Confirmation of *Salmonella* Isolates by Loop-Mediated Isothermal Amplification (LAMP)

The LAMP method can be used to confirm presumptive *Salmonella* isolates from culture isolation of human food and animal food samples. The LAMP assay runs at a constant temperature (1) and exhibits high tolerance to assay inhibitors (2-4). The assay specifically targets the *Salmonella* invasion gene *invA* (GenBank accession number M90846) (5) and is rapid, reliable, and robust (3, 6-12). The limits of detection of the LAMP assay when tested using different *Salmonella* serovars in pure culture ranged from 1.3 to 28 cells per rection (10).

LAMP assembly and data analysis protocols described below are for three instrument platforms: Genie II, Genie III, and 7500 Fast (13). A video article (14) demonstrating the entire protocol on Genie II (from sample preparation to result interpretation) was published, which can be accessed through this link for the text and another link for the video. Contact: Beilei Ge, FDA-CVM (Beilei.Ge@fda.hhs.gov). Use of other platforms and protocols must be first validated per FDA's Microbiological Methods Validation Guidelines (15), AOAC's Appendix J (16) or ISO 16140-6 (17), and approved for use by FDA's Microbiological Methods Validation Subcommittee.

A. Equipment and Materials

Items 1-3 are needed for assay runs on Genie II or Genie III, whereas items 4-8 are needed for assay runs on 7500 Fast. Items 9-12 are common equipment.

1. Genie II or Genie III (OptiGene Ltd., West Sussex, United Kingdom; also available through select U.S. distributors) capable of temperature control up to 100°C with ± 0.1°C accuracy and simultaneous fluorescence detection via the FAM channel
2. Genie 8-well strips (OptiGene Ltd. #OP-0008)
3. Genie strip holder (OptiGene Ltd. #GBLOCK)
4. 7500 Fast (Thermo Fisher Scientific, Waltham, MA) capable of temperature control/cycling and simultaneous fluorescence detection via the FAM channel
5. MicroAmp Fast optical 96-well reaction plates (Thermo Fisher Scientific #4346906) or MicroAmp Fast 8-tube strips (Thermo Fisher Scientific #4358293)
6. MicroAmp optical adhesive film (Thermo Fisher Scientific #4311971) or MicroAmp optical 8-cap strips (Thermo Fisher Scientific #4323032)
7. MicroAmp 96-well support base (Thermo Fisher Scientific #4379590) or equivalent
8. Mini plate spinner centrifuge
9. Pipettes (0.5-10 µl, 2-20 µl, 20-200 µl, and 200-1000 µl) and tips (aerosol-resistant)
10. Vortex mixer
11. Microcentrifuge (capable of spinning at 16,000 × *g*) and tubes (0.5 to 2 ml)
12. Heat block capable of maintaining 100 ± 1°C

B. Media and Reagents

1. Trypticase soy broth (TSB) or brain heart infusion (BHI) broth
2. Peptone water (0.1%)

3. Isopropanol (70%)
4. DNA AWAY (Thermo Fisher Scientific #7010PK) or equivalent
5. LAMP master mix: three choices, which are GspSSD isothermal master mix (OptiGene Ltd. #ISO-001 for 400 reactions or #ISO-002 for 2,000 reactions), GspSSD2.0 isothermal master mix (**RECOMMENDED**; OptiGene Ltd. #ISO-004 for 300 reactions or ISO-005 for 1,500 reactions), and WarmStart LAMP kit (New England Biolabs, Ipswich, MA; #E1700S for 100 reactions or #E1700L for 500 reactions), among which GspSSD2.0 had the best performance (13)
6. Molecular grade water
7. *Salmonella* LAMP primers and worksheet for preparing the LAMP primer mix (10×)

   LAMP primers (**Table 1**) are synthesized by Integrated DNA Technologies (Coralville, IA) or equivalent with standard desalting purification. Prepare stock solutions of each primer (100 µM) by rehydrating it 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).

Table 1. LAMP primers for confirming *Salmonella* from culture isolation

Primer name	Sequence (5′-3′)	Length (bp)
Sal4-F3	GAACGTGTCGCGGAAGTC	18
Sal4-B3	CGGCAATAGCGTCACCTT	18
Sal4-FIP	GCGCGGCATCCGCATCAATA-TCTGGATGGTATGCCCGG	38
Sal4-BIP	GCGAACGGCGAAGCGTACTG-TCGCACCGTCAAAGGAAC	38
Sal4-LF	TCAAATCGGCATCAATACTCATCTG	25
Sal4-LB	AAAGGGAAAGCCAGCTTTACG	21

Prepare the LAMP primer mix (10×) 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. Aliquot to 500 µl per microcentrifuge tube and store at -20°C.

Table 2. Worksheet for preparing the LAMP primer mix (10×)

Component	Volume (µl)
Sal4-F3 primer (100 µM)	10
Sal4-B3 primer (100 µM)	10
Sal4-FIP primer (100 µM)	180
Sal4-BIP primer (100 µM)	180
Sal4-LF primer (100 µM)	100
Sal4-LB primer (100 µM)	100
Molecular grade water	420
Total	1,000

8. LAMP controls. Always include a positive control (PC) and a no template control (NTC) in every LAMP run.
    a. DNA extracted from a *Salmonella* reference strain, e.g., *Salmonella enterica* serovar Typhimurium ATCC 19585 (LT2), may be used as the PC. Inoculate the bacterium on a nonselective agar plate and incubate 24 ± 2 h at 35 ± 2°C. Transfer several single colonies to 5 ml of trypticase soy broth (TSB) or brain heart infusion (BHI) broth and incubate 16 ± 2 h at 35 ± 2°C to reach *ca.* 10^8 to 10^9 CFU/ml. Serially dilute the overnight culture in 0.1% peptone water to obtain *ca.* 10^6 to 10^7 CFU/ml. Transfer 500 µl of this dilution to a microcentrifuge tube and heat 10 min at 100 ± 1°C in a dry heat block. Cool to room temperature, centrifuge again 2 min at 12,000 × *g* and transfer supernatant to a new microcentrifuge tube. Store extracted DNA at -20°C.
    b. Sterile molecular grade water is used as the NTC.

C. DNA Extraction from Presumptive *Salmonella* Isolates

Go to this link (Preparation of DNA Templates; 2:00 to 2:24) for a quick demonstration.

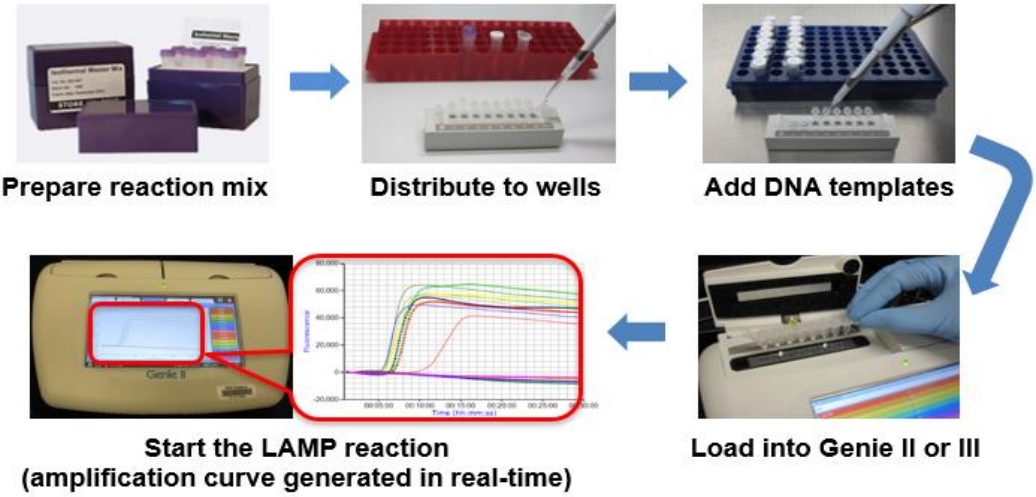
1. Inoculate presumptive *Salmonella* isolates on a nonselective agar plate and incubate 24 ± 2 h at 35 ± 2°C.

   NOTE: This step can be optional if the presumptive *Salmonella* culture is pure.
2. Transfer several single colonies to 5 ml of TSB or BHI broth and incubate 16 ± 2 h at 35 ± 2°C.
3. Transfer 500 µl of the overnight culture to a microcentrifuge tube and heat 10 min at 100 ± 1°C in a dry heat block.
4. Cool to room temperature, centrifuge again 2 min at 12,000 × *g* and transfer supernatant to a new microcentrifuge tube. Store extracted DNA at -20°C.

D. LAMP Assembly

1. Overview

   Go to this link (Assembly of a LAMP Reaction; 2:25 to 3:49) for a quick demonstration.



2. LAMP reaction assembly

IMPORTANT: Before starting to assemble a LAMP reaction, first set up a new LAMP run and enter sample information on Genie or 7500 Fast following **Section E.1 or E.2, respectively, through step c**.

To prevent cross-contamination, it is highly recommended to physically separate the areas used for preparing the LAMP reaction mix and adding DNA templates, either in different areas of the same laboratory or in different laboratories.

a. Clean bench with isopropanol and a DNA- and DNase-degrading solution such as DNA AWAY and clean pipettes and plate/strip holders with DNA AWAY.

b. Thaw LAMP isothermal master mix (GspSSD, GspSSD2.0, or WarmStart), 10× primer mix, molecular grade water, PC *Salmonella* DNA, and isolate DNA extracts at room temperature.

c. Prepare the LAMP reaction mix according to a worksheet (**Tables 3a or 3b**). After all components have been added to a microcentrifuge tube or other appropriate tube, vortex gently then flick contents down.

   Always include a PC and an NTC in every LAMP run. When using only one Genie II block or running LAMP on Genie III (8 samples total), prepare the LAMP reaction mix according to the column for 9 reactions (1 extra for pipetting loss). When using both Genie II blocks (A and B, 16 samples total), prepare the LAMP reaction mix according to the column for 18 reactions (2 extras for pipetting loss). When running LAMP on 7500 Fast, prepare the LAMP reaction mix for up to 100 reactions (96 samples plus 4 extras for pipetting loss). For all other sample numbers, adjust the volumes accordingly.

Table 3a. Worksheet for preparing the LAMP reaction mix using GspSSD or GspSSD2.0

Component	Volume (µl) for 1 reaction	Volume (µl) for 9 reactions	Volume (µl) for 18 reactions	Volume (µl) for 100 reactions
GspSSD or GspSSD2.0 isothermal master mix	15	135	270	1,500
Primer mix (10×)	2.5	22.5	45	250
Molecular grade water	5.5	49.5	99	550
Total	23	207	414	2,300

Table 3b. Worksheet for preparing the LAMP reaction mix using WarmStart

Component	Volume (µl) for 1 reaction	Volume (µl) for 9 reactions	Volume (µl) for 18 reactions	Volume (µl) for 100 reactions
WarmStart isothermal master mix	12.5	112.5	225	1,250
LAMP fluorescent dye	0.5	4.5	9	50
Primer mix (10×)	2.5	22.5	45	250
Molecular grade water	7.5	67.5	135	750
Total	23	207	414	2,300

d. Place the Genie strip or MicroAmp reaction plate/strip in the holder and distribute 23 µl of the LAMP reaction mix to each well.

e. Vortex all DNA templates, add 2 µl of DNA template to each well and cap tightly. When using the MicroAmp optical adhesive film, apply it after all DNA templates have been added.

f. Remove the Genie strip or MicroAmp reaction plate/strip from the holder and flick wrist to ensure all reagents have pooled at the bottom of the tube. Centrifuge the MicroAmp reaction plate briefly in a mini plate spinner as needed.

g. Load the Genie strip into block(s) of Genie II or Genie III and the MicroAmp plate/strip into the plate holder of 7500 Fast, ensuring caps are secure and a good seal before closing the lid.

E. LAMP Run

1. Run on Genie
   a. Turn on the Genie II or Genie III instrument and tap once to access the home screen.
   b. Set up a new run by tapping the default [LAMP & Anneal] profile and select [Edit].

Isothermal amplification phase	Anneal phase
65°C, 30 min	98°C to 80°C with 0.05°C decrement per sec

   c. Enter sample names using the touchscreen keyboard and tap the [✓] icon to save.
   d. Click the [▶] icon in the upper right of the screen and select the block(s) containing Genie strip(s) to start the run.
   e. OPTIONAL: While the LAMP reaction is in progress, tap the [Temperature], [Amplification], and [Anneal] tabs to view real-time results (~ 40 min).
   f. The run will automatically save when complete.
2. Run on 7500 Fast
   a. Turn on 7500 Fast and launch 7500 software (v2.3) from a connected computer.
   b. Set up a LAMP experiment template by clicking [Advanced Setup] from the home screen and save it as “Salmonella LAMP.edt” for all future LAMP runs.

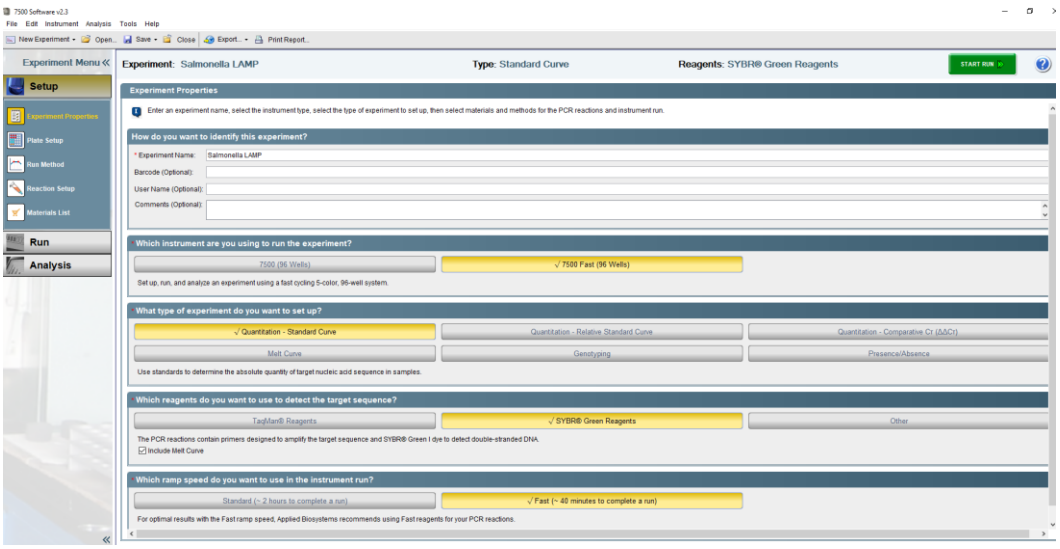
i. In the [Experimental Properties] tab, select the following parameters:

a) Instrument: 7500 Fast (96 Wells)

b) Type of experiment: Quantitation – Standard Curve

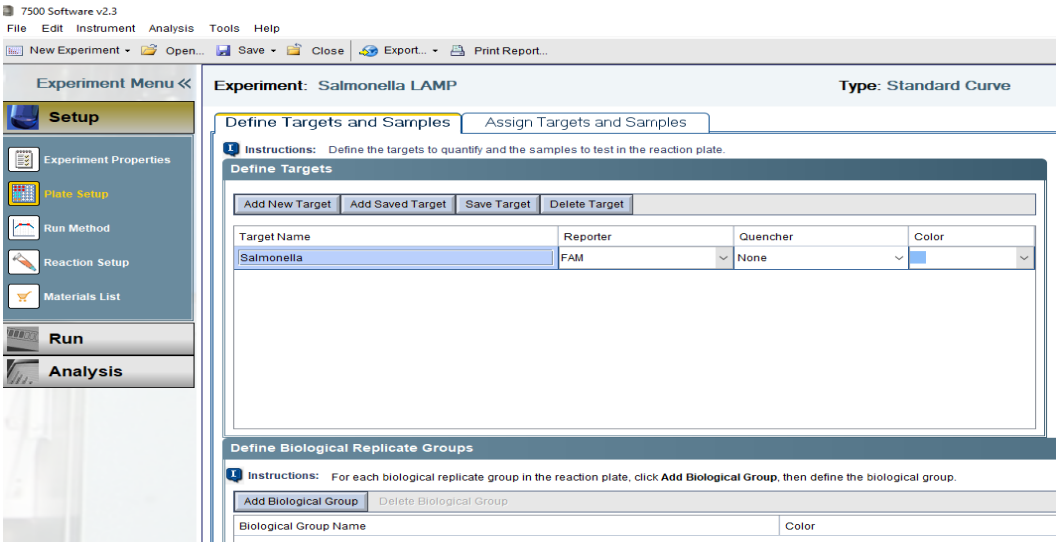
c) Reagents: SYBR® Green Reagents and check [Include Melt Curve]

d) Ramp speed: Fast (~ 40 minutes to complete a run)



ii. In the [Plate Setup] tab, under [Define Targets]:

a) Add a new target “Salmonella” and select “FAM” as the Reporter and Quencher is “None.”



iii. In the [Run Method] tab, select either [Graphical View] or [Tabular View] and set up the following:

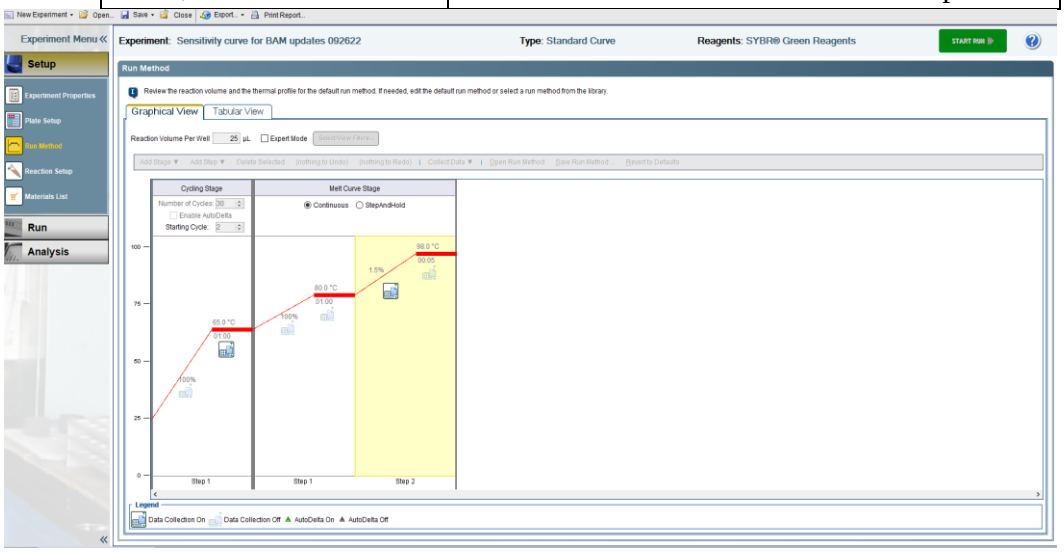
a) Reaction Volume Per Well: 25 µl

b) Delete all default stages but [Cycling Stage] and [Melt Curve Stage].

c) Set up [Cycling Stage] (1 step): Number of Cycles “30,” Starting Cycle “2,” Ramp Rate “100%,” Temperature “65°C,” Time “1:00,” Collect Data on Hold. This enables a LAMP reaction of 30 min at 65°C with fluorescence readings collected every min.

d) Set up [Melt Curve Stage] (2 steps, “Continuous”): Step 1 - Ramp Rate “100%,” Temperature “80°C,” Time “1:00;” Step 2 - Ramp Rate “1.5%,” Temperature “98°C,” Time “0:05,” Collect Data on Ramp.

Isothermal amplification	Melt curve analysis
65°C, 30 min	80°C to 98°C with 0.05°C increment per sec



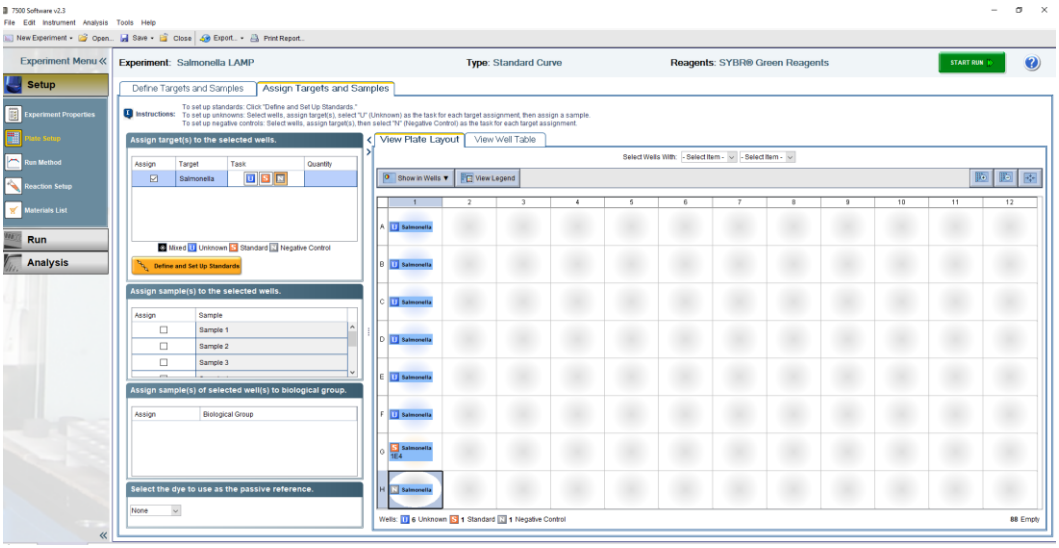
c. Set up a new run and enter experiment name and sample information.

i. Open the “Salmonella LAMP.edt” template from the home screen.

ii. In the [Experimental Properties] tab, type in an “Experiment Name.”

iii. In the [Plate Setup] tab, under [Define Samples], click [Add New Sample] and type in sample names. View tab [Assign Targets and Samples] and assign targets and samples to the plate layout. **IMPORTANT**: Make sure that under “select the dye as the passive reference” on the bottom left, select “None.”

iv. Save experiment (.eds) file, using the experiment name.



d. Click "Start Run" from the top right corner of the screen.

e. OPTIONAL: While the LAMP reaction is in progress, click the [Temperature Plot], [Amplification Plot], and [Melt Curve] tabs in the [Run] tab to view real-time results (~ 40 min).

f. The run will automatically save when complete.

F. LAMP Result Interpretation

Go to this link (LAMP Results Interpretation; 3:50 to 5:48) for a quick demonstration.

In the video, the instrument firmware is v2.25.5 and the Genie Explorer software is v2.0.6.3. Check the manufacturer's site periodically for Genie instrument firmware and software updates.

1. Results on the instrument panel of Genie II (firmware v2.34.13)

   a. Tap the [🗀] icon in the bottom left corner of the home screen and select [log] to navigate to the LAMP run of interest.

   b. Observe the five tabs associated with each run: [Profile], [Temperature], [Amplification], [Anneal], and [Results] (**Figure 1**).

   c. Tap the [Results] tab for a tabular view of the results (**Figure 1e**). There are five columns (Well, Type, Result, Peak Ratio, and Anneal Peak). The "Peak Ratio" column shows the time-to-peak values (min:sec) for each sample ("Well") and the "Anneal Peak" column shows the melting/annealing temperatures (°C) for any amplified product in that well. **NOTE: Acceptable abbreviations for "Peak Ratio" and "Anneal Peak" are T_p and T_a, respectively**.

   d. Examine the control wells first. The NTC well should be blank for "Peak Ratio (T_p)" while "Anneal Peak (T_a)" can be either blank (both Genie II and Genie III) or < 83°C (Genie II only). The PC well should have a "Peak Ratio (T_p)" less than 10 min and "Anneal Peak (T_a)" at 89 ± 2°C. All samples with "Peak Ratio (T_p)" (≤ 20 min) and the correct "Anneal Peak (T_a)" (89 ± 2°C) are considered LAMP-positive for *Salmonella*. For wells with the correct "Anneal Peak (T_a)" (89 ± 2°C) but "Peak Ratio (T_p)" > 20 min or blank, manually observe amplification curves and repeat LAMP on

sample DNA extracts having valid amplifications (typical LAMP sigmoid amplification curve as shown in **Figures 1c and 2a**). If the same results are obtained for the repeat run, the isolates are considered LAMP-positive for *Salmonella*.

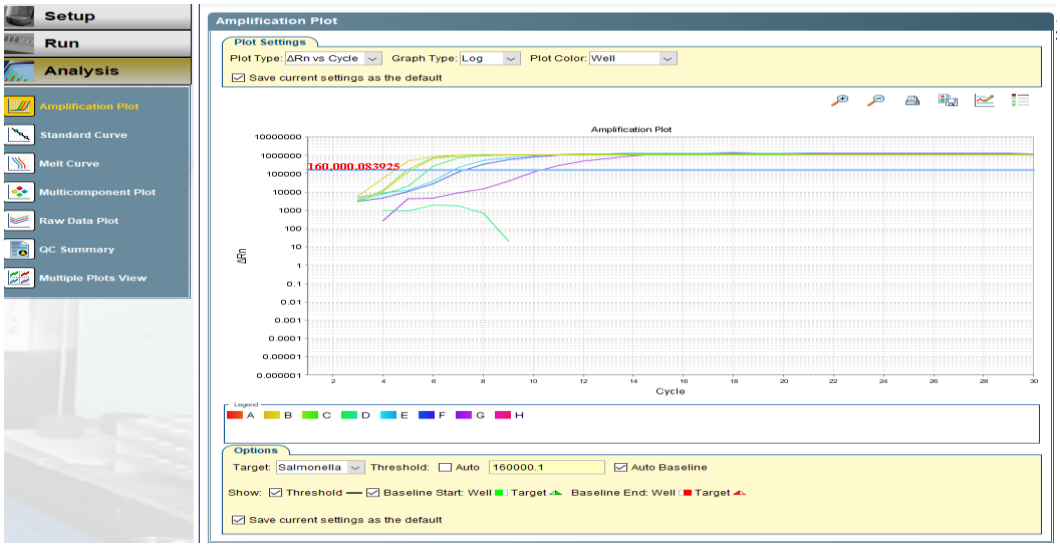
e. In the [Results] tab, save data to a single page pdf report [ ], export to a CSV file [ ], or print directly [ ]. These outputs can also be copied to a USB stick and transferred to a computer.

2. Results on the 7500 software (v2.3)

The LAMP runs on 7500 Fast can be analyzed using the 7500 software v2.3, which can be used as a stand-alone software for remote analysis.

a. Open the experiment file (.eds) for the LAMP run of interest and click the [Analysis] tab on the left panel.

b. In the [Amplification Plot] tab, choose "log" as the graph type to view a graphic display of ΔRn over cycle in log scale. Uncheck "Auto" for [Threshold] and adjust it to be within the exponential phase of the amplification curve according to the instrument manual.



After setting the threshold, choose "linear" as the graph type to view a graphic display of ΔRn over cycle in linear scale (**Figure 2a**).

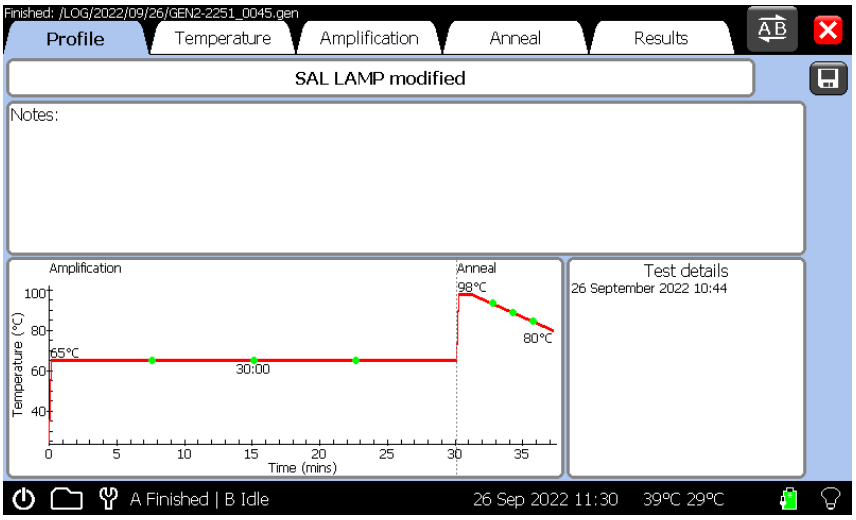
c. In the [Melt Curve] tab, select "Derivative Reporter" as the plot type to view a graphic display of "Derivative Reporter (−Rn′)" over temperature (**Figure 2b**). In the [View Plate Layout] tab, select wells to see corresponding C_T values (**Figure 2c**) and [Amplification Plot] and [Melt Curve] for assigned sample(s). The [View Well Table] tab shows a tabular view of this data, including C_T and T_m (**Figure 2d**).

d. Examine the control wells first. The NTC well should report "Undetermined" for C_T while T_m can be < 83°C. The PC well should have a C_T less than 10 cycles (one min per cycle) and T_m at 89 ± 2°C. All samples with C_T (≤ 20 cycles) and the correct T_m (89 ± 2°C) are considered LAMP-positive for *Salmonella*. For wells with the correct T_m (89 ± 2°C) but C_T > 20 cycles or "undetermined," manually observe amplification

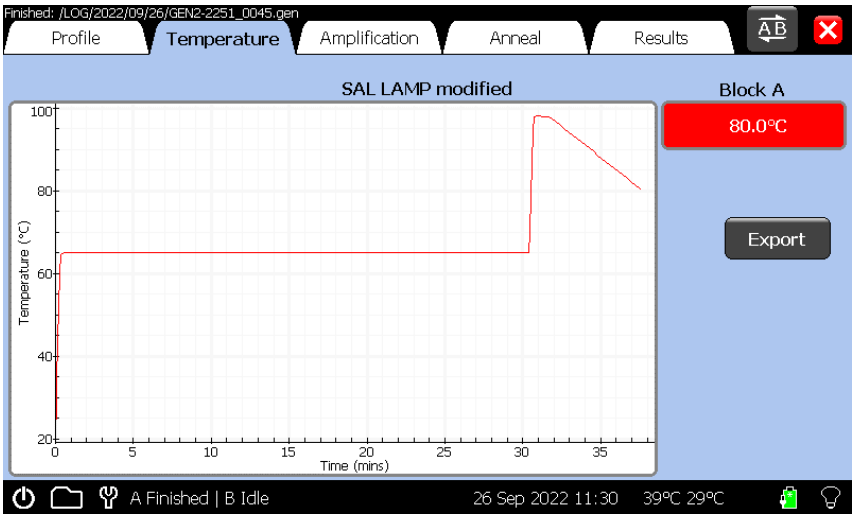
curves and repeat LAMP on sample DNA extracts having valid amplifications (typical LAMP sigmoid amplification curve as shown in **Figures 1c and 2a**). If the same results are obtained for the repeat run, the samples are considered LAMP-positive for *Salmonella*.

Figure 1. Representative LAMP results displayed on the Genie II instrument panel (firmware v2.34.13). In this LAMP run, samples A1 to A6 are 10-fold serial dilutions of *Salmonella enterica* serovar Typhimurium ATCC 19585 (LT2) ranging from 2.6×10^5 cells to 2.6 cells per reaction. PC is *S.* Typhimurium ATCC 19585 (LT2) at 1.7×10^4 cells per reaction and NTC is molecular grade water. The master mix is GspSSD2.0. (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.

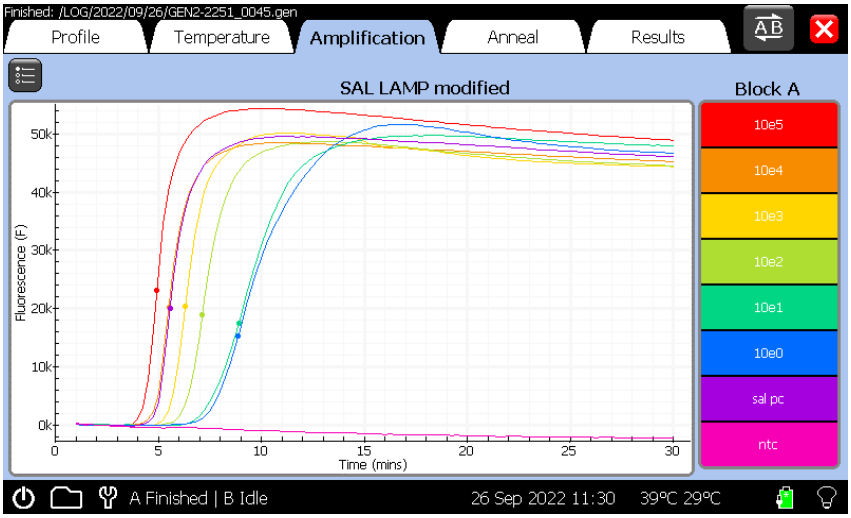
a. The [Profile] tab



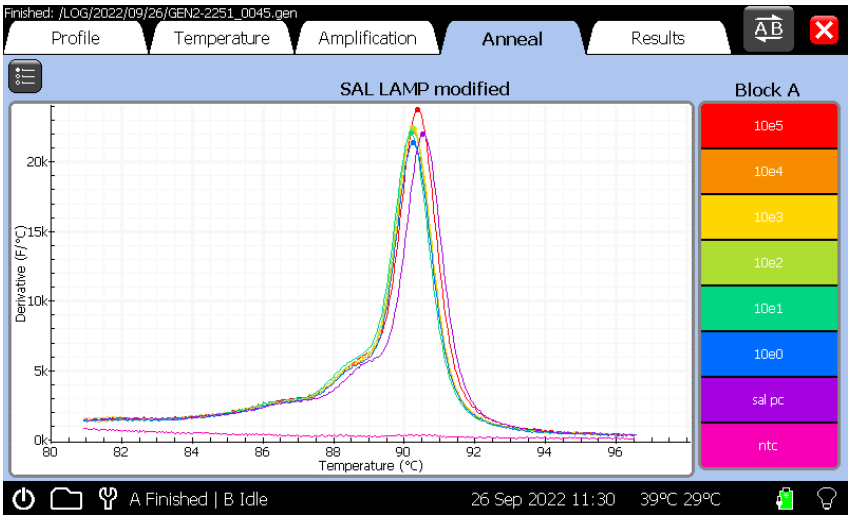
b. The [Temperature] tab



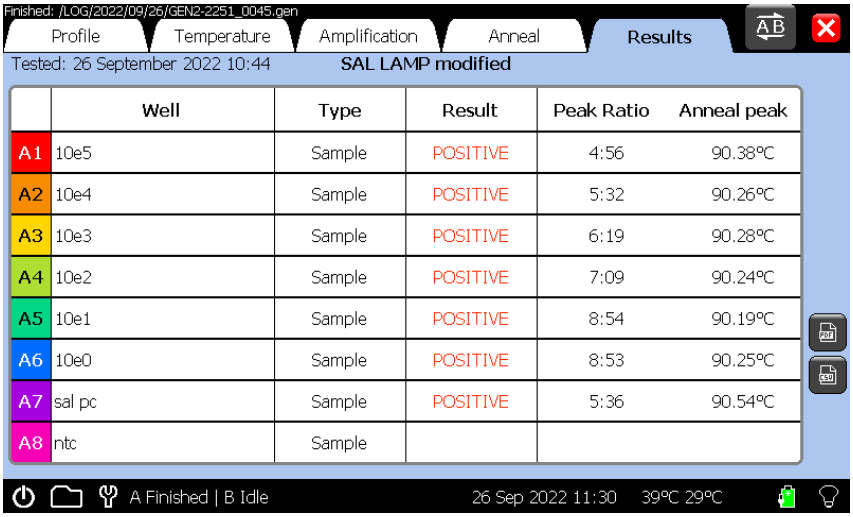
c. The [Amplification] tab



d. The [Anneal] tab



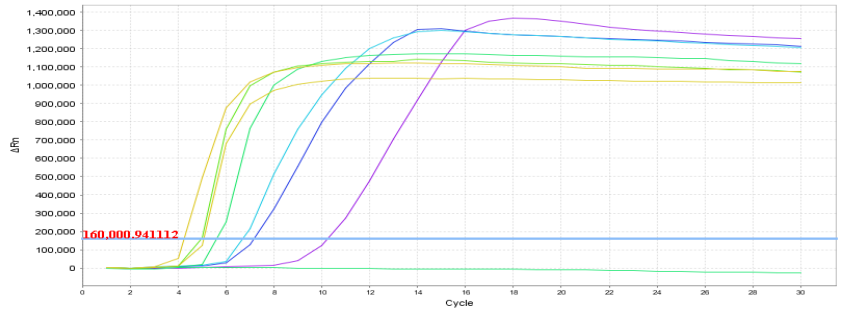
e. The [Results] tab



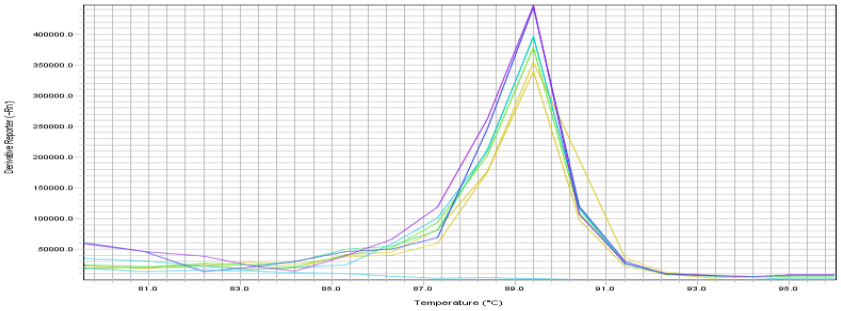
	Well	Type	Result	Peak Ratio	Anneal peak
A1	10e5	Sample	POSITIVE	4:56	90.38°C
A2	10e4	Sample	POSITIVE	5:32	90.26°C
A3	10e3	Sample	POSITIVE	6:19	90.28°C
A4	10e2	Sample	POSITIVE	7:09	90.24°C
A5	10e1	Sample	POSITIVE	8:54	90.19°C
A6	10e0	Sample	POSITIVE	8:53	90.25°C
A7	sal pc	Sample	POSITIVE	5:36	90.54°C
A8	ntc	Sample			

Figure 2: Representative LAMP results viewed in the 7500 software (v2.3). This is a run using the same master mix and samples as in **Figure 1**. (a) The [Amplification Plot] tab shows fluorescence readings during LAMP amplification. (b) The [Melt Curve] tab shows fluorescence readings during the melt curve stage. (c) The [View Plate Layout] tab shows C_T values for each well on the plate. (d) The [View Well Table] tab shows a tabular view of the LAMP results.

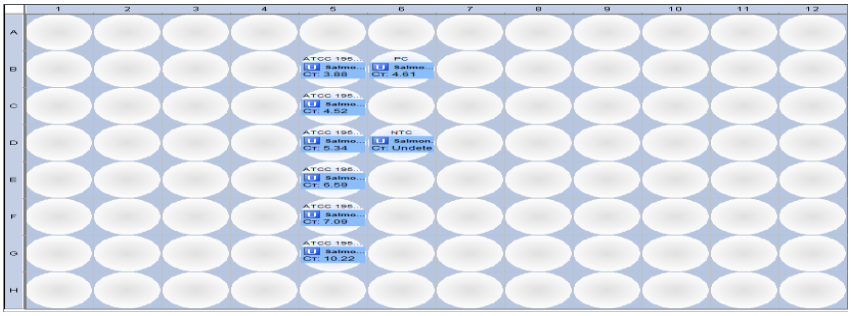
a. The [Amplification Plot] tab



b. The [Melt Curve] tab



c. The "View Plate Layout" tab



d. The "View Well Table" tab

#	Well	Sample Name	Target Name	Dyes	Cт	Tm1
1	B6	PC	Salmonella	FAM-None	04.612	90.8
2	D6	NTC	Salmonella	FAM-None	Undetermined	79.4
3	B5	ATCC 19585 10-5	Salmonella	FAM-None	03.884	90.8
4	C5	ATCC 19585 10-4	Salmonella	FAM-None	04.519	91
5	D5	ATCC 19585 10-3	Salmonella	FAM-None	05.343	91
6	E5	ATCC 19585 10-2	Salmonella	FAM-None	06.594	90.8
7	F5	ATCC 19585 10-1	Salmonella	FAM-None	07.093	90.6
8	G5	ATCC 19585 10-0	Salmonella	FAM-None	10.223	90.6

G. References

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