

LAMP Screening of *Salmonella* from Animal Food

Animal food may be screened for *Salmonella* using the loop-mediated isothermal amplification (LAMP) method on the Genie II or Genie III platform (1). Samples are enriched in buffered peptone water prior to DNA extraction for LAMP. Only LAMP-positive samples should continue with the isolation of *Salmonella* as described in the FDA's *Bacteriological Analytical Manual* Chapter 5, Section D (2). The LAMP assay specifically targets the *Salmonella* invasion gene *invA* (GenBank accession number M90846) (3) and is rapid, reliable, and robust in multiple food matrices (1, 4-8). The method is capable of detecting < 1 CFU/25 g in animal food (1). The LAMP assay has been validated in six animal food matrices via a single laboratory validation (SLV) study (1) and in dry dog food via a multi-laboratory validation (MLV) study (9), with the latter being performed by seven FDA, state, and academic laboratories. The FDA's Microbiological Methods Validation Subcommittee has approved both SLV and MLV reports.

A. Equipment and Materials

1. Genie II or Genie III (OptiGene, Ltd., West Sussex, United Kingdom; also available through Pro-Lab Diagnostics Inc., Round Rock, TX) or other instrument capable of temperature control up to 100°C with $\pm 0.1^\circ\text{C}$ 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. Vortex mixer
5. Microcentrifuge (capable of spinning at 16,000 $\times g$)
6. Microcentrifuge tubes (0.5 to 2 ml)
7. Pipettes (0.5 to 10 μl , 2-20 μl , 20-200 μl , and 200-1000 μl) and tips (aerosol resistant)
8. Heat block capable of maintaining 100°C
9. Whirl-Pak sterile filter bags with resealable tape and wire (19 \times 30 cm and 38 \times 38 cm)

B. Media and Reagents

1. Peptone water (0.1%)
2. Isopropanol (70%)
3. DNA AWAY (Thermo Fisher Scientific, Waltham, MA) or equivalent
4. PrepMan Ultra sample preparation reagent (Thermo Fisher Scientific)
5. Sterile molecular grade water
6. Positive control *Salmonella* DNA (PC)

Any *Salmonella* reference strains, e.g., *Salmonella enterica* serovar Typhimurium ATCC 19585 (LT2), may be used as the positive control. Grow the bacteria on a nonselective agar plate, transfer several single colonies to 5 ml of trypticase soy broth or brain heart infusion broth, and incubate 16 ± 2 h at 35°C with shaking to reach *ca.* 10^9 CFU/ml.

Serially dilute the overnight culture in 0.1% peptone water to obtain *ca.* 10⁷ CFU/ml (10⁻² dilution). Transfer 500 µl of this dilution to a new microcentrifuge tube, heat 10 min at 100°C in a dry heating block, cool to room temperature, and store at -20°C.

7. ISO-001 isothermal master mix (400 reactions; OptiGene Ltd.)
8. *Salmonella* LAMP primers (**Table 1**) and worksheet for making 5× primer mix (**Table 2**)

Table 1. LAMP primers for use on the Genie II or Genie III platform based on the *Salmonella invA* sequence (GenBank accession number M90846)

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

Stock solutions of primers in **Table 1** (F3 and B3 at 40 µM each and FIP, BIP, LF, and LB at 100 µM each) are prepared from commercially synthesized primers with standard desalting purification (Integrated DNA Technologies, Coralville, IA or equivalent) by rehydrating with appropriate amount of sterile molecular grade water, and store at -20°C.

Table 2. Worksheet for making 1 ml of primer mix (5×)

Component	Stock concentration (µM)	Concentration in 5× primer mix (µM)	Volume (µl)
Sal4-F3 primer	40	0.5	12.5
Sal4-B3 primer	40	0.5	12.5
Sal4-FIP primer	100	9	90
Sal4-BIP primer	100	9	90
Sal4-LF primer	100	5	50
Sal4-LB primer	100	5	50
Molecular grade water	N/A	N/A	695
Total	N/A	N/A	1,000

Primer mix (5×) is prepared by diluting the stock solutions above in sterile molecular grade water. Mix all reagents well by vortexing for 10 s, aliquot to 200 µl per microcentrifuge tube, and store at -20°C.

C. Sample Preparation, Enrichment, and DNA Extraction

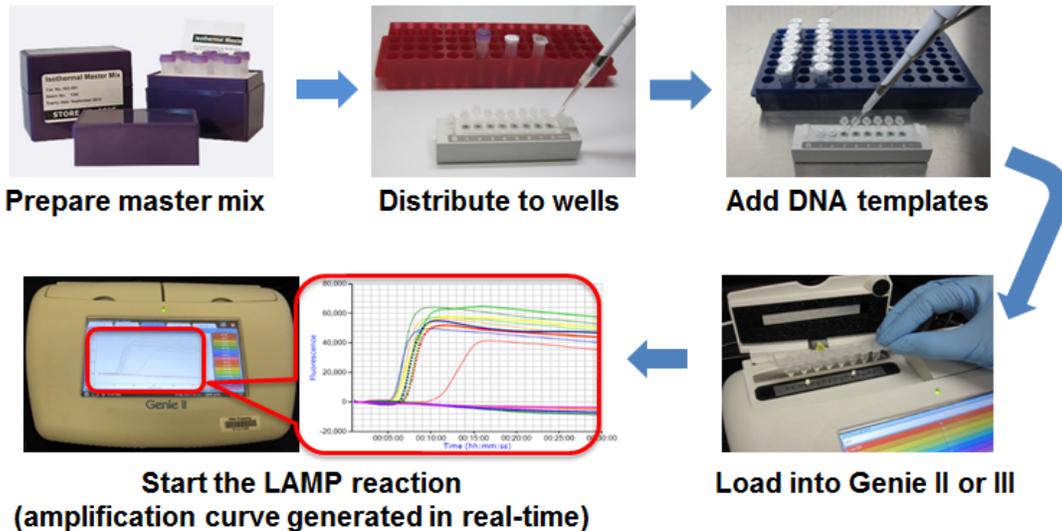
1. Aseptically weigh 25 g sample into a sterile Whirl-Pak filter bag. Add 225 ml sterile buffered peptone water and mix 2 min by hand or in a stomacher.
2. Incubate the sample mixture 24 ± 2 h at 35°C.
3. Gently shake incubated sample. Take 1 ml liquid from the filtered side of the bag and transfer to a microcentrifuge tube. Vortex briefly.
4. Extract DNA using the PrepMan Ultra sample preparation reagent:

Centrifuge 1 min at $900 \times g$ to remove large particles and transfer supernatant to a new microcentrifuge tube. Centrifuge 2 min at $16,000 \times g$ and discard supernatant. Suspend the pellet in 100 µl of PrepMan Ultra sample preparation reagent, heat 10 min at 100°C, cool to room temperature, and centrifuge again 2 min at $16,000 \times g$. Transfer supernatant to a new microcentrifuge tube and store sample DNA extracts at -20°C.

D. LAMP Assembly and Data Analysis

1. Overview

Go to <https://youtu.be/zJwnfyikWRO> for a quick demonstration of the LAMP assembly.



2. Preparation

- a. Clean bench with isopropanol and a DNA and DNase degrading solution such as DNA AWAY and clean pipettes and Genie strip holder also with DNA AWAY.
- b. Thaw LAMP ISO-001 isothermal master mix, primer mix (5×), molecular grade water, positive control *Salmonella* DNA (PC), and sample DNA extracts at room temperature.

- c. Turn on the Genie II or Genie III instrument and tap on the opening screen to access the home screen.

Create a run by tapping on [LAMP+Anneal] (a default profile consisting of 30 min at 65°C and an annealing step from 98°C to 80°C with 0.05°C decrement per second) and selecting [Edit]. Enter sample information (8 samples in each block) by tapping on each sample row to activate the cursor and using the [AB] icon to switch between the blocks. Tap on the [√] icon when all sample information has been entered.

Optional: You can find all run profiles by tapping on the folder icon at the bottom left side of the home screen and selecting [Profile].

3. LAMP reaction assembly

- a. Always include a positive control *Salmonella* DNA (PC) and a no template control (NTC, molecular grade water) in every LAMP run. It is recommended to perform duplicate testing in independent LAMP runs for each sample.

Note: It is highly recommended to physically separate the areas for preparing the LAMP master mix and adding DNA templates to prevent cross-contamination.

- b. Prepare LAMP master mix (**Table 3**) by adding ISO-001 isothermal master mix, primer mix, and sterile molecular grade water into a microcentrifuge tube and vortex for 10 s. Centrifuge briefly.

Note: When using both blocks A and B (a total of 16 samples), prepare the master mix for 18 samples. If using only one block (8 samples), prepare the master mix for 9 samples. For other sample numbers, adjust the volume accordingly to accommodate pipetting loss.

Table 3. Components in a LAMP reaction

Component	Working concentration	Final concentration	Volume per sample (µl)	Volume (µl) in master mix for 18 samples	Volume (µl) in master mix for 9 samples
ISO-001 isothermal master mix	1.67×	1×	15	270	135
Primer mix	5×	1×	5	90	45
Molecular grade water	N/A	N/A	3	54	27
Master mix subtotal	N/A	N/A	23	414	207
DNA template	N/A	N/A	2	N/A	N/A

- c. Place the Genie strip in the Genie strip holder and distribute 23 μl of the master mix to each well.
 - d. Add 2 μl of sample DNA extracts or control template and cap tightly. Flick wrist while holding the Genie strip to ensure all reagents have pooled at the tip of the tube.
 - e. Load the Genie strip into block A and/or B of Genie II or the single block in Genie III.
4. LAMP run on Genie II or Genie III

- a. After the Genie strip(s) have been loaded, close the lid and click the [▶] icon in the upper right corner of the screen, select the block or blocks containing Genie strips to start the 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 annealing temperature values (T_m ; °C) are obtained automatically for the temperature point when fluorescence derivative reaches the maximum value of the anneal derivative curve.

- b. While the reaction is in progress, you may tap on the [Temperature], [Amplification], and [Anneal] tabs to see dynamic changes of these parameters during the LAMP run.
- c. Once the run is complete, tap on the [Results] tab to view the final results and tap on [Amplification] and [Anneal] tabs to see complete amplification and anneal curves.
- 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.”

5. Interpretation of LAMP results on the instrument panel

- a. Open the LAMP run of interest on the instrument panel. There are five tabs associated with each run: [Profile], [Temperature], [Amplification], [Anneal], and [Results] (**Figure 1**).

Note: To open previous runs, tap on the folder icon at the bottom left of the home screen and select [log]. The runs are organized by date.

- b. Tap on the [Results] tab for a tabular view of the results (**Figure 1e**). There are three columns (Wells, Amplification, and Anneal). The column entitled “Amplification” shows the time-to-peak values (T_{max}) in minutes and seconds, and the column entitled “Anneal” shows the annealing temperature values (T_m) in °C.

The NTC well should have no T_{max} values while T_m can be either blank (both Genie II and Genie III) or *ca.* 81°C (Genie II only). The PC well should have T_{max} between 5 and 10 min and T_m around 90°C.

All sample DNA extracts with the correct T_m (approximately 90°C) and T_{max} values between 5-30 min are positive for *Salmonella*.

- c. As duplicate runs have been performed for each sample, when the sample has consistent results in both runs, final LAMP results can be reported. If two repeats

generate inconsistent results, one more time repeat (duplicate runs independently) is recommended.

6. Interpretation of LAMP results using the Genie Explorer software (version 2.0.6.3 or higher)
 - a. Open the Genie Explorer software and the file containing the LAMP run of interest. There are two additional tabs, [Amplification Rate] and [Anneal Derivative], for a total of 7 tabs (**Figure 2**).

Note: A Genie experimental report can be generated from the run by clicking on the blue notebook icon at the bottom right of the software screen.

- b. Tap on the [Result] tab for a tabular view of the results (**Figure 2g**).

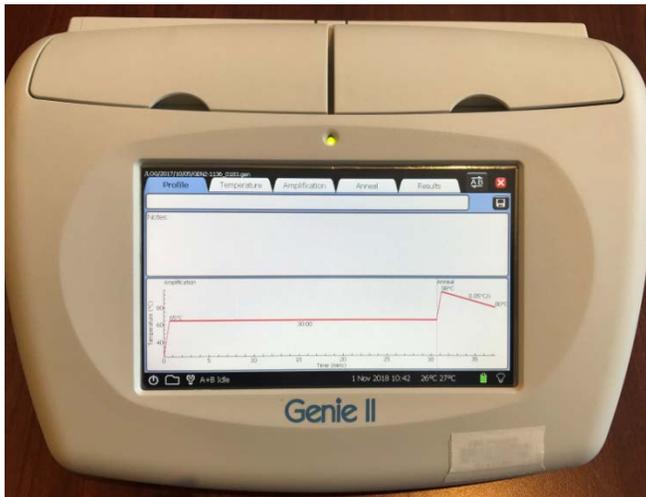
The NTC well should have no T_{max} values and blank T_m as the Genie Explorer software has a refinement step that removes noise in detecting annealing peaks (approximately 81°C), which may result from low threshold setting on the Genie II instrument. The PC well should have T_{max} between 5 and 10 min and T_m around 90°C.

All sample DNA extracts with the correct T_m (approximately 90°C) and T_{max} values between 5-30 min are positive for *Salmonella*.

- c. As duplicate runs have been performed for each sample, when the sample has consistent results in both runs, final LAMP results can be reported. If two repeats generate inconsistent results, one more time repeat (duplicate runs independently) is recommended.

Figure 1. Examples of LAMP run information and results shown on the Genie II instrument panel when testing 10-fold serial dilutions of *S. enterica* serovar Infantis ATCC 51741 ranging from 1.1×10^6 to 11 CFU per reaction. PC is *S. Typhimurium* ATCC 19585 (LT2) at 1.7×10^4 CFU per reaction and NTC is molecular grade water.

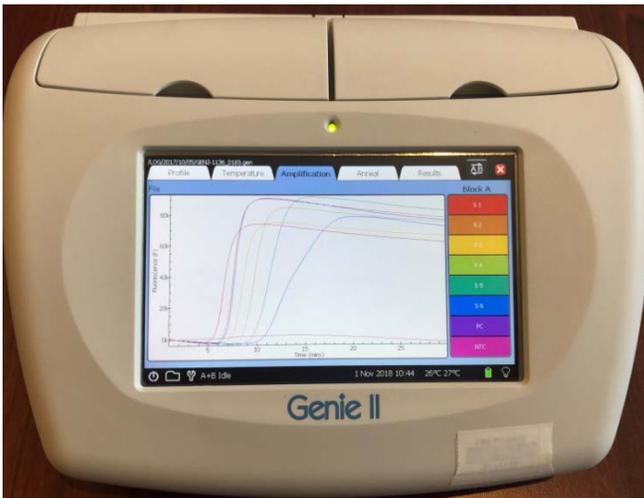
- a. The [Profile] tab



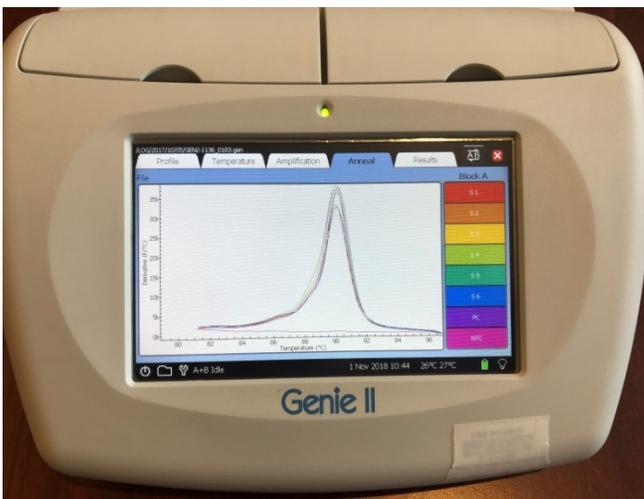
b. The [Temperature] tab



c. The [Amplification] tab



d. The [Anneal] tab



e. The [Results] tab

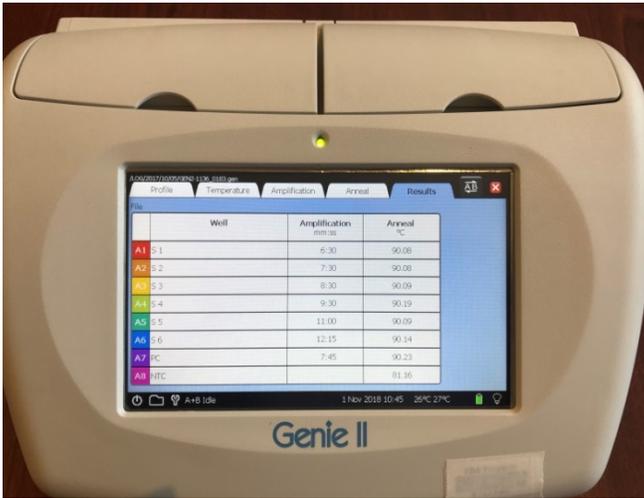
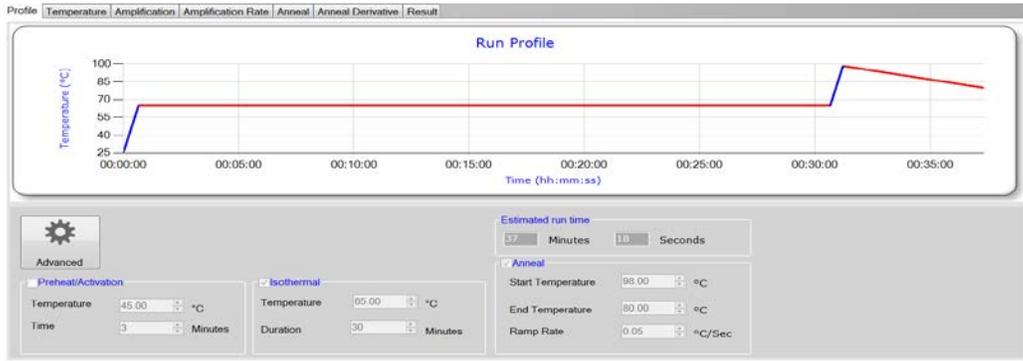
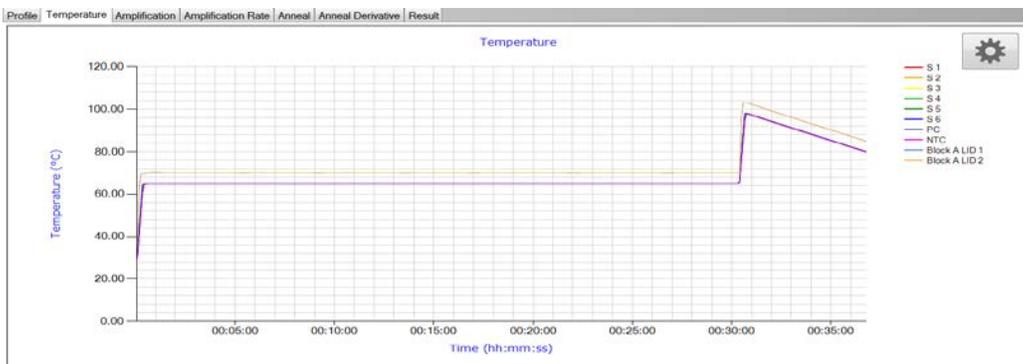


Figure 2. Examples of LAMP run information and results shown in the Genie Explorer software (for the same run as in Figure 1)

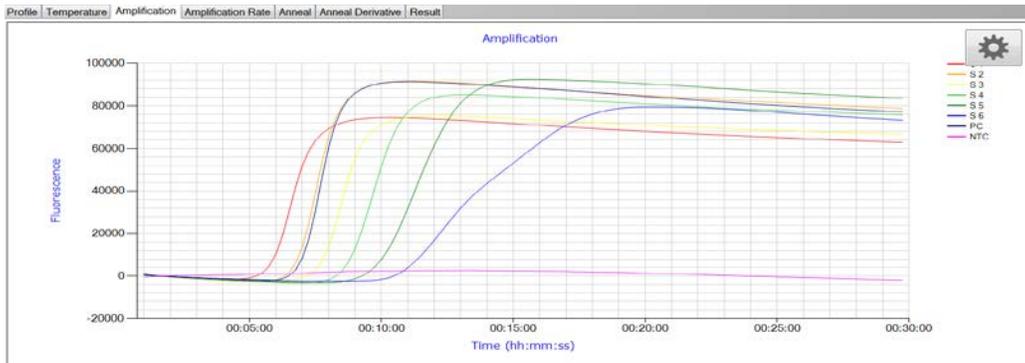
a. The [Profile] tab



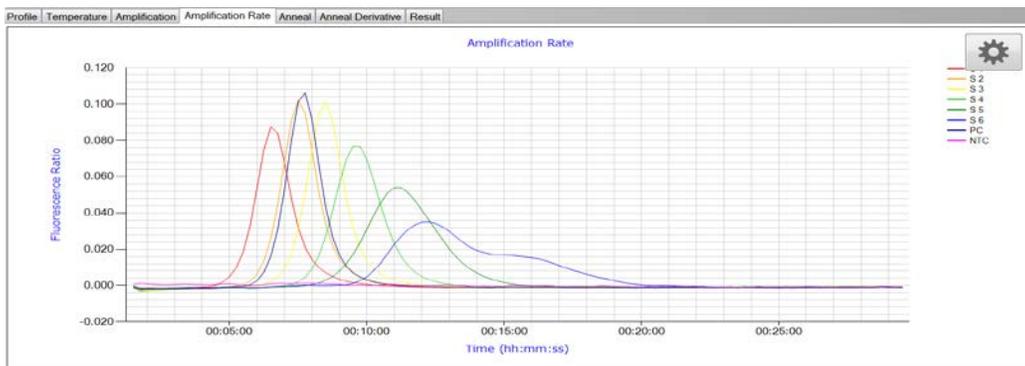
b. The [Temperature] tab



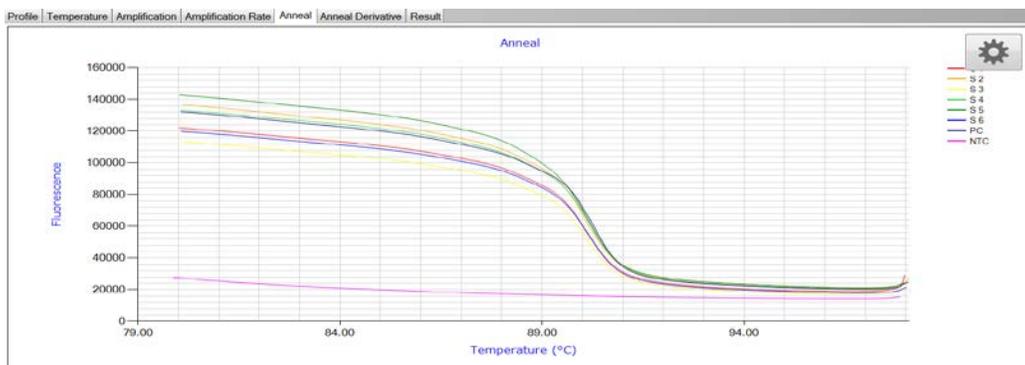
c. The [Amplification] tab



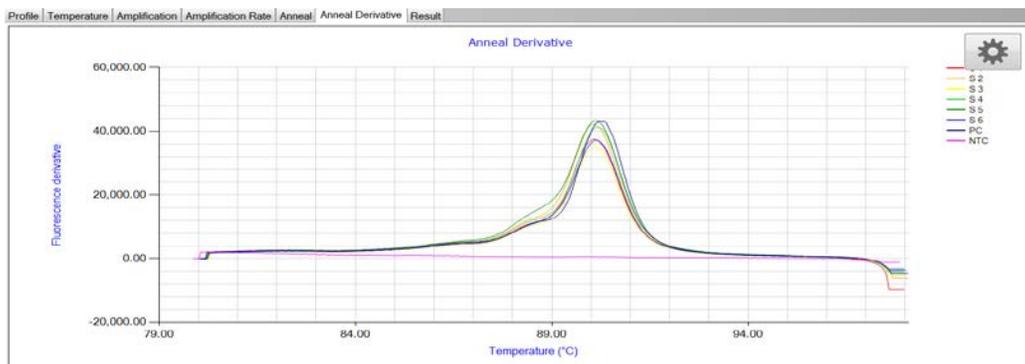
d. The [Amplification Rate] tab



e. The [Anneal] tab



f. The [Anneal Derivative] tab



g. The [Result] tab

Profile	Temperature	Amplification	Amplification Rate	Anneal	Anneal Derivative	Result
Graph Name				Well Number		Well Name
						Peak Value
						mm:ss
Amp Time				1		S 1
Amp Time				2		S 2
Amp Time				3		S 3
Amp Time				4		S 4
Amp Time				5		S 5
Amp Time				6		S 6
Amp Time				7		PC
Amp Time				8		NTC
						°C
Anneal Derivative				1		S 1
Anneal Derivative				2		S 2
Anneal Derivative				3		S 3
Anneal Derivative				4		S 4
Anneal Derivative				5		S 5
Anneal Derivative				6		S 6
Anneal Derivative				7		PC
Anneal Derivative				8		NTC

References

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