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Gold Nanoparticle-Based Loop-Mediated Isothermal Amplification for Rapid, Low-Cost, and Simple Detection of *Salmonella* in Coffee and Cream

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Abstract

The critical public health risk caused by *Salmonella* necessitates the need for efficient detection methods. Conventional techniques based on plate counting and real-time polymerase chain reaction are time-consuming, costly, and labor-intensive, thus mainly performed in centralized laboratories. To meet the emerging need for decentralized/on-site testing, here we describe a rapid, low-cost, and simple method for *Salmonella* detection in coffee and cream by using gold nanoparticle-based loop-mediated isothermal amplification.

Keywords: *Salmonella* detection, loop-mediated isothermal amplification, gold nanoparticles, coffee, and cream

1 Introduction

Salmonella infection imposes a considerable global health and economic burden [1]. Increased public awareness related to food-borne contamination has led to greater efforts to develop faster, cheaper, and simpler methods of *Salmonella* detection for an effective food safety control response. The gold standard detection method is bacteria culture based on plate counting, which typically involves pre-enrichment, selective enrichment, and isolation on selective agar media, followed by confirmation through additional biochemical or serological tests. The process is excessively time-consuming (5–7 days), costly, and labor-intensive, which is mainly performed in centralized microbiology laboratories [2]. In recent decades, a variety of molecular methods have

been extensively utilized for *Salmonella* detection, offering significant advantages over traditional culture techniques. Real-time polymerase chain reaction (real-time PCR or quantitative PCR "qPCR", in short PCR) is the current nucleic acid testing gold standard, which has been reported for *Salmonella* detection in food samples with a pre-enrichment step in buffered peptone water followed by a deoxyribonucleic acid (DNA) isolation/purification step [3]. The turnaround time is around 24 hours, much faster than that for the culture method. However, the pre-PCR procedure is complicated while PCR requires bulky and expensive instrument, Hence, PCR-based nucleic acid testing is still limited in centralized settings. Isothermal nucleic acid amplification techniques are promising candidates for decentralized testing thanks to their simple temperature control and fast amplification [4]. Among them, loop-mediated isothermal amplification (LAMP) has the advantages of: (1) simple isothermal temperature control at ~65 °C; (2) fast amplification in 15–30 min; (3) high specificity by means of 4–6 primers that recognize 6–8 distinct regions of the target sequence; and (4) high tolerance to unpurified samples for direct amplification (without nucleic acid isolation/purification steps) [5]. LAMP result readout can be achieved by real-time turbidity measurement (production of magnesium pyrophosphate crystals in a positive LAMP sample; endpoint visual readout by the naked eye is also possible but only under special lighting condition [6]). Another LAMP result readout can be achieved by colorimetric method based on the use of pH-sensitive dye (e.g., phenol red; production of hydrogen ions in a positive LAMP sample; under unbuffered or weakly buffered condition [7]). It should be pointed out that the assay accuracy is affected by the turbidity and pH of the specimen for these 2 LAMP result readout methods. To address this issue, gold nanoparticle precipitation readout method for LAMP (Gold-LAMP) was developed [8]. The gold nanoparticles are surface-functionalized with thiolated poly(ethylene glycol) and 11-mercaptoundecanoic acid (PEG/MUA-AuNPs). For a negative Gold-LAMP sample (absence of a target nucleic acid sequence), the gold nanoparticles appear as a red dispersion. On the other hand, for a positive Gold-LAMP sample (presence of a target nucleic acid sequence), the gold nanoparticles turn to a red precipitate as a result of the coprecipitation with magnesium pyrophosphate crystals (Fig. 1). Herein, *Salmonella* detection in coffee (containing tannic acid) [9] and cream (containing fat) [10] is demonstrated, which is very challenging with PCR due to enzyme inhibition. Together with a simple and low-cost sample preparation method by filtration (Fig. 2; without the need for nucleic acid isolation/purification as well as complicated operation steps/instruments), *Salmonella* can be detected in enriched coffee and cream samples by Gold-LAMP assay (65 °C for 25 min) with a clear result readout by the naked eye (Fig. 3).

2 Materials

All solutions are prepared using UltraPure DNase/RNase-free distilled water (UltraPure water) from Invitrogen and analytical grade reagents. Unless otherwise stated, all reagents are prepared and stored at room temperature. All disposable consumables used are sterile grade. Waste materials are disposed following waste disposal regulations.

2.1 Salmonella Sample Enrichment and Spike Test [11]

1. Blender (Interscience BagMixer 400 SW or equivalent) for blending sample containing lactose broth.
2. Sampling bag (Interscience Stomacher bag 400 Polysilk blender bags or equivalent).
3. Balance, with weights 2,000 g capacity and sensitivity of 0.1 g.
4. Incubator, with temperature of 35 ± 2 °C.
5. Heat block, 56 ± 2 °C.
6. Centrifuge (up to 20,000 × g) for 1.5 mL microcentrifuge tubes.
7. Autoclave for sterilizing broths and utensils.
8. Sterile spoons for transferring food samples. Sterilize by autoclaving at 121 °C for 15 min.
9. Pipettes and pipette tips.
10. Vortex mixer.
11. Coffee and cream samples.
12. Lactose broth (Thermo Scientific Oxoid CM0137B, or equivalent). Dissolve 13 g of lactose broth powder in 1 L of distilled water and mix well. Sterilize by autoclaving at 121 °C for 15 min. This broth can be kept at 4 °C for 2 weeks.
13. *Salmonella enterica* subsp. enterica serovar Typhimurium strain (ATCC 14028 or equivalent) is kept at −80 °C as stock. Grow the strain on tryptone soya agar plate or in tryptone soya broth for 48 ± 4 h at 35 °C incubator.
14. QIAGEN DNeasy Blood and Tissue Kit and absolute ethanol (*See* **Note 1**).

2.2 Filtration of Enriched Coffee and Cream Samples

1. Enriched coffee and cream samples.
2. 40 µm cell strainers from Biosharp.
3. 50 mL centrifugation tubes.
4. 20 mL and 1 mL syringes.
5. 0.45 µm PES syringe filters (13 mm in diameter).
6. Female to female coupler luer syringe connectors.
7. UltraPure DNase/RNase-free distilled water.
8. 1.5 mL microcentrifuge tubes.

2.3 Gold-LAMP Assay

1. Metal cold racks for 1.5 mL centrifuge tubes and 0.2 mL PCR tubes.
2. Ice and ice box.
3. 0.1–10 µL, 2–20 µL, and 20–200 µL pipettes and compatible filtered pipette tips.
4. 1.5 mL microcentrifuge tubes and 0.2 mL PCR tubes.
5. Mini centrifuge for brief centrifugation.
6. Vortex mixer.
7. Marker pens.
8. Weighing balance with sensitivity of 0.001 g.
9. Heat block (65 °C) for 0.2 mL PCR tubes.
10. NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific) for absorbance measurement.
11. PEG/MUA-AuNPs solution (20 nM) prepared as described before [8]. Store at room temperature.
12. 10× isothermal amplification reaction buffer (200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 500 mM KCl, 20 mM $MgSO_4$, 1% Tween 20, pH 8.8 at 25 °C; New England Biolabs B0537S). Store at −20 °C.
13. Betaine solution (5 M; PCR reagent; Merck). Store at 4 °C.
14. Magnesium sulfate ($MgSO_4$) solution (1 M). Dissolve 12.037 g of $MgSO_4$ (Merck M7506) in a final volume of 100 mL of UltraPure water. Sterilize by autoclaving at 121 °C for 15 min. Store at −20 °C.
15. Six specific LAMP primers targeting enterotoxin (*stn*) gene of *Salmonella spp.* [12]:
    FIP: 5′-ACCGGGTGGTAAGCGAATTGCGAGGTTAACCGTCTGGAGC-3′;
    BIP: 5′-TCGGCCTCTTTGGCCATCACTGGCGAAATACTTTGCCGAG-3′;
    F3: 5′-ACCAGATTCAGGGAGTGAGT-3′;
    B3: 5′-CGCGCACGAAATTCGTAAC-3′;
    Loop F: 5′-TGGTAAAGCCCGCGCATCTG-3′;
    Loop B: 5′-GCGCCAGTTCATGCGACTCG-3′.
    Lyophilized and HPLC-purified primers (Integrated DNA Technologies) are reconstituted by adding IDTE buffer (1× TE solution, pH 7.5) to 100 µM according to the manufacturer's instructions. The ready-to-use LAMP primer mixture (220 µL) is prepared by mixing 80 µL of 100 µM FIP/BIP, 10 µL of 100 µM F3/B3, and 20 µL of 100 µM Loop F/Loop B. Store at −20 °C.
16. *Bst* 2.0 DNA polymerase (120,000 units/mL, New England Biolabs M0537M). Store at −20 °C.
17. Deoxyribonucleoside triphosphates (dNTPs) set (25 mM each prepared from 100 mM stock, Thermo Fisher Scientific R0186). Store at −20 °C (*See* **Note 2**).
18. UltraPure water (Invitrogen). Store at room temperature.

19. Purified *Salmonella* DNA prepared from Section 2.1. Store at −20 °C.
20. Mineral oil (BioReagent, for molecular biology, light oil; Merck M5904). Store at room temperature.

3 Methods

3.1 Salmonella Sample Enrichment and Spike Test [11]

1. Place the lactose broth at ambient temperature.
2. Add an amount of sample test portion (mass or volume) to a quantity of lactose broth (mass or volume) to yield a tenfold dilution. Place a sterile sampling bag in a container, aseptically weigh 25 g coffee or cream into the sterile sampling bag, followed by the addition of *Salmonella* ATCC14028 (spiked samples) and 225 mL sterile lactose broth to the bag.
3. Put the bag containing the mixture to blender and blend for 2 min.
4. Incubate the mixture for 24 ± 2 h at 35 °C incubator. The resultant enriched coffee and cream samples are ready for subsequent filtration steps.

3.2 DNA Extraction by QIAGEN DNeasy Blood and Tissue Kit

This section is to obtain purified *Salmonella* DNA as a positive control for subsequent Gold-LAMP assay.

1. Harvest *Salmonella* ATCC 14028 in a 1.5 mL microcentrifuge tube by centrifugation for 10 min at 5,000 × g. Then, discard the supernatant.
2. Resuspend the pellet in 180 µL Buffer ATL (provided by the kit). Add 20 µL proteinase K. Mix thoroughly by vortexing and incubate with a heat block at 56 °C for 30 min.
3. Short spin the mixture, add 200 µL Buffer AL, and mix well by vortexing.
4. Short spin the mixture, add 200 µL absolute ethanol, mix well by vortexing, and short spin the mixture.
5. Pipette the mixture into DNeasy mini spin column placed in a 2 mL collection tube (provided by the kit). Centrifuge at 6,000 × g for 1 min. Discard flow-through and the collection tube.
6. Place the DNeasy mini spin column in a new 2 mL collection tube, add 500 µL Buffer AW1. Centrifuge for 1 min at 6,000 × g. Discard flow-through and collection tube.
7. Place the DNeasy mini spin column in a new 2 mL collection tube, add 500 µL Buffer AW2. Centrifugate at 20,000 × g for 3 min. Discard flow-through and collection tube (*see* **Note 3**).
8. Place the DNeasy mini spin column in a new 1.5 mL centrifuge tube, pipette 200 µL Buffer AE. Centrifuge at 6000 × g for 1 min to elute DNA form the spin column

(*see* **Note 4**).

9. The purified DNA sample is stored at −20 °C (*see* **Note 5**).
10. Measure the absorbances at 260 nm (A_{260}) and 280 nm (A_{280}) of the eluted DNA sample with NanoDrop One Microvolume UV-Vis Spectrophotometer.
11. Calculate the mass concentration (ng/μL) based on A_{260} of 1.0 = 50 μg/mL pure double-stranded DNA.
12. Calculate the copy concentration based on 1 ng *Salmonella* ATCC 14028 DNA (genome length: 4,964,087) corresponds to 1.52×10^7 copies of the entire genome [13].

3.3 Filtration of Enriched Coffee and Cream Samples

1. Please refer to Fig. 2 for this section (*see* **Note 6**).
2. Place a 40 μm cell strainer on top of a 50 mL centrifuge tube. Pour 10 mL of the enriched coffee or cream sample through the cell strainer. Allow the filtrate to pass through. Discard any residue caught in the strainer.
3. Draw the filtrate collected from the previous step into a 10 mL syringe. Attach the syringe to a 0.45 μm PES syringe filter. Push the filtrate through the filter slowly using the syringe. Keep the syringe filter for the following backflushing (*see* **Note 7**).
4. Connect a female to female luer connector to the opposite opening of the syringe filter. Put the syringe filter on top of a 1.5 mL centrifuge tube (the luer connector at the top).
5. Draw 0.2 mL of UltraPure water with a 1 mL syringe. Backflush the syringe filter through the luer connector with 0.2 mL UltraPure water into the 1.5 mL centrifuge tube. Draw 1 mL air with the 1 mL syringe and flush again (*see* **Note 8**).
6. The backflushed bacteria suspension from coffee or cream is ready for the Gold-LAMP assay. Store at 4 °C for up to several hours (*see* **Note 9**).

3.4 Gold-LAMP Assay

1. Set up a clean work area.
2. Prepare an ice box with ice. Chill metal cold racks on ice.
3. Thaw all frozen reagents (10× isothermal amplification buffer, 25 mM each dNTPs, ready-to-use LAMP primers mixture, and 1 M $MgSO_4$ solution) on a metal cold rack. Mix each thawed reagent well by vortexing. Short spin down the mixture to the bottom of the tube (*see* **Note 10**).
4. Prepare a Gold-LAMP master mix for 4.5 reactions (15 μL for each 25 μL reaction, i.e., 67.5 μL) in a 0.2 mL PCR tube on a cold rack according to the following order: 7.123 μL of Ultrapure water, 11.25 μL of 10× isothermal amplification buffer, 4.5

μL of 5 M betaine, 0.675 μL of 1 M $MgSO_4$, 6.3 μL of dNTPs mixture (25 mM each), 4.95 μL of ready-to-use LAMP primers mixture, 32.4 μL of 20 nM PEG/MUA-AuNPs, and 0.302 μL of *Bst* 2.0 polymerase (*see* **Note 11**).

5. Put 4 PCR tubes on a cold rack. Aliquot 15 μL of the Gold-LAMP master mix into each PCR tube (*see* **Note 12**).
6. Label the 4 tubes as "NTC", "Coffee", "Cream", and "PC".
7. Add 10 μL of UltraPure water into "NTC" as the no template control. Mix well by vortexing or pipetting. Add 20 μL of mineral oil on top of the mixture.
8. Add 10 μL of the backflushed bacteria suspension from coffee and cream into "Coffee" or "Cream", respectively. Mix well by vortexing or pipetting. Add 20 μL of mineral oil on top of the mixtures.
9. Add 10 μL of purified *Salmonella* DNA (diluted to 10^2 copies/μL from the stock in Section 3.1, i.e., 10^3 copies per reaction) into "PC" as the positive control. Mix well by vortexing or pipetting. Add 20 μL of mineral oil on top of the mixture.
10. Incubate the 4 samples at 65 °C for 25 min (*see* **Note 13**).
11. Take out the samples from the heat block. Interpret the results by the naked eye (Fig. 3) (*see* **Note 14**).

4 Notes

1. Proteinase K provided by the kit is ready to use. For buffer AW1 and AW2 provided, add appropriate volume of absolute ethanol as indicated on the bottle label and shake thoroughly. If there is precipitate in Buffer AL, incubate the buffer at 56 °C until it disappears.
2. Avoid repeated freezing/thawing by preparing aliquots.
3. Carefully remove the DNeasy mini spin column from the collection tube. If there is ethanol carryover in the spin column, empty the collection tube, then reuse it, centrifuge at 20000 × g for 1 min to remove the remaining ethanol.
4. The DNA yield can be increased by 2 times elution, each with 100 μL of Buffer AE.
5. Avoid repeated freeze–thaw cycles by preparing aliquots.
6. Make sure proper handling of samples and reagents to prevent contamination. Follow safety protocols while working with bacteria and chemicals.
7. Make sure the filter is attached tightly to the syringe and keep the pushing slow.
8. Make sure thorough rinsing to collect all the bacteria.
9. Mix the suspended bacteria well before adding to the Gold-LAMP reagents.
10. All frozen reagents must be completely thawed and mixed before use.
11. Mix quickly by mild vortexing and spin down the mixture after adding each reagent.
12. Close the PCR tube lid securely to prevent contamination.
13. Put the samples from cold rack to heat block immediately to avoid nonspecific

amplification.

14. Do not open the tube of amplified sample, especially in the sample preparation area, to avoid potential carryover contamination.

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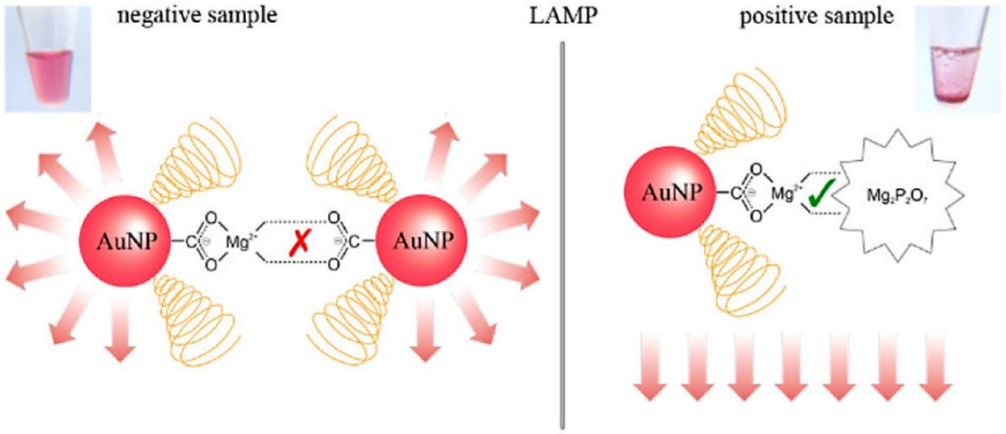

Fig. 1 Schematic illustration of the gold nanoparticle-based loop-mediated isothermal amplification (Gold-LAMP) assay (reproduced from ref. 8 with permission from American Chemical Society, Copyright 2017).

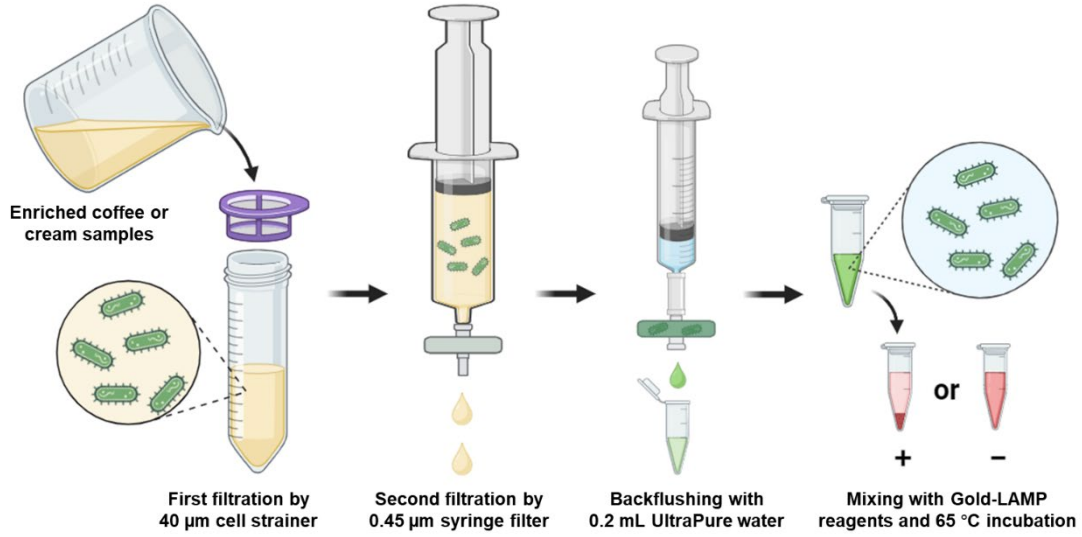

Fig. 2 Schematic illustration of the filtration-based sample preparation steps. First, 10 mL of enriched coffee or cream sample is briefly filtered through a 40 µm cell strainer, followed by a second filtration with 10 mL syringe through a 0.45 µm syringe filter. The syringe filter with remained bacteria is then backflushed with 0.2 mL of ultrapure water using a 1 mL syringe through a female to female luer connector. 10 µL of the 0.2 mL bacteria suspension is then mixed with 15 µL of Gold-LAMP reagent mix and incubated at 65 °C. After incubation for 25 min, a negative sample remains as a red dispersion while a positive sample appears as a red precipitate.

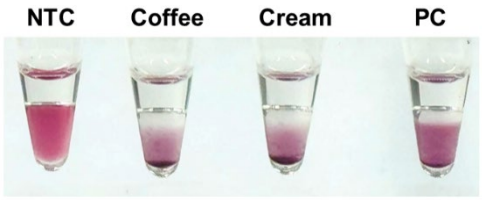

Fig. 3 *Salmonella* detection in coffee and cream with filtration-based sample preparation and Gold-LAMP system. NTC: no template control (no *Salmonella* DNA). PC: positive control (10^3 copies of purified *Salmonella* DNA). The photo was taken after LAMP (65 °C for 25 min).