DNA polymerase is a commercially available enzyme suitable for LAMP reactions; Store according the manufacturer's instructions.

6.17 **Oligonucleotides**. Commercial preparation (*Table B*); the lyophilized product is reconstituted with reagent grade H_2O (5.1), at a concentration of 100 pmol/µL according to the manufacturer's recommendations; the lyophilized product can be stored frozen for up to 20 years; the reconstituted product can be stored frozen up to 10 years.

6.18 **LAMP Primers Mix**. Oligonucleotides mixture used for LAMP reactions (6.7). The mixture is obtained combining a defined volume of the oligonucleotides reported in Table A in reagent grade H2O (5.1); the final concentration in the reaction corresponds to 40 pmol for BIP and FIP, 5 pmol for F3 and B3, 20 pmol of LF and LB. 100µL aliquots are prepared and stored frozen up to 10 years.

6.19 **Loading buffer**. Commercial product allowing DNA molecule electrophoresis to be performed; store according to the manufacturer's recommendations.

6.20 **Agarose**. Commercial product suitable for performing DNA molecule electrophoresis; store at room temperature according to the manufacturer's recommendations.

6.21 **TAE solution 50x**. Commercial product (2M Tris-acetate, 50mM EDTA, pH 8.2–8.4 at 25°C); store according to the manufacturer's recommendations.

6.22 **TAE solution 1x**. 1000 mL preparation: take 20 mL of the 50x solution and bring to 1000 mL with water; store at room temperature for up to 1 month.

6.23 **DNA intercalating agent**. Commercial product able to fit in the DNA double helix, used to view the amplification product on agarose gel. Store according to the manufacturer's recommendations

6.24 **L1000**. Commercial product containing markers for DNA molecular size multiple of 50 bp; all commercial products containing molecules in the range 250-1.000 bp and 250-10.000 bp can be used; store refrigerated according to manufacturer's recommendations.

6.25 **Reagent grade water or Milli-Q**.

6.26 **Reference sample**. Baby lettuce pellet (900 µl ± 50 µl) spiked with 100 ± 10 *T. gondii* oocysts. It is analysed in the same working session of test samples, to verify the efficacy of the DNA extraction session. Store at -20 °C (5.6) up to 5 years.

6.27 **Reference DNA**. Purified DNA from *Toxoplasma gondii* tachizoytes (10ng/µL); store at -20 °C (5.4) up to 10 years.

7. Procedure

7.1 Sample preparation

The sample (50 g±2g in sealed plastic bags) is inspected to check the integrity and no trace of content spread out. If the sample is considered not suitable, the test is not performed.

Samples are then stored in a refrigerator (5.11) for a maximum of 3 days before being tested.

7.2 Method

7.2.1 *Recovery of* T. gondii *oocysts from test sample*

- If not otherwise specified, the procedure is carried out at room temperature.
- Before starting the procedure, prepare a sufficient volume of Glycine buffer (6.1).

a) Transfer the sample in the paddle-filtered bag (6.2) and mark it with a progressive number.

b) Put the bag on the stand (6.3).

c) Add 200 ml glycine buffer pH 5.5. (6.1).

d) Seal the bag (6.2) and fold the upper edge.

e) Accommodate the bag (6.2) in the paddle homogenizer (5.4).

f) Process sample for 30 s at 300 rpm.

g) Repeat step (f).

h) Carefully, transfer the bag from the paddle to the stand (6.3).

i) Prepare four 50 ml conical tubes (6.13) for each test sample and mark them with the number corresponding to the test sample.

j) Collect homogenate by pipetting into the 50 ml conical tubes (6.13), ensuring that all the vegetable matter is retained in the filter. Squeeze the bag and the filter tightly in order to ensure that all the eluate is obtained from the sample. Equally distribute the homogenate solution into the four 50 ml conical tubes.

k) Centrifuge (5.5) the tubes at 2 500 x g maximum for 10 min.

l) After centrifugation, remove the supernatant from the 50 ml tubes leaving 1-2 ml of solution and ensuring the pellet is not disturbed. If no pellet is visible, extra care shall be taken to ensure that parasites are not lost during aspiration.

m) Re-suspend the pellet in the residual liquid left in the bottom of each tube and combine the pellets into a single centrifuge tube.

n) Add 10 ml of glycine buffer (6.1) to bag and rinse by manipulating the sample from outside the bag. Add the rinsate from the sample to the 50 ml tube (6.12) of step “m”.

o) Centrifuge (5.5) the tubes at 2 500 x g maximum for 10 min.

p) After centrifugation, remove the supernatant from the 50 ml tubes leaving 1-2 ml of solution and ensuring the pellet is not disturbed. If no pellet is visible, extra care shall be taken to ensure that parasites are not lost during aspiration.

q) Repeat from step “n” to “p”.

r) Add 50 ml of reagent grade water or Milli-Q (6.25).

s) Centrifuge (5.5) the tube at 2 500 x g maximum for 10 min.

t) After centrifugation, remove the supernatant from the 50 ml tube leaving 1-2 ml of solution and ensuring the pellet is not disturbed. If no pellet is visible, extra care shall be taken to ensure that parasites are not lost during aspiration.

u) Transfer the resuspended pellet in a new 2 ml tube (6.13) correctly numbered.

v) Centrifuge (5.8) the tube at 2 500 x g maximum for 10 min.

w) After centrifugation, remove all the supernatant from the 2 ml tube ensuring the pellet is not disturbed. If no pellet is visible, extra care shall be taken to ensure that parasites are not lost during aspiration.

x) Resuspend the pellet in 900 ml of resuspension buffer (6.4). Samples are then store frozen (5.6) for at

least 48h before to proceed with the test. Frozen sample can be stored up to 5 years.

7.2.2 DNA extraction

- If not otherwise specified, the procedure is carried out at room temperature.
- Each working session requires the DNA extraction of a "reference sample" (6.26) identified as "positive control for the extraction".

a) Thaw the tube containing the sample and the "reference sample" (6.26) at room temperature.
b) Add 125 µL of homogenization buffer to the sample (6.5).
c) Transfer the sample to a lysis tube properly numbered (6.6).
d) Homogenate the sample at an amplitude of 6.0 for 40 sec. on benchtop beat beater instrument (5.7).
e) Repeat step "d".
f) Centrifuge (5.8) the tube at 14.000 x g for 10 minutes to pellet debris
g) Transfer the supernatant to a clean 2.0 ml microcentrifuge tube (6.13) properly numbered.
h) Add 250 µL of lysis buffer (6.7) and mix by inverting the tube for 10 times.
i) Centrifuge (5.8) the tube at 14.000 x g (12.000 rpm) for 5 minutes.
j) Transfer the supernatant to a clean 2.0 ml microcentrifuge tube (6.13) properly numbered.
k) Resuspend the silica matrix (6.8) by vortex for 30 sec.
l) Add 1 ml of resuspended silica matrix (6.8) to the sample tube and mix gently on a rotor (5.10) for 2 min.
m) Place the microcentrifuge tube in a rack for 3 min to allow setting of silica matrix (6.8).
n) Carefully remove and discard 500 µl of supernatant without disturbing the silica matrix (6.8).
o) Resuspend by pipetting (5.16) the silica matrix (6.8) in the remaining supernatant.
p) Transfer 500 µl of the mixture to a properly number collection column (6.9) positioned on a collecting tube.
q) Centrifuge (5.8) at 14.000 x g for 1 minute.
r) Empty the collecting column and repeat steps from "p" to "r" until all the matrix is transferred (6.8) to the collection column (6.9).
s) Add 500 µl of washing buffer (6.10) to the collection column (6.9) and gently resuspend matrix by pipetting (5.16).
t) Centrifuge (5.8) the collection column (6.9) at 14.000 x g for 1 minute.
u) Empty the collecting tube.
v) Centrifuge (5.8) the collection column (6.9) at 14.000 x g for 2 minutes to dry the matrix.
w) Transfer the collection column (6.9) to a new collecting tube (6.11) properly numbered.
x) Air dry the collection column (6.9) for 5 minutes.
y) Gently resuspend the silica matrix with 100 µl of elution buffer (6.12) by pipetting (5.16).
z) Incubate 55°C (±3°C) for 5 minutes in thermoblock (5.9).
aa) Elute DNA by centrifugation (5.8) at 14.000 x g for 1 minute, discard the collection column (6.9) and keep the collecting tube with DNA (6.11).
bb) The obtained DNA will be defined 'DNA/sample' and store frozen (5.4) for up to 10 years.

7.2.3 LAMP amplification

- Unless otherwise clearly stated, store tubes on ice; use tips with barrier and wear disposable gloves.
- At each working session, use a positive and a negative amplification control. Use reference DNA (6.27) as positive control and water (6.20) as negative control.
- The following procedure use a 2X concentrated LAMP reaction mix. If different concentration are used adjust the protocol according to manufacturer.

a) Thaw DNA/sample, 2x LAMP reaction mix (6.14), dNTP Mix (6.15), LAMP primer mix (6.118), Bst DNA polymerase (6.16) and positive amplification control (Reference DNA 6.27).

b) Mark with a progressive number an adequate number of 0.2 mL tubes (6.13).

c) Prepare an adequate cumulative volume of the amplification mix. Calculate the volume on the basis of a single sample amplification mix (*Table B*) and of the total number of samples added of three reactions (one for the positive amplification control, one for the negative one and an extra reaction).

Table B – single sample amplification mix: components and volumes

LAMP reaction mix 2X (6.9)	12.5 µL
LAMP Primers Mix (6.13)	6 µL
Bst DNA polymerase (6.11)	1 µL
dNTP mix (6.10)	1.4 µL
H_2O (6.20)	0.1 µL
Total	21 µL

d) Mix the amplification mix by vortexing (5.17) and, if necessary, centrifuge (5.8) at maximum speed for a few sec.

e) Transfer 21 µL of the cumulative amplification mix to each tube (point “b”).

f) Add 4 µL of the DNA/sample to be tested to each tube, or 2 µL of reference DNA (6.27) plus 2 µL of reagent grade water (6.25).

g) Close the tubes, mix and centrifuge (5.3) at maximum speed for a few sec.

h) Start the amplifying cycle (*Table C*) on the thermocycler (5.12); wait for the temperature to reach 63 °C and insert the tubes in the thermoblock by pausing the instrument.

Table D – amplification cycles

Amplification	120 min/63 °C
Inactivation	2 min/80 °C

i) At the end of the amplification phase, centrifuge (5.8) the tubes at maximum speed for a few sec.

l) Keep tubes on ice or refrigerated (5.11).

7.2.4 Result display and interpretation

Results are visualized by electrophoresis on agarose gel.

a) Add, to each vial, the loading buffer (6.19) according to the manufacturer’s instructions.

b) Keep tubes on ice or refrigerated (5.11) until gel loading.

c) Assemble the electrophoresis apparatus (5.13) according to the manufacturer’s recommendations. For the gel preparation, use a comb suited for the number of samples.

d) Weight (5.2) 1.5 g of agarose (6.20) and add 100 mL TAE 1x (6.22) in a glass beaker.

e) Weight (5.2) the solution.

f) Gently resuspend the powder by rotation.

g) Boil the agarose suspension for 30 sec. If the solution is not homogeneous, boil for further 30 sec.

h) Restore with water the volume lost by boiling.

i) Allow the agarose solution to cool.

j) Before it solidifies, add DNA intercalating agent (6.23) according to the manufacturer’s instructions

k) Shake gently to dissolve uniformly the DNA intercalating agent and pour the agarose in the gel tray previously prepared (point “c”).

l) Wait for the gel to solidify, which requires at least 30 min.

m) Place the tray with the gel in the electrophoresis apparatus.

n) Cover the gel with TAE 1x buffer (6.22) and gently pull out the comb.

o) Load in each well 16 µL of the amplification product (point 7.2.3 “l”), respecting the progressive numbering of the tubes (point 7.2.3 “b”).

p) Load the first or last well with 15 µL of the L50 solution (6.24).

q) Connect the electrophoresis apparatus with the power supply and set 10 V/cm of gel.

r) Run the gel for about 30 min or until the fastest dye, contained in the loading buffer (6.19), reaches a distance of 1 cm from the gel border.

s) After 30 min, switch off the power supply, place the gel under UV illumination and check the band separation; the electrophoresis run is adequate if it is possible to easily distinguish all bands of molecular weight marker ranging from 50 to 2000 bp; if the separation is incomplete, continue the run.

t) At the end of the run, transfer the gel to the imaging system and print the result.

The amplification profile after gel electrophoresis is evaluated directly by comparison with the molecular size marker L1000 (6.24) and the positive control of extraction and amplification. The visual evaluation is considered sufficient and appropriate.

The amplification test is considered valid if:

i. The positive control of amplification shows a typical ladder of bands.

ii. The negative control shows no ladder of bands or eventually shows only bands related to oligonucleotides excess or primer dimers.

iii. The positive control of extraction shows a typical ladder of bands.

8. Results

If the test is considered valid, the results are expressed in the test report as follow:

- POSITIVE for the presence of *T. gondii* DNA, if the amplification product shows a typical ladder of bands,
- NEGATIVE if no amplification is shown or an amplification profile not comparable with the positive controls is shown, even after the amplification.

In case of a NEGATIVE result, the presence of *T. gondii* DNAin the matrix cannot be excluded, since the amount of parasite DNA could be below the limit of sensitivity of the method (1,000 tachizoytes/5 g meat).

9. Characteristics of the method

This method has been characterised in terms of repeatability and specificity. The results of the validation protocol are used to confirm that the method is suitable for the expected aim and are reported in the validation report, which can be sent upon request.

10. Safety measures

This method has to be carried out only by authorized personnel. The operator should wear individual protection devices during the test performance. For general safety measures, refer to the CDC guidelines.