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

The LAMP method can be used to confirm presumptive *Salmonella* isolates from culture isolation. The LAMP assay runs at a constant temperature (1) and exhibits high tolerance to assay inhibitors (2-4). The LAMP assay specifically targets the *Salmonella* invasion gene *invA* (GenBank accession number M90846) (5) and is rapid, sensitive, and highly specific (3, 6-12).

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

A. Equipment and Materials

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 strips (8-well microtube strips with integral locking caps, working volume of 10 to 150 µl; OptiGene Ltd.) or other equivalent LAMP reaction tubes for selected instrument
3. Genie strip holder (OptiGene Ltd.) or equivalent
4. Pipettes (0.5 to 10 µl, 2-20 µl, 20-200 µl, and 200-1000 µl) and tips (aerosol resistant)
5. Vortex mixer
6. Microcentrifuge (capable of spinning at 16,000 × *g*) and tubes (0.5 to 2 ml)
7. Heat block capable of maintaining 100 ± 1°C

B. Media and Reagents

1. Peptone water (0.1%)
2. Isopropanol (70%)
3. DNA AWAY (Thermo Fisher Scientific, Waltham, MA) or equivalent
4. ISO-001 isothermal master mix (400 reactions; OptiGene Ltd.)
5. Sterile molecular grade water
6. *Salmonella* LAMP primers (**Table 1**) and worksheet for the 10× primer mix (**Table 2**)

   LAMP primers (**Table 1**) are synthesized commercially with standard desalting purification. 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).

   Prepare the 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 1. LAMP primers for confirming *Salmonella* from culture isolation

Primer name	Description	Sequence (5′-3′)	Length (bp)
Sal4-F3	Forward outer primer	GAACGTGTCGCGGAAGTC	18
Sal4-B3	Backward outer primer	CGGCAATAGCGTCACCTT	18
Sal4-FIP	Forward inner primer	GCGCGGCATCCGCATCAATA-TCTGGATGGTATGCCCGG	38
Sal4-BIP	Backward inner primer	GCGAACGGCGAAGCGTACTG-TCGCACCGTCAAAGGAAC	38
Sal4-LF	Loop forward primer	TCAAATCGGCATCAATACTCATCTG	25
Sal4-LB	Loop backward primer	AAAGGGAAAGCCAGCTTTACG	21

The primers are designed based on the *Salmonella invA* sequence (GenBank accession number M90846).

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

Component	Concentration of stock solution (µM)	Concentration in 10× primer mix (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	1,000

The primers are listed in **Table 1**.

7. 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*. 10^9 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 and 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.

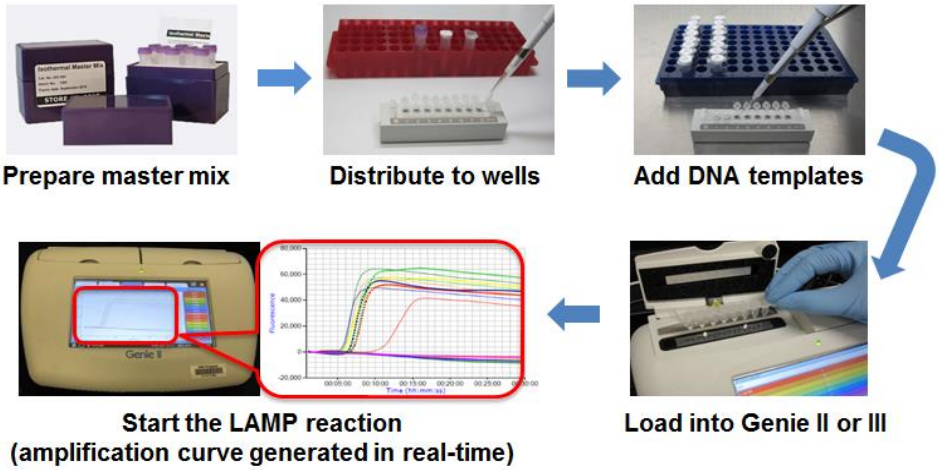
   NOTE: This step can be optional if the presumptive *Salmonella* culture is pure.

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 and store isolate DNA extracts 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. Genie instrument setup

   a. Turn on the Genie II or Genie III instrument and tap the opening screen to access the home screen. The default [LAMP & Anneal] run profile consists of amplification 30 min at 65°C and an anneal phase from 98°C to 80°C with 0.05°C decrement per second.

   IMPORTANT: For Genie II, a recent instrument software update (firmware v2.34.3; October 2020) modified the default setting on positive sample calls from 30 min to 20 min. To adjust it back to 30 min, tap [LAMP & Anneal], select [Edit], and click on

the [ ]button to enter the "Detection Targets" screen. Select [Edit] to enter the "Target 1" screen. Select [Regions] to enter the "Sample Regions" screen where [Peak Ratio] is highlighted by default. Select [Edit] to enter the "Sample, Region 1" screen, navigate to [Range X], and change the maximum value from 1200 to 1800. Click on the [ ] icons to save this new profile as [SAL LAMP] and use for all LAMP runs.

b. Set up a new LAMP run by tapping [SAL LAMP] and selecting [Edit] to enter sample information. Genie II has 2 blocks (A and B) with 8 samples in each block. Genie III has a single block that accommodates 8 samples. Enter sample names using the touchscreen keyboard and tap the [ ] icon to save sample information.

   You may save this profile containing sample names and run conditions by tapping the [ ] icon and giving the profile a unique name. To initiate another run using this profile, tap the [ ] icon at the bottom left of the home screen and select [Profile] to load saved profiles.

3. LAMP reaction assembly

   IMPORTANT: To prevent cross-contamination, it is highly recommended to physically separate the areas used for preparing the LAMP master mix and adding DNA templates.

   NOTE: A LAMP reaction mix contains DNA polymerase, buffer, $MgSO_4$, dNTPs, primers, DNA template, and water. The first four reagents are contained in the ISO-001 isothermal master mix. Primers are pre-mixed in-house to become a 10× primer mix (see **section B.6**). When using both Genie II blocks (A and B, 16 samples total), prepare the LAMP master mix for 18 samples. If using only one Genie II block or running LAMP on Genie III (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 PC and an NTC in every LAMP run. Duplicate testing of each sample in independent LAMP runs (i.e., using different master mixes) is recommended.

   a. Clean bench with isopropanol and a DNA- and DNase-degrading solution such as DNA AWAY and clean pipettes and the Genie strip holder with DNA AWAY.

   b. Thaw LAMP ISO-001 isothermal master mix, 10× primer mix, molecular grade water, PC *Salmonella* DNA, and isolate DNA extracts at room temperature.

   c. Prepare the LAMP master mix according to a worksheet (**Table 3**). Add appropriate volumes of ISO-001 isothermal master mix, primer mix, and molecular grade water into a microcentrifuge tube and vortex gently for 3 s. Centrifuge briefly.

Table 3. Worksheet for preparing the LAMP reaction mix

Component	Working concentration	Final concentration	Volume per sample (µl)	Volume (µl) in master mix for 10 samples	Volume (µl) in master mix for 18 samples
ISO-001 isothermal master mix	1.67×	1×	15	150	270

Primer mix	10×	1×	2.5	25	45
Molecular grade water	N/A	N/A	5.5	55	99
Master mix subtotal	N/A	N/A	23	230	414
DNA template	N/A	N/A	2	N/A	N/A

The primer mix (10×) is prepared according to **Table 2** using stock solutions of primers listed in **Table 1** (see **section B.6**).

d. Place the Genie strip in the Genie strip holder and distribute 23 µl of the LAMP master mix to each well.

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

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

g. Load the Genie strip into block(s) of Genie II or Genie III, ensuring caps are secure before closing the lid.

4. LAMP run on Genie II or Genie III

NOTE: During a LAMP run, fluorescence readings are acquired using the FAM channel. The "Peak Ratio" values (min:sec) are determined automatically by the instrument for the time point when fluorescence ratio reaches the maximum value of the amplification rate curve. The "Anneal Peak" values (°C) are the melting/annealing temperatures of the final amplified product.

a. Click on the [▶] icon at the upper right of the screen and select the block(s) containing Genie strip(s) to start the LAMP run.

b. OPTIONAL: While the reaction is in progress, tap the [Temperature], [Amplification], and [Anneal] tabs to see dynamic changes of various parameters during the LAMP run.

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

d. 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."

E. LAMP Result Interpretation

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

NOTE: In the video, the instrument firmware is v2.25.5 and the Genie Explorer software is v2.0.6.3.

1. Interpretation of LAMP results on the instrument panel of Genie II (firmware v2.34.3)

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

   NOTE: The runs are organized by date, starting with year.

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.

d. Interpret the LAMP results as follows: The control wells are examined first. The NTC well should be blank for "Peak Ratio" while "Anneal Peak" can be either blank (both Genie II and Genie III) or approximately 81°C (Genie II only). The PC well should have a "Peak Ratio" between 6 and 9 min and "Anneal Peak" is 90 ± 1°C. All samples with the correct "Anneal Peak" (90 ±1°C) and "Peak Ratio" (between 4-30 min) are considered positive for *Salmonella*.

e. On the Results tab, data can be generated into a single page pdf report [], exported as a CSV file [], or directly printed [].

f. If the duplicate runs have consistent results, a final LAMP results can be reported. If duplicate runs are inconsistent, repeat both runs independently (i.e., using different master mixes). If results are still inconsistent, the sample should be considered presumptive positive for *Salmonella* and will need to go through culture confirmation.

2. Interpretation of LAMP results using the Genie Explorer software (v2.0.7.11)

   IMPORTANT: The Genie Explorer software uses a default "Peak Detection Threshold Ratio" of 0.020, which is different from the Genie II instrument. To ensure that all valid peaks are identified, and the results obtained using the software match with those displayed on the instrument panel, click on the [] icon at the top right of the "Amplification Rate" tab and adjust the "Peak Detection Threshold Ratio" from 0.020 to 0.010.

   NOTE: The files are transferred from the Genie instrument to the computer via a USB cord that comes with the instrument. Once the files are transferred, the Genie Explorer software does not need to be connected to the instrument to analyze LAMP results, i.e., remote access is available.

   a. Click on the [] icon on the left panel and navigate to the LAMP run of interest. Runs are organized by date.

   b. Besides the five tabs displayed on the instrument panel, observe two additional tabs within the software, [Amplification Rate] and [Anneal Derivative], for a total of 7 tabs (**Figure 2**).

   c. Tap the [Amplification Rate] tab to view a graphic display of the fluorescence ratios by time (**Figure 2d**).

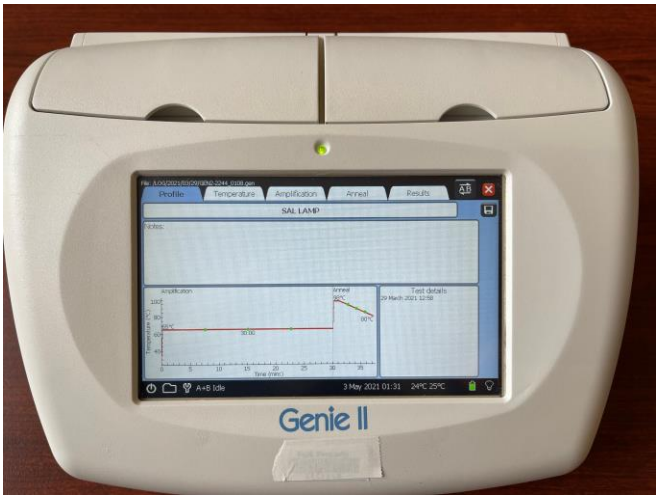
d. Tap the [Result] tab for a tabular view of the results (**Figure 2g**). There are four columns (Graph Name, Well Number, Well Name, and Peak Value). The "Amp Time" (min:sec) is equivalent to the "Peak Ratio" on the Genie II instrument while the "Anneal Derivative" is equivalent to the "Anneal Peak" on the Genie II instrument (°C).

e. Generate a Genie experiment report from the run by clicking on the [ ] icon at the bottom right of the software screen. Alternatively, export data as a text file by clicking on the [ ] icon or as an image file by clicking on the [ ] icon.

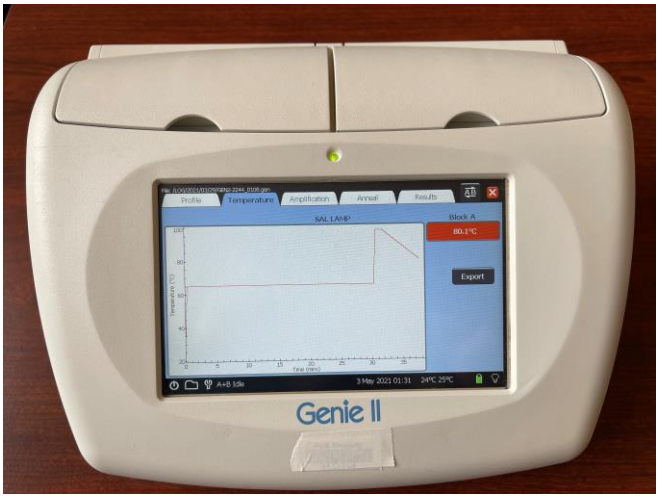
f. Interpret the LAMP results and decide when to repeat samples following the same rules used for the Genie II instrument panel (see **section E.1**) with the exception that the NTC well and other negative samples should have blank "Anneal Peak" as the Genie Explorer software settings eliminate those with approximately 81°C results.

Figure 1. Representative LAMP results displayed on the Genie II instrument panel (firmware v2.34.3). In this LAMP run, samples S1 to S6 are 10-fold serial dilutions of *S. enterica* serovar Typhimurium ATCC 19585 (LT2) ranging from 2.6×10^6 CFU to 26 CFU per reaction. PC is *S.* Typhimurium ATCC 19585 (LT2) at 1.7×10^4 CFU per reaction and NTC is molecular grade water. (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.

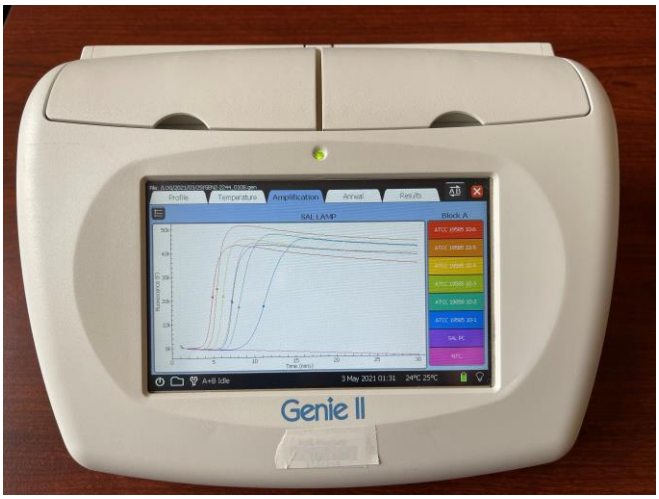
a. The [Profile] tab



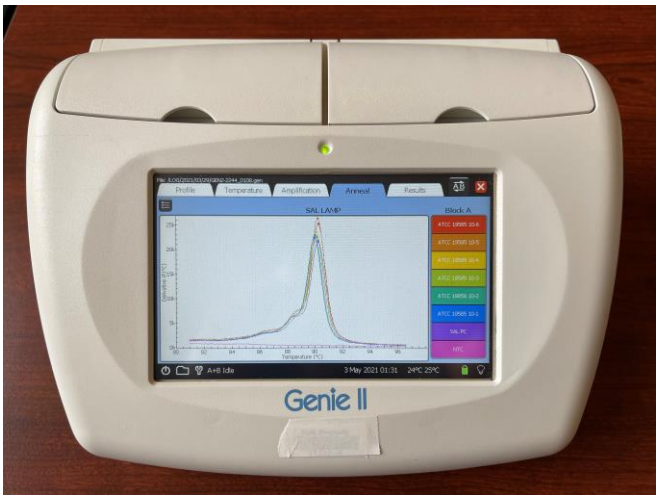
b. The [Temperature] tab



c. The [Amplification] tab



d. The [Anneal] tab



e. The [Results] tab

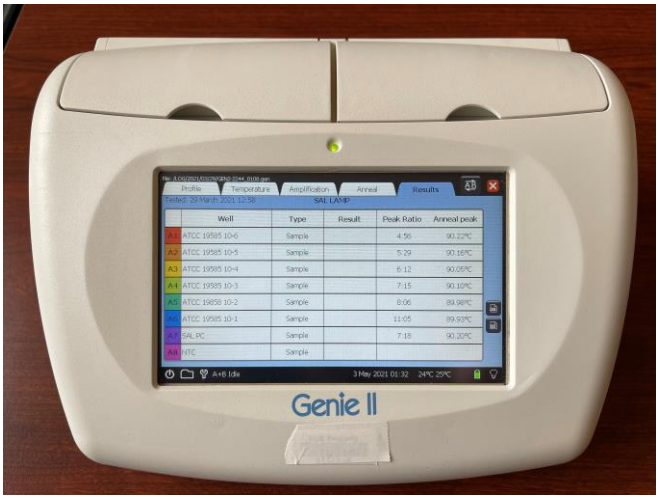
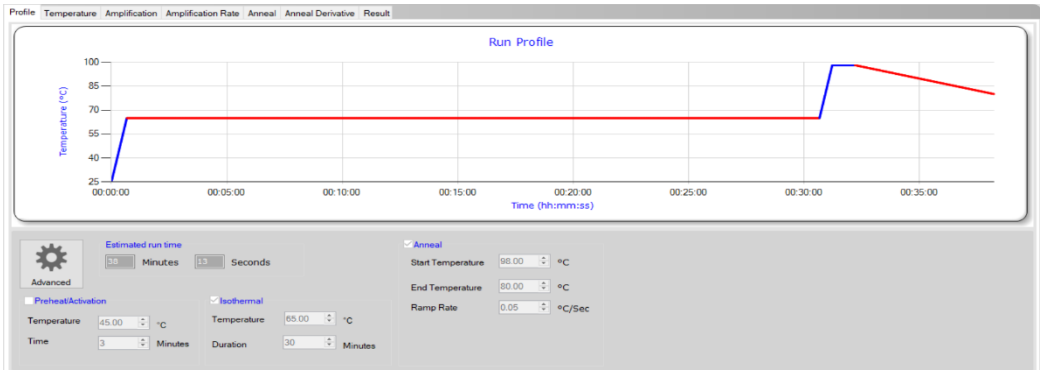
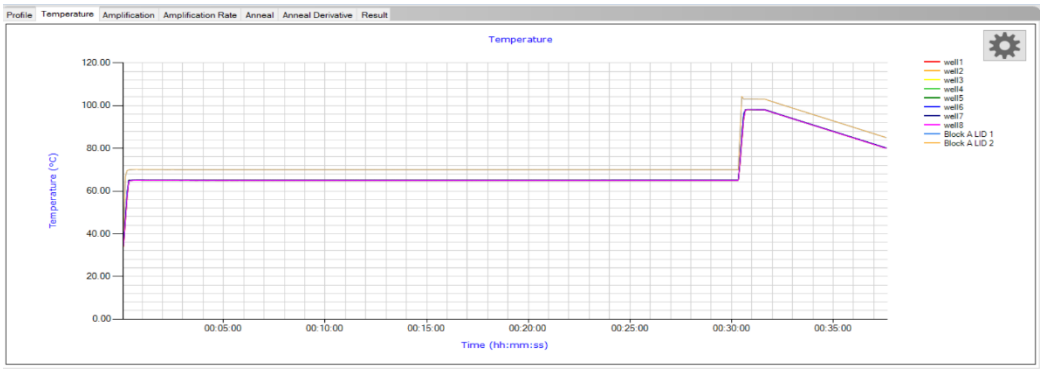


Figure 2: Representative LAMP results viewed in the Genie Explorer software (v 2.0.7.11). This is the same run as in **Figure 1**. (a) The [Profile] tab shows the programmed temperature profile. (b) The [Temperature] tab shows actual temperatures in the sample wells as LAMP reaction proceeds. (c) The [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 [Results] tab shows a tabular view of the LAMP results.

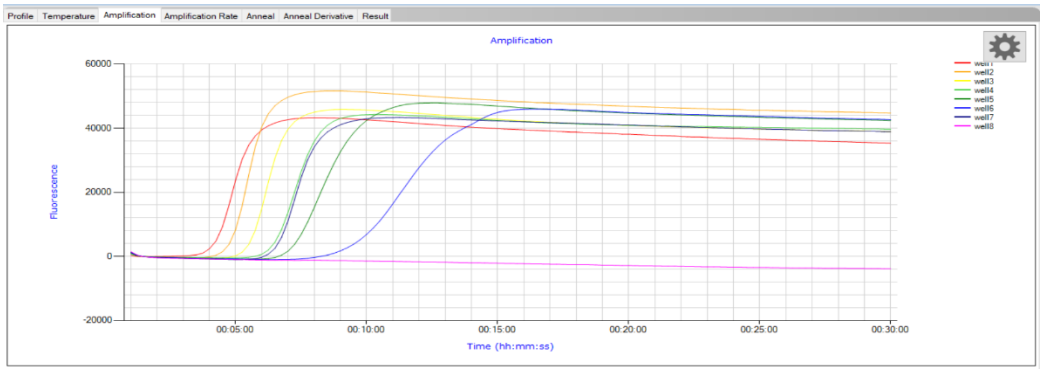
a. The [Profile] tab



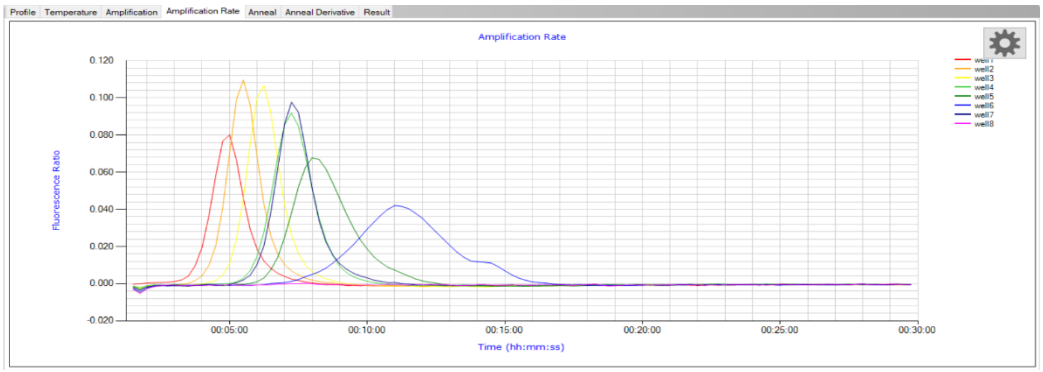
b. The [Temperature] tab



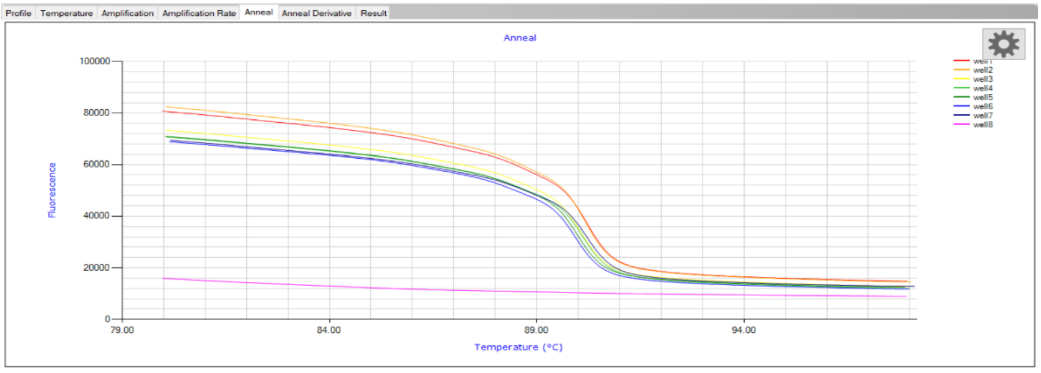
c. The [Amplification] tab



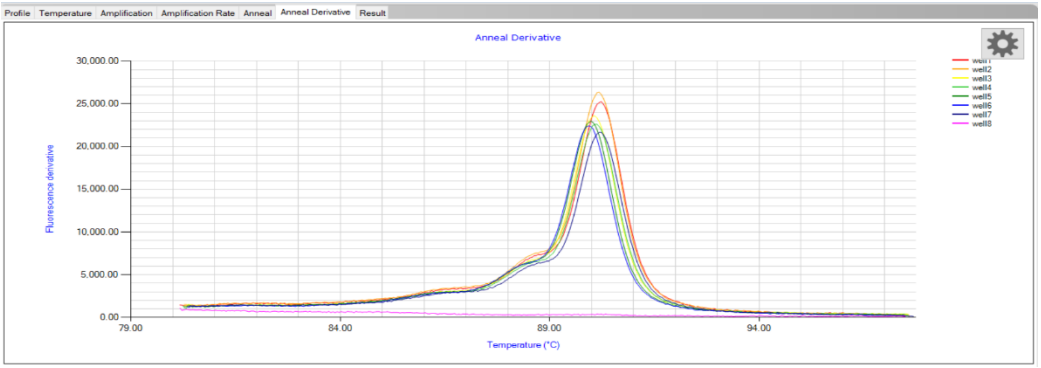
d. The [Amplification Rate] tab



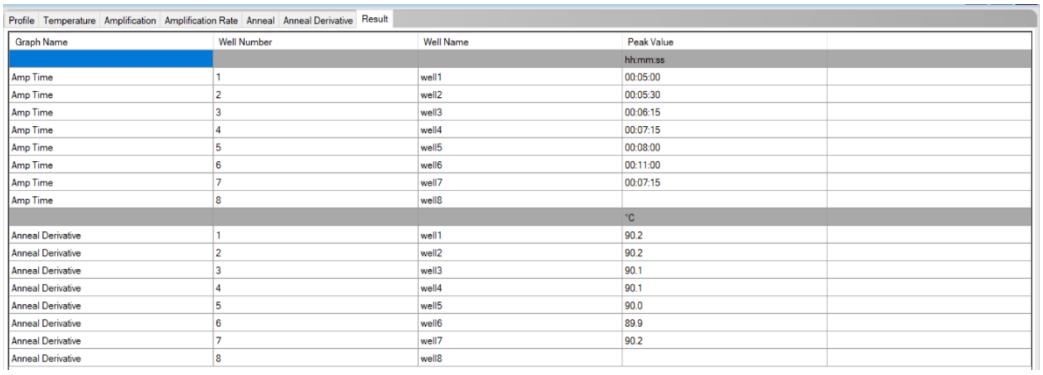
e. The [Anneal] tab



f. The [Anneal Derivative] tab



g. The [Result] tab



Graph Name	Well Number	Well Name	Peak Value	
			hh:mm:ss	
Amp Time	1	well1	00:05:00	
Amp Time	2	well2	00:05:30	
Amp Time	3	well3	00:06:15	
Amp Time	4	well4	00:07:15	
Amp Time	5	well5	00:08:00	
Amp Time	6	well6	00:11:00	
Amp Time	7	well7	00:07:15	
Amp Time	8	well8		
			°C	
Anneal Derivative	1	well1	90.2	
Anneal Derivative	2	well2	90.2	
Anneal Derivative	3	well3	90.1	
Anneal Derivative	4	well4	90.1	
Anneal Derivative	5	well5	90.0	
Anneal Derivative	6	well6	89.9	
Anneal Derivative	7	well7	90.2	
Anneal Derivative	8	well8		

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