Journal of Visualized Experiments

Loop-Mediated Isothermal Amplification for Screening Salmonella in Animal Food and Confirming Salmonella from Culture Isolation

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61239R2
Full Title:	Loop-Mediated Isothermal Amplification for Screening Salmonella in Animal Food and Confirming Salmonella from Culture Isolation
Section/Category:	JoVE Biology
Keywords:	Loop-mediated isothermal amplification, Salmonella, molecular method, screening, animal food, confirmation
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Order of Authors:	Beilei Ge
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Laurel, Maryland

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Xiaoyan Cao, Ph.D.
Review Editor
Journal of Visualized Experiments (JoVE)

February 28, 2020

Dear Cao:

I am submitting to JoVE our revised manuscript, JoVE61239-R1, entitled "A Loop-Mediated Isothermal Amplification Method for Screening *Salmonella* in Animal Food and Confirming *Salmonella* from Culture Isolation." All changes are tracked and a separate rebuttal letter is attached.

We hope these modifications resulted in a manuscript that is suitable for publication in JoVE. We greatly appreciate your time and assistance in refining this manuscript, and look forward to hearing from you soon.

Sincerely,

Beilei Ge, Ph.D.
Research Microbiologist
Center for Veterinary Medicine
U.S. Food and Drug Administration

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TITLE:

Loop-Mediated Isothermal Amplification for Screening *Salmonella* in Animal Food and Confirming *Salmonella* from Culture Isolation

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KEYWORDS:

loop-mediated isothermal amplification, *Salmonella*, molecular method, screening, animal food, confirmation

SUMMARY:

Loop-mediated isothermal amplification (LAMP) is an isothermal nucleic acid amplification test (iNAAT) that has attracted broad interest in the pathogen detection field. Here, we present a multi-laboratory-validated *Salmonella* LAMP protocol as a rapid, reliable, and robust method for screening *Salmonella* in animal food and confirming presumptive *Salmonella* from culture isolation.

ABSTRACT:

Loop-mediated isothermal amplification (LAMP) has emerged as a powerful nucleic acid amplification test for the rapid detection of numerous bacterial, fungal, parasitic, and viral agents. *Salmonella* is a bacterial pathogen of worldwide food safety concern, including food for animals. Presented here is a multi-laboratory-validated *Salmonella* LAMP protocol that can be used to rapidly screen animal food for the presence of *Salmonella* contamination and can also be used to confirm presumptive *Salmonella* isolates recovered from all food categories. The LAMP assay specifically targets the *Salmonella* invasion gene (*invA*) and is rapid, sensitive, and highly specific. Template DNAs are prepared from enrichment broths of animal food or pure cultures of presumptive *Salmonella* isolates. The LAMP reagent mixture is prepared by combining an isothermal master mix, primers, DNA template, and water. The LAMP assay runs at a constant temperature of 65 °C for 30 min. Positive results are monitored via real-time fluorescence and can be detected as early as 5 min. The LAMP assay exhibits high tolerance to inhibitors in animal food or culture medium, serving as a rapid, reliable, robust, cost-effective, and user-friendly method for screening and confirming *Salmonella*. The LAMP method has recently been incorporated into the U.S. Food and Drug Administration's *Bacteriological Analytical Manual*

(BAM) Chapter 5.

INTRODUCTION:

Loop-mediated isothermal amplification (LAMP) is a novel isothermal nucleic acid amplification test (iNAAT) invented in 2000 by a group of Japanese scientists[1]. Through the formation of a target-specific stem-loop DNA structure during initial steps, LAMP uses a strand-displacing DNA polymerase to efficiently amplify this starting material quasi-exponentially, resulting in 10^9 copies of target in less than 1 h[1]. Compared to polymerase chain reaction (PCR), a widely used NAAT, LAMP possesses several advantages. First, LAMP reactions are carried out under isothermal conditions. This obviates the need for a sophisticated thermal cycling instrument. Second, LAMP is highly tolerant to culture media and biological substances[2] with robustness demonstrated for both clinical and food applications[3,4]. This simplifies sample preparation and minimizes false negative results[5]. Third, LAMP is amenable to multiple detection platforms, such as turbidity, colorimetry, bioluminescence, fluorescence, and microfluidics[6]. Fourth, LAMP is highly specific as it uses four to six specially designed primers to target six to eight specific regions[1,7]. Fifth, LAMP is ultrasensitive and numerous studies have reported its superior sensitivity to PCR or real-time PCR[8]. Finally, LAMP is faster with many assays now adopting a 30 min standard run time while PCR-type assays usually take 1−2 h[8].

These attractive features fueled the application of LAMP in broad pathogen detection areas, including in vitro diagnostics[9], animal disease diagnostics[10], and food and environmental testing[11]. Notably, a TB-LAMP (LAMP for *Mycobacterium tuberculosis*) has been recommended by WHO as a valid replacement test for sputum-smear microscopy for pulmonary tuberculosis diagnoses in peripheral settings[12]. LAMP application also expands beyond microbial identification to include the detection of allergens, animal species, drug resistance, genetically modified organisms, and pesticides[13].

Nontyphoidal *Salmonella* is a zoonotic pathogen of substantial food safety and public health concern worldwide[14]. It has also been identified as an important microbial hazard in food for animals (i.e., animal food)[15,16]. To prevent *Salmonella* illnesses/outbreaks from contaminated human food and animal food, it is imperative to have rapid, reliable, and robust methods for testing *Salmonella* in a variety of matrices. In the past decade, considerable efforts have been made internationally on the development and application of *Salmonella* LAMP assays in a wide array of food matrices, as recently summarized in an extensive review[8]. Several *Salmonella* LAMP assays, including the one presented here, have successfully completed multi-laboratory validation following well-established international guidelines[17-20].

Our *Salmonella* LAMP assay specifically targets the *Salmonella* invasion gene *invA* (GenBank accession number M90846)[21] and is rapid, reliable, and robust in multiple food matrices[4,22-26]. The method has been validated in six animal food matrices in a precollaborative study[26] and in dry dog food in a multi-laboratory collaborative study[19]. As a result, the *Salmonella* LAMP method presented here has recently been incorporated into the U.S. Food and Drug Administration (FDA)'s *Bacteriological Analytical Manual* (BAM) Chapter 5 *Salmonella*[27] to serve two purposes, one as a rapid screening method for the presence of *Salmonella* in animal food and two as a

reliable confirmation method for presumptive *Salmonella* isolated from all foods.

PROTOCOL:

NOTE: A LAMP reaction mix contains DNA polymerase, buffer, $MgSO_4$, dNTPs, primers, DNA template, and water. The first four reagents are contained in an isothermal master mix (**Table of Materials**). Primers are premixed in-house to become a primer mix (10x) (see section 1.2). DNA templates can be prepared from enrichment broths of animal food samples for screening purpose or from cultures of presumptive *Salmonella* isolates for confirmation purpose. In addition, a positive control (DNA extracted from any *Salmonella* reference strains, e.g., *Salmonella enterica* serovar Typhimurium ATCC 19585 [LT2]) and a no template control (NTC; sterile molecular grade water) are included in every LAMP run.

1. Preparation of DNA templates

1.1. To prepare DNA templates from animal food enrichments, follow these steps.

1.1.1. Aseptically weigh 25 g of animal food sample (e.g., dry cat food, dry dog food, cattle feed, horse feed, poultry feed, and swine feed) into a sterile filter bag (**Table of Materials**), or equivalent. Place the bag into a large container or rack for support during incubation.

1.1.2. Add 225 mL of sterile buffered peptone water (BPW). Mix well by swirling and brief hand-massage. Let stand at room temperature for 60 ± 5 min.

1.1.3. Mix well by swirling and determine pH with a test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1 N NaOH or 1 N HCl. Incubate at 35 ± 2 °C for 24 ± 2 h.

1.1.4. Mix well by swirling the bag containing animal food enrichment broths. Transfer 1 mL from the filtered side of the bag to a microcentrifuge tube. Vortex briefly.

1.1.5. Extract DNA using a sample preparation reagent (**Table of Materials**) as follows.

1.1.5.1. Centrifuge at 900 x *g* for 1 min to remove large particles and transfer supernatant to a new microcentrifuge tube.

1.1.5.2. Centrifuge at 16,000 x *g* for 2 min and discard supernatant.

1.1.5.3. Suspend the pellet in 100 µL of the sample preparation reagent and heat at 100 ± 1 °C for 10 min in a dry heat block.

1.1.5.4. Cool to room temperature and store sample DNA extracts at -20 °C.

1.2. To prepare DNA templates from presumptive *Salmonella* cultures, follow these steps.

1.2.1. Obtain presumptive *Salmonella* isolates from culture isolation in all foods following FDA's BAM Chapter 5 *Salmonella* section D: Isolation of *Salmonella*[27].

1.2.2. Inoculate presumptive *Salmonella* isolates on a nonselective agar plate (e.g., blood agar, nutrient agar, and trypticase soy agar) and incubate at 35 ± 2 °C for 24 ± 2 h.

1.2.3. Transfer several single colonies to 5 mL of trypticase soy broth (TSB) or brain heart infusion (BHI) broth and incubate at 35 ± 2 °C for 16 ± 2 h.

NOTE: This step can be optional if the presumptive *Salmonella* culture is pure. In that case, DNA templates can be prepared by suspending several single colonies in 5 mL of TSB and heat at 100 ± 1 °C for 10 min in a dry heat block, i.e., also skipping step 1.2.4.

1.2.4. Transfer 500 µL of the overnight culture to a microcentrifuge tube and heat at 100 ± 1 °C for 10 min in a dry heat block.

1.2.5. Cool to room temperature and store isolate DNA extracts at -20 °C.

1.3. To prepare positive control DNA, follow similar steps as in section 1.2 with one extra dilution step (step 1.3.3).

1.3.1. Inoculate *S.* Typhimurium ATCC 19585 (LT2) or any *Salmonella* reference strains on a nonselective agar plate (e.g., blood agar, nutrient agar, and trypticase soy agar) and incubate at 35 ± 2 °C for 24 ± 2 h.

1.3.2. Transfer several single colonies to 5 mL of TSB or BHI broth and incubate at 35 ± 2 °C for 16 ± 2 h to reach ~10^9 CFU/mL.

1.3.3. Serially dilute the overnight culture in 0.1% peptone water to obtain ~10^7 CFU/mL.

1.3.4. Transfer 500 µL of this dilution to a microcentrifuge tube and heat at 100 ± 1 °C for 10 min in a dry heat block.

1.3.5. Cool to room temperature and store positive control DNA at -20 °C.

2. Preparation of primer mix (10x)

2.1. Obtain commercially synthesized LAMP primers (Sal4-F3, Sal4-B3, Sal4-FIP, Sal4-BIP, Sal4-LF, and Sal4-LB) with standard desalting purification (**Table 1**).

2.2. Prepare stock solutions of each primer (100 µM) by rehydrating the primer with appropriate amount of sterile molecular grade water. Mix well by vortexing for 10 s and store at -20 °C (-80 °C for long-term storage).

2.3. Prepare the primer mix (10x) according to a worksheet (**Table 2**). Add appropriate volumes of primer stock solutions and sterile molecular grade water into a microcentrifuge tube. Mix all reagents well by vortexing for 10 s.

2.4. Aliquot the 10x primer mix to 500 µL per microcentrifuge tube and store at -20 °C.

3. Assembly of a LAMP reaction

NOTE: To prevent cross-contamination, it is highly recommended to physically separate the areas used for preparing the LAMP master mix and adding DNA templates. **Figure 1** is a LAMP diagram.

3.1. Preparation and run setup

3.1.1. Clean bench with isopropanol and a DNA- and DNase-degrading solution (**Table of Materials**). Clean pipettes and tube strip holders (**Table of Materials**) with the DNA- and DNase-degrading solution.

3.1.2. Thaw the isothermal master mix, primer mix (10x), molecular grade water, positive control DNA, and DNA templates at room temperature.

3.1.3. Turn on the LAMP instrument (**Table of Materials**) and tap the opening screen to access the home screen. Follow these steps to create a run.

NOTE: One model of the LAMP instrument has 2 blocks (A and B) with 8 samples in each block and another model has a single block that accommodates 8 samples (**Table of Materials**).

3.1.3.1. Tap **LAMP+Anneal** and select **Edit** to enter sample information.

NOTE: The default LAMP run profile consists of amplification at 65 °C for 30 min and an anneal phase from 98 °C to 80 °C with 0.05 °C decrement per second.

3.1.3.2. Tap each sample row to activate the cursor and enter relevant sample information, using the **AB block** icon to switch between the two LAMP instrument blocks.

3.1.3.3. Tap the **check** icon when all sample information has been entered.

NOTE: Optionally, the run setup (termed “profile,” which contains sample information along with the default LAMP run profile) may be saved for later use. Tap the **save** icon and give the profile a unique name. When testing this same set of samples next time, a new run can be initiated using the saved profile. Tap the **folder** icon at the bottom left of the home screen and select **Profile** to load saved profiles.

3.2. LAMP reaction assembly

NOTE: When using both LAMP instrument blocks (A and B, a total of 16 samples), prepare the LAMP master mix for 18 samples. If using only one LAMP instrument block (8 samples total), prepare the LAMP master mix for 10 samples. For other sample numbers, adjust the volume accordingly to accommodate pipetting loss. Always include a positive control and an NTC in every LAMP run. Duplicate testing of each sample in independent LAMP runs is recommended.

3.2.1. Prepare the LAMP master mix according to a worksheet (**Table 3**). Add appropriate volumes of the isothermal master mix, primer mix, and molecular grade water into a microcentrifuge tube and vortex gently for 3 s. Centrifuge briefly.

3.2.2. Place the tube strip in the strip holder and distribute 23 µL of the LAMP master mix to each well.

3.2.3. Vortex all DNA templates and centrifuge briefly. Add 2 µL of DNA template to the appropriate well and cap tightly.

3.2.4. Remove the tube strip from the holder and flick wrist to ensure all reagents have pooled at the bottom of the tube.

3.2.5. Load the tube strip into the LAMP instrument block(s), ensuring caps are secure before closing the lid.

4. LAMP Run

NOTE: During a LAMP run, fluorescence readings are acquired using the FAM channel. The time-to-peak values (T_{max}; min) are determined automatically by the instrument for the time point when fluorescence ratio reaches the maximum value of the amplification rate curve. The T_m (°C) is the melting/annealing temperature of the final amplified product.

4.1. Click on the **run** icon at the upper right of the screen and select the block(s) containing tube strip(s) to start the LAMP run.

4.2. Optionally, while the reaction is in progress, tap the **Temperature**, **Amplification**, and **Anneal** tabs to see dynamic changes of various parameters during the LAMP run.

4.3. Once the run is complete, tap the **Amplification** and **Anneal** tabs to see complete amplification and anneal curves and tap the **Results** tab to view the results.

4.4. Optionally, for record keeping, record the run number located at the top left of the screen, using the format of "Instrument serial number_run number," e.g., "GEN2-2209_0030."

REPRESENTATIVE RESULTS:

LAMP results can be viewed on the LAMP instrument panel directly and/or using a LAMP software (**Table of Materials**). **Figure 2** and **Figure 3** show representative LAMP graphs/tables displayed

on both platforms. In this LAMP run, samples S1 to S6 are 10-fold serial dilutions of *S. enterica* serovar Infantis ATCC 51741 ranging from 1.1 x 10^6 CFU to 11 CFU per reaction. Positive control is *S. enterica* serovar Typhimurium ATCC 19585 (LT2) at 1.7 x 10^4 CFU per reaction and NTC is molecular grade water.

Both platforms have five tabs associated with the run: **Profile**, **Temperature**, **Amplification**, **Anneal**, and **Results**, and the software view has two additional tabs, **Amplification Rate** and **Anneal Derivative**, for a total of 7 tabs (**Figure 2** and **Figure 3**). Specifically, the **Profile** and **Temperature** tabs (**Figure 2A,B** and **Figure 3A,B**) show programmed and actual temperatures, respectively, in the sample wells as the LAMP reaction proceeds. The **Amplification**/**Amplification Rate** and **Anneal**/**Anneal Derivative** tabs (**Figure 2C,D** and **Figure 3C–F**) show fluorescence readings or changes in fluorescence during the amplification and anneal phases, respectively. The **Results** tab (**Figure 2E** and **Figure 3G**) shows a tabular view of the LAMP results with slight differences. On the LAMP instrument panel, there are three columns (Well, Amplification, and Anneal). The "Amplification" column shows the time-to-peak values (T_{max}; min:sec) for each sample ("Well") and the "Anneal" column shows the melting/annealing temperatures (T_m; °C) for any amplified product in that well (**Figure 2E**). In the LAMP software, there are four columns (Graph Name, Well Number, Well Name, and Peak Value). The top portion of the "Peak Value" column shows "Amp Time" (T_{max}; min:sec) for each sample ("Well Name") while the bottom portion shows "Anneal Derivative" (T_m; °C) for any amplified product in that well (**Figure 3G**).

LAMP results shown in **Figure 2E** and **Figure 3G** are interpreted following these rules. The control wells are examined first. On the LAMP instrument panel, the NTC well should have blank T_{max} while T_m can be either blank (both LAMP instrument models) or < 83 °C (only for the LAMP instrument model with two blocks). The positive control well should have T_{max} between 5 and 10 min and T_m around 90 °C. All samples with the correct T_m (approximately 90 °C) and T_{max} (between 5−30 min) are considered positive for *Salmonella*. Based on these rules, samples S1 to S6 in this LAMP run are *Salmonella*-positive, and both positive control and NTC are valid (**Figure 2E**). When using the LAMP software, first adjust the "Peak Detection Threshold Ratio" from 0.020 to 0.010. This option is accessible under the **Amplification Rate** tab by clicking on the setting icon. The adjustment is necessary to ensure that all valid peaks are identified, and the results obtained using the software match with those displayed on the instrument panel. After this adjustment, the LAMP results are interpreted following the same rules used for the LAMP instrument panel with the exception that the NTC well and other negative samples should have blank T_m as the LAMP software settings eliminate those T_m < 83 °C results. The same results are obtained for samples S1−S6, as well as positive control and NTC (**Figure 3G**).

If the duplicate runs have consistent results, final LAMP results can be reported. If duplicate runs are inconsistent, repeat both runs independently. If results are still inconsistent, the sample should be considered presumptive positive for *Salmonella* and will need to go through culture confirmation.

FIGURE AND TABLE LEGENDS:

Figure 1: A schematic diagram of the *Salmonella* LAMP workflow.

Figure 2: Representative LAMP results displayed on the LAMP instrument panel. (**A**) The **Profile** tab shows the programmed temperature profile. (**B**) The **Temperature** tab shows actual temperatures in the sample wells as LAMP reaction proceeds. (**C**) The **Amplification** tab shows fluorescence readings during LAMP amplification. (**D**) The **Anneal** tab shows changes in fluorescence (derivative) during the anneal phase. (**E**) The **Results** tab shows a tabular view of the LAMP results.

Figure 3: Representative LAMP results viewed in the LAMP software. (**A**) The **Profile** tab shows the programmed temperature profile. (**B**) The **Temperature** tab shows actual temperatures in the sample wells as LAMP reaction proceeds. (**C**) The **Amplification** tab shows fluorescence readings during LAMP amplification. (**D**) The **Amplification Rate** tab shows changes in fluorescence (fluorescence ratio) during LAMP amplification. (**E**) The **Anneal** tab shows fluorescence readings during the anneal phase. (**F**) The **Anneal Derivative** tab shows changes in fluorescence (derivative) during the anneal phase. (**G**) The **Result** tab shows a tabular view of the LAMP results.

Table 1: LAMP primers for screening *Salmonella* in animal food and **confirming *Salmonella* from culture isolation.** The primers are designed based on the *Salmonella invA* sequence (GenBank accession number M90846).

Table 2: Worksheet for preparing the LAMP primer mix (10x). The primers are listed in **Table 1**.

Table 3: Worksheet for preparing the LAMP reaction mix. The primer mix (10x) is prepared according to **Table 2** using stock solutions of primers listed in **Table 1**.

DISCUSSION:

We have presented here a simple, rapid, specific, and sensitive LAMP method for screening and confirming *Salmonella* in animal food and pure culture, respectively. With the convenience of an isothermal master mix that contains four key reagents, and a ready-to-use, in-house prepared primer mix, assembling a LAMP reaction requires only a few pipetting steps (**Figure 1**). The total run time including amplification and anneal phases is less than 38 min (**Figure 2A,B** and **Figure 3A,B**). Positive results are monitored via real-time fluorescence (**Figure 2C** and **Figure 3C,D**) and can be detected as early as 5 min[26]. The anneal phase serves as an extra confirmation of LAMP specificity since only samples with correct T_m (around 90 °C) are reported as positive (**Figure 2D,E** and **Figure 3E–G**). Sensitivities of 1 *Salmonella* cell in pure culture and <1 CFU/25 g in animal food have been reported previously[26].

As LAMP is quite effective and generates a large quantity of DNA[1], it is critical that best laboratory practices are used to prevent cross-contamination, which may include physically separating the areas for preparing the LAMP master mix and adding DNA templates, avoiding generating aerosols, using filter pipette tips, changing gloves often, and refraining from opening LAMP

reaction tubes post-amplification.

The specificity of this *Salmonella* LAMP method was previously tested using 300 bacterial strains (247 *Salmonella* of 185 serovars and 53 non-*Salmonella*) and demonstrated to be 100% specific[26]. Notably, significant differences in T_{max} were between the two *Salmonella* species, *S. enterica* and *Salmonella bongori*, and among *S. enterica* subspecies, especially subsp. Arizonae (IIIa)[26]. Nonetheless, these were still valid positive results per the rules for interpreting LAMP results. In our multi-laboratory collaborative study in dry dog food which involved 14 analysts[19], samples having inconsistent results in duplicate LAMP runs were occasionally observed. These usually involved samples with delayed positive results (T_{max} > 15 min). Repeating both runs independently usually resolved the issue. More rarely, we observed samples with correct T_m but no or irregular T_{max} values (<5 min). This was usually caused by air bubbles in the reaction tube.

Throughout the lifecycle of LAMP method development, evaluation, precollaborative study, and multi-laboratory validation, we have observed high tolerance of LAMP to inhibitors in various animal food or food matrices and culture medium[4,19,22-24], highlighting the robustness of the method and collaborating numerous other studies on a global scale[8]. This is superior compared to PCR or real-time PCR, which usually requires an internal amplification control to ensure that negative results are not due to matrix inhibition[28]. Further, LAMP demonstrated similar (or superior) specificity and sensitivity compared to PCR or real-time PCR in the vast majority of studies[8]. The cost of LAMP reagents is at about $1 per reaction. The LAMP instruments used in this protocol are small, low-maintenance, and portable. They can handle any isothermal amplification method that employs target detection by fluorescence measurement, LAMP included. Using the LAMP software, comprehensive reports can be generated in multiple format (pdf, text, and image).

Method validation is a critical step before a new method can be adopted for routine use. It is noteworthy that the LAMP protocol reported here has successfully completed multi-laboratory validation[19]. With the incorporation of this LAMP protocol into the U.S. FDA's BAM Chapter 5 *Salmonella*[27], it is expected that the method will gain much wider use, both as a rapid screening method in animal food and as a reliable confirmation method for presumptive *Salmonella* isolates from all food categories.

ACKNOWLEDGMENTS:

The authors thank members of the FDA's Microbiology Methods Validation Subcommittee (MMVS) and Bacteriological Analytical Manual (BAM) Council for critically reviewing *Salmonella* LAMP method validation studies.

DISCLOSURES:

The authors declare that they have no competing financial interests. The views expressed in this manuscript are those of the authors and do not necessarily reflect the official policy of the Department of Health and Human Services, the U.S. Food and Drug Administration, or the U.S. Government. Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the Food and Drug Administration.

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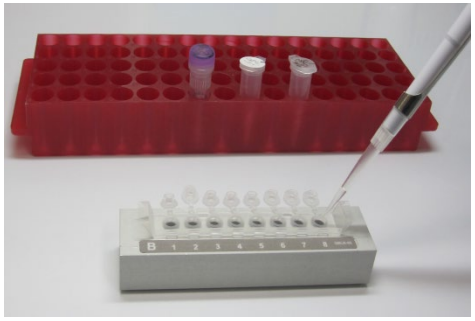
Figure 1

Click here to access/download;Figure;Fig 1-LAMP workflow.pdf

Prepare master mix



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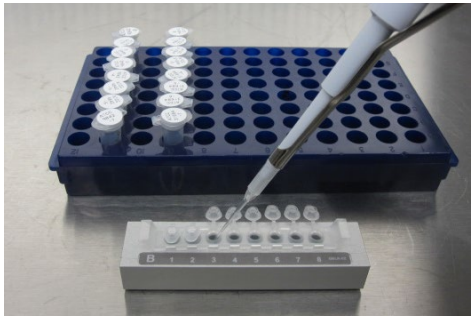
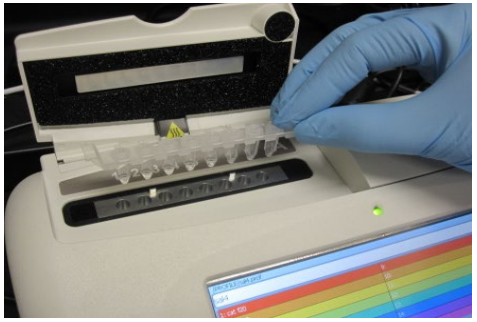
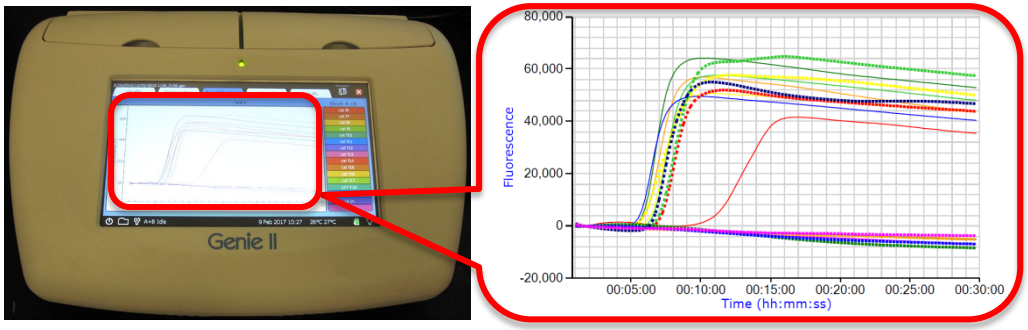


Add DNA templates*



Load into the LAMP instrument



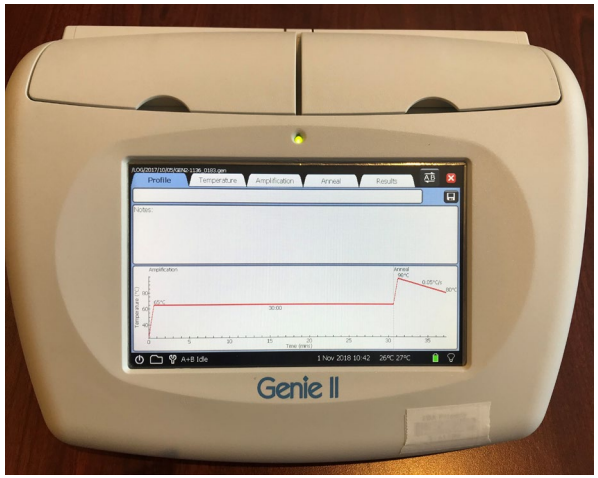
Run LAMP
(amplification graph generated in real-time)

*DNA templates can be prepared from enrichment broths of animal food samples for screening purpose or cultures of presumptive *Salmonella* isolates for confirmation purpose.

Figure 2

Click here to access/download;Figure;Fig 2-Instrument panel display.pdf

A



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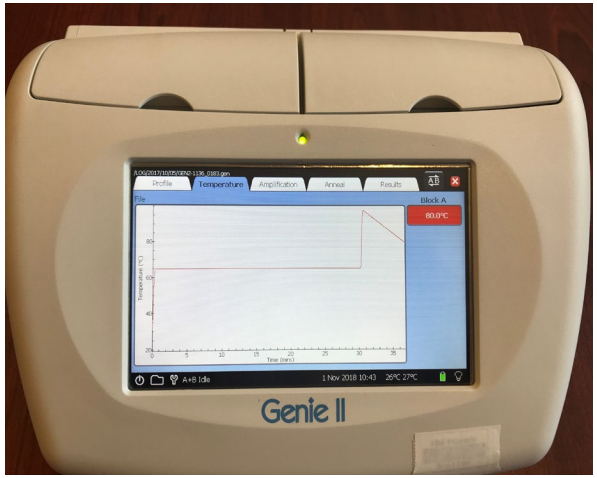

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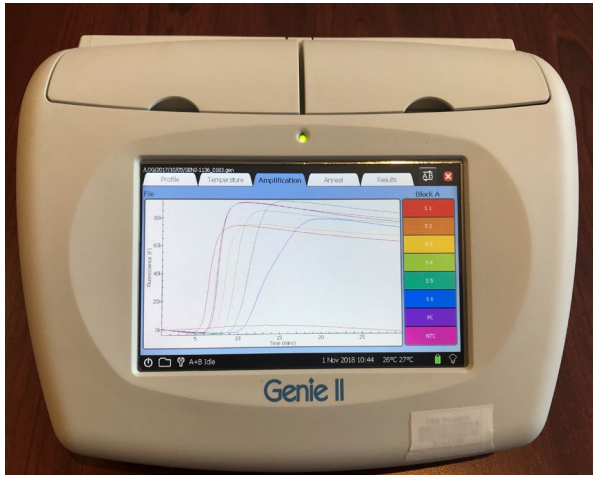

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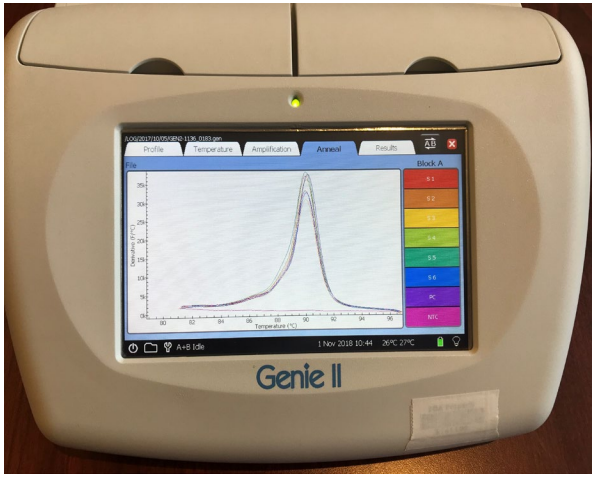

E

Figure 3

Click here to access/download;Figure;Fig 3-Software display.pdf

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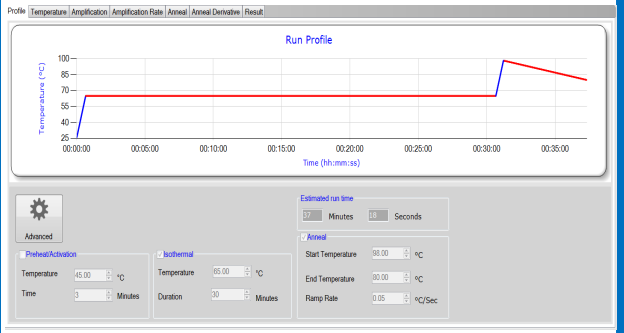

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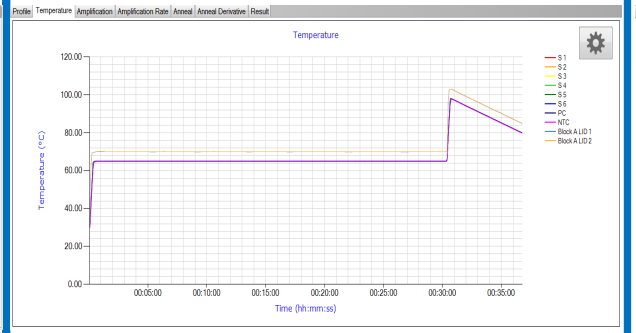

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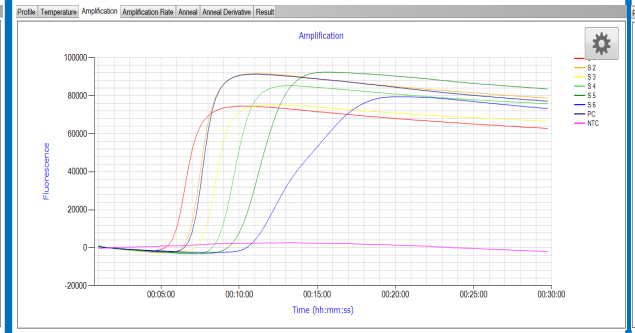

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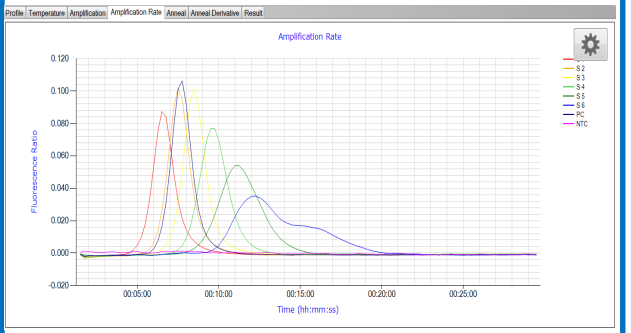

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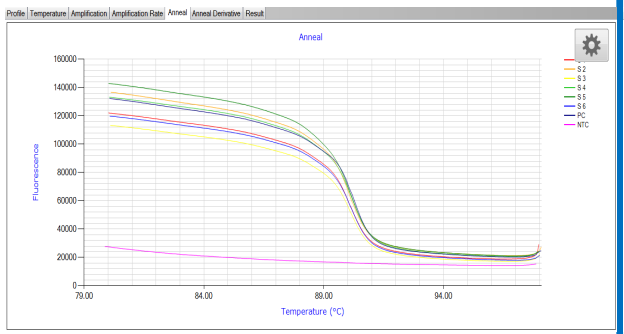

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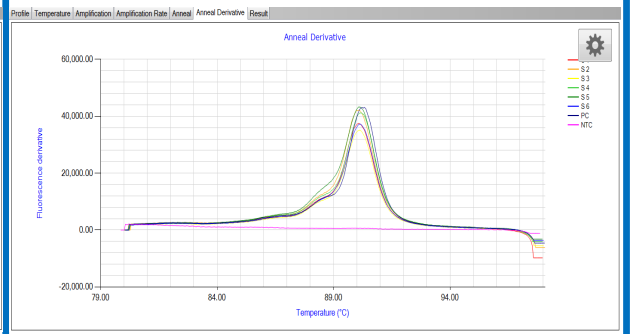

G

Graph Name	Well Number	Well Name	Peak Value
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Amp Time	1	S 1	06:30
Amp Time	2	S 2	07:30
Amp Time	3	S 3	08:30
Amp Time	4	S 4	09:30
Amp Time	5	S 5	11:00
Amp Time	6	S 6	12:15
Amp Time	7	PC	07:45
Amp Time	8	NTC	
			°C
Anneal Derivative	1	S 1	90.1
Anneal Derivative	2	S 2	90.1
Anneal Derivative	3	S 3	90.1
Anneal Derivative	4	S 4	90.2
Anneal Derivative	5	S 5	90.1
Anneal Derivative	6	S 6	90.1
Anneal Derivative	7	PC	90.3
Anneal Derivative	8	NTC	

Table 1

Click here to access/download;Table;Table 1-LAMP primers.xlsx

Primer name	Description	Sequence (5´-3´)
Sal4-F3	Forward outer primer	GAACGTGTCGCGGAAGTC
Sal4-B3	Backward outer primer	CGGCAATAGCGTCACCTT
Sal4-FIP	Forward inner primer	GCGCGGCATCCGCATCAATA-TCTGGATGGTATGCCCGG
Sal4-BIP	Backward inner primer	GCGAACGGCGAAGCGTACTG-TCGCACCGTCAAAGGAAC
Sal4-LF	Loop forward primer	TCAAATCGGCATCAATACTCATCTG
Sal4-LB	Loop backward primer	AAAGGGAAAGCCAGCTTTACG

Length (bp)
18
18
38
38
25
21

Table 2

Click here to access/download;Table;Table 2-LAMP primer mix.xlsx

Component	Stock conc. (µM)	Primer mix conc. (µM)	Volume (µL)
Sal4-F3 primer	100	1	10
Sal4-B3 primer	100	1	10
Sal4-FIP primer	100	18	180
Sal4-BIP primer	100	18	180
Sal4-LF primer	100	10	100
Sal4-LB primer	100	10	100
Molecular grade water	N/A	N/A	420
Total	N/A	N/A	1000

Table 3

Click here to access/download;Table;Table 3-LAMP reaction mix.xlsx

Component	Working conc.	Final reaction conc.	Volume per sample (µL)
ISO-001 isothermal master mix	1.67x	1x	15
Primer mix	10x	1x	2.5
Molecular grade water	N/A	N/A	5.5
Master mix subtotal	N/A	N/A	23
DNA template	N/A	N/A	2

Volume for 18 samples (µL)	Volume for 10 samples (µL)
270	150
45	25
99	55
414	230
N/A	N/A

Table of Materials

Click here to access/download;Table of Materials;Table of materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Brain heart infusion (BHI) broth	BD Diagnostic Systems, Sparks, MD	299070	Liquid growth medium used in the cultivation of *Salmonella* .
Buffered peptone water (BPW)	BD Diagnostic Systems, Sparks, MD	218105	Preenrichment medium for the recovery of *Salmonella* from animal food samples.
DNA AWAY	Thermo Fisher Scientific, Waltham, MA	7010	Eliminates unwanted DNA and DNase from laboratory bench, glassware, and plasticware without affecting subsequent DNA samples.
Genie Explorer software	OptiGene Ltd., West Sussex, United Kingdom	Version 2.0.6.3	Supports remote operation of Genie instruments including LAMP runs and data analysis.
Genie II or Genie III (LAMP instrument)	OptiGene Ltd., West Sussex, United Kingdom	GEN2-02 or GEN3-02	A small instrument capable of temperature control up to 100 °C with ± 0.1 °C accuracy and simultaneous fluorescence detection via the FAM channel. Genie II has 2 blocks (A and B) with 8 samples in each block. Genie III has a single block that accommodates 8 samples.
Genie strip	OptiGene Ltd., West Sussex, United Kingdom	OP-0008	8-well microtube strips with integral locking caps and a working volume of 10 to 150 µl.
Genie strip holder	OptiGene Ltd., West Sussex, United Kingdom	GBLOCK	Used to hold Genie strips when setting up a LAMP reaction, the aluminum holder can also be used as a cool block.
Hydrochloric acid (HCl) solution, 1 N	Thermo Fisher Scientific, Waltham, MA	SA48-500	Adjusts pH of animal food samples after adding BPW and prior to overnight enrichment.
Heat block	Thermo Fisher Scientific, Waltham, MA	88-860-022	Heats samples at 100 ± 1 °C for DNA extraction.
Incubator	Thermo Fisher Scientific, Waltham, MA	3960	Standard laboratory incubator.
ISO-001 isothermal master mix	OptiGene Ltd., West Sussex, United Kingdom	ISO-001	An optimized master mix to simplify the assembly of a LAMP reaction, containing a strand-displacing *Gsp* SSD DNA polymerase large fragment from *Geobacillus* spp., thermostable inorganic pyrophosphatase, reaction buffer, $MgSO_4$, dNTPs, and a double-stranded DNA binding dye (FAM detection channel).
Isopropanol	Thermo Fisher Scientific, Waltham, MA	A416	Disinfects work surfaces.
LAMP primers	Integrated DNA Technologies Inc., Coralville, IA	Custom	LAMP primers with detailed information in Table 1.
Microcentrifuge	Eppendorf North America, Hauppauge, NY	22620207	MiniSpin plus personal microcentrifuge.
Microcentrifuge tubes	Thermo Fisher Scientific, Waltham, MA	05-408-129	Standard microcentrifuge tubes.
Molecular grade water	Thermo Fisher Scientific, Waltham, MA	AM9938	Used in making primer stocks, primer mix, and LAMP reaction mix.
Sodium hydroxide (NaOH) solution, 1 N	Thermo Fisher Scientific, Waltham, MA	SS266-1	Adjusts pH of animal food samples after adding BPW and prior to overnight enrichment.
Nonselective agar (e.g., blood agar, nutrient agar, and trypticase soy agar)	Thermo Fisher Scientific, Waltham, MA	R01202	Solid growth medium used in the cultivation of *Salmonella.*
Peptone water	BD Diagnostic Systems, Sparks, MD	218071	Dilutes overnight *Salmonella* cultures to make positive control DNA.
Pipettes and tips	Mettler-Toledo Rainin LLC, Oakland CA	Pipet Lite LTS series	Standard laboratory pipettes and tips.
PrepMan Ultra sample preparation reagent	Thermo Fisher Scientific, Waltham, MA	4318930	A simple kit used for the rapid preparation of DNA templates for use in a LAMP reaction.
Salmonella reference strain LT2	ATCC, Manassas, VA	700720	*Salmonella* reference strain used as positive control.
Trypticase soy broth (TSB)	BD Diagnostic Systems, Sparks, MD	211768	Liquid growth medium used in the cultivation of *Salmonella* .
Vortex mixer	Scientific Industries, Inc., Bohemia, NY	SI-0236	Standard laboratory vortex mixer.
Whirl-pak filter bag	Nasco Sampling Brand, Fort Atkinson, WI	B01318	Filter bags to hold animal food samples for preenrichment.

Rebuttal Letter
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RESPONSE TO EDITOR COMMENTS

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

Response: Thank you very much for your detailed formatting of the manuscript.

2. Please address specific comments marked in the attached manuscript.

Response: All comments noted have been addressed. Thank you.

3. Please upload Table 1 to your Editorial Manager account.

Response: Table 1 is now uploaded.

Figure 1

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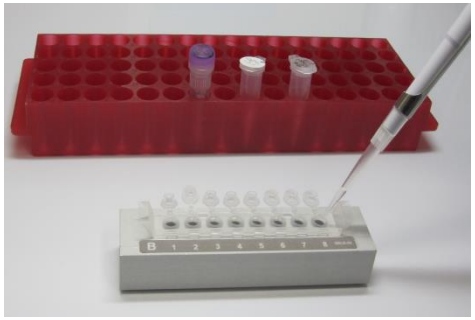
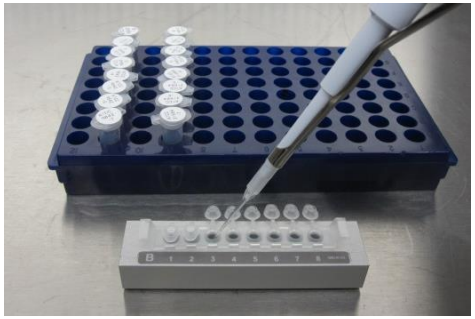
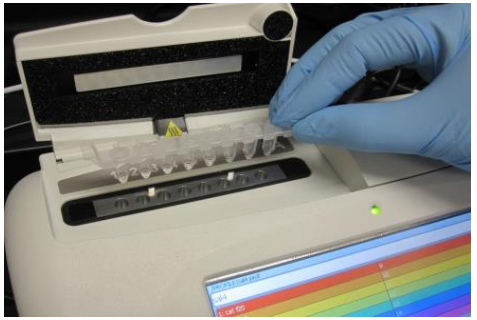
Prepare master mix



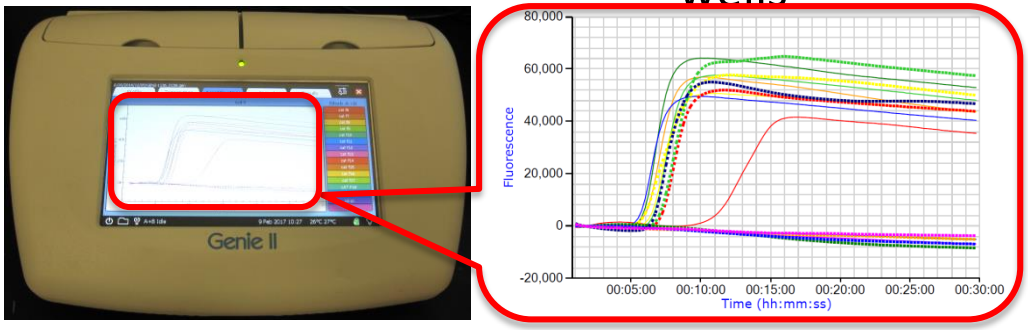
Distribute to wells



Add DNA templates*



Load into the LAMP instrument



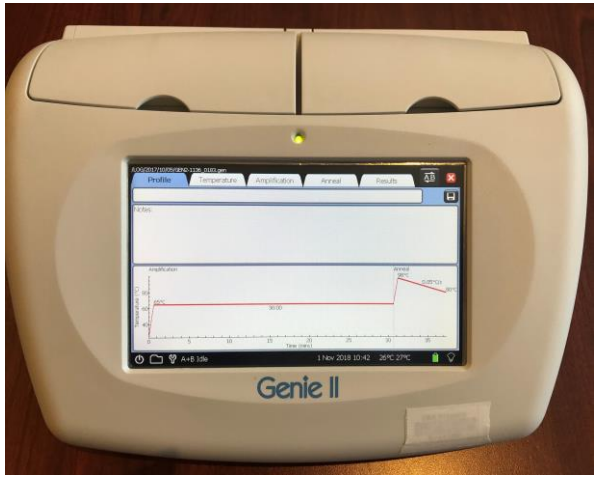
Run LAMP
(amplification graph generated in real-time)

*DNA templates can be prepared from enrichment broths of animal food samples for screening purpose or cultures of presumptive *Salmonella* isolates for confirmation purpose.

Figure 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Fig 2-Instrument panel display.pptx

A



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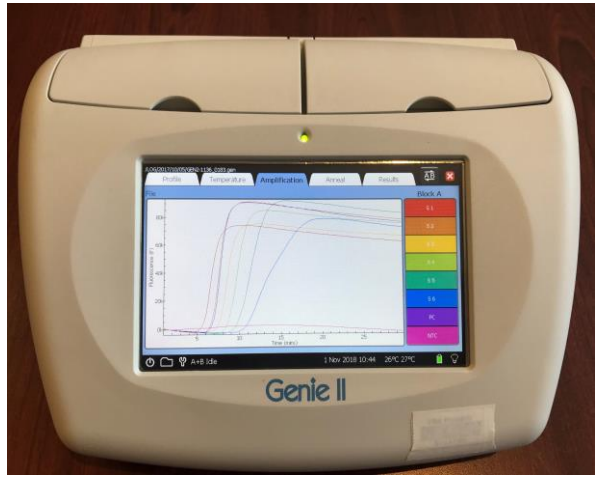

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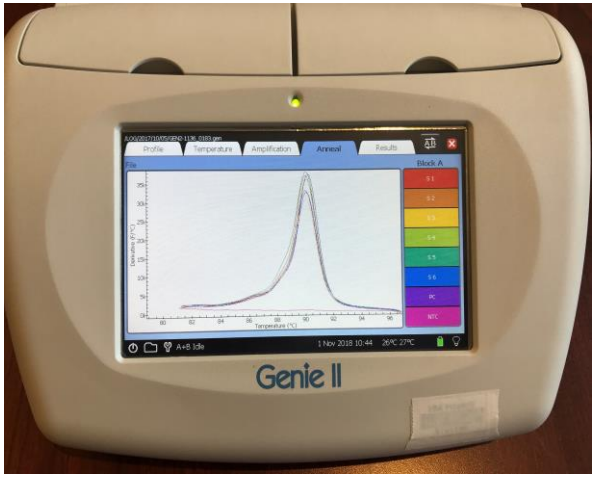

E

Figure 3

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Fig 3-Software display.pptx

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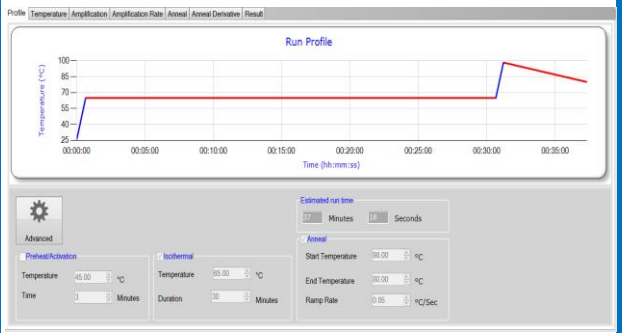

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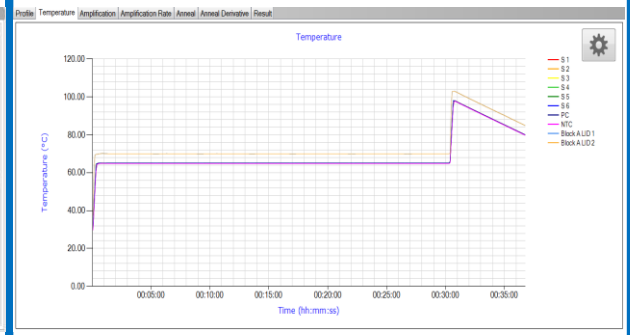

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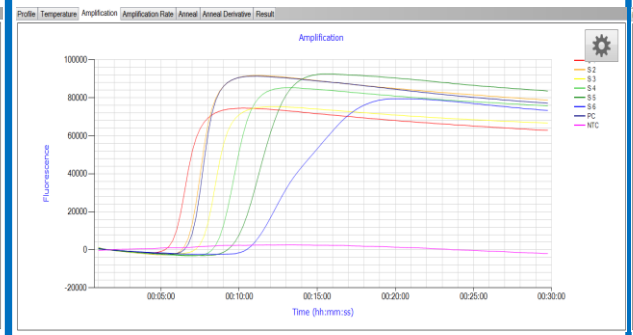

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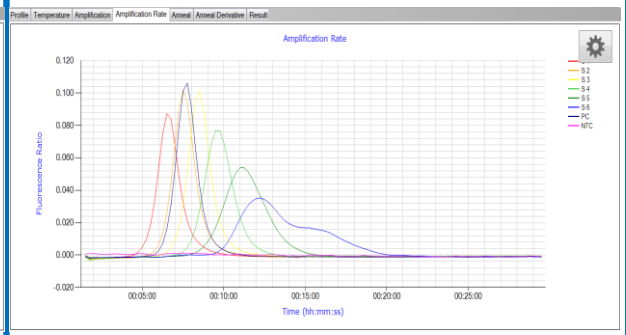

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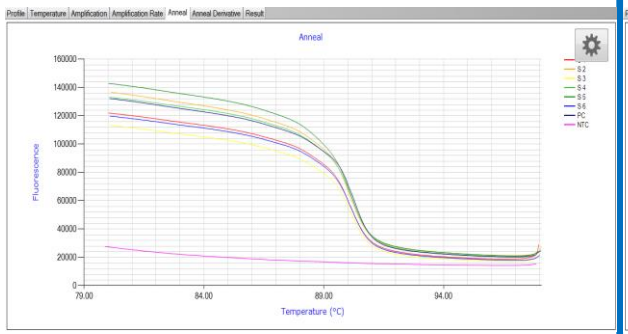

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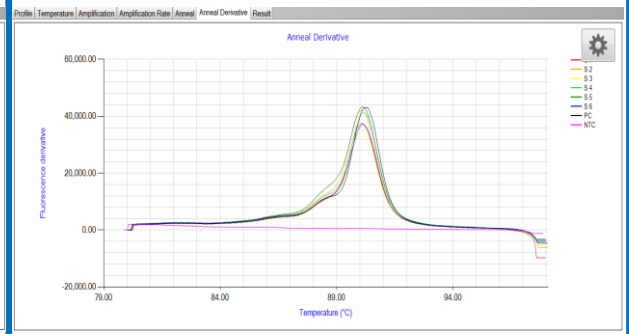

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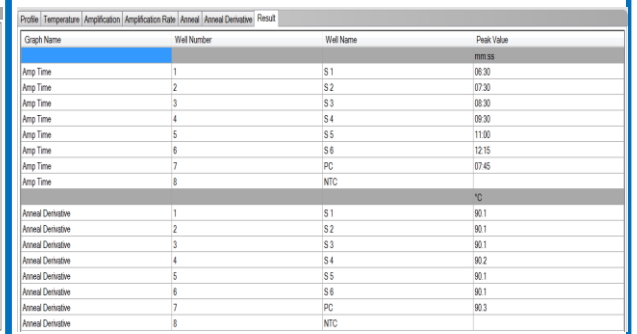

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Amp Time	2	S 2	07:30
Amp Time	3	S 3	08:30
Amp Time	4	S 4	09:30
Amp Time	5	S 5	11:00
Amp Time	6	S 6	12:15
Amp Time	7	PC	07:45
Amp Time	8	NTC	
			°C
Anneal Derivative	1	S 1	90.1
Anneal Derivative	2	S 2	90.1
Anneal Derivative	3	S 3	90.1
Anneal Derivative	4	S 4	90.2
Anneal Derivative	5	S 5	90.1
Anneal Derivative	6	S 6	90.1
Anneal Derivative	7	PC	90.3
Anneal Derivative	8	NTC	