

## 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, 6-11).

LAMP assembly and data analysis protocols described below are for two instrument platforms: Genie II and Genie III. Contact: Beilei Ge, FDA-CVM ([Beilei.Ge@fda.hhs.gov](mailto:Beilei.Ge@fda.hhs.gov)). Use of other platforms and protocols must first be validated per FDA's Microbiological Method Validation Guidelines (12), AOAC's Appendix J (13) or ISO 16140-6 (14) and approved for use by FDA's Microbiology 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  $\pm 0.1^\circ\text{C}$  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  $\mu\text{l}$ ; OptiGene Ltd.) or other equivalent LAMP reaction tubes for selected instrument
3. Genie strip holder (OptiGene Ltd.) or equivalent
4. Microcentrifuge (capable of spinning at 16,000  $\times g$ ) and tubes (0.5 to 2 ml)
5. Pipettes (0.5 to 10  $\mu\text{l}$ , 2-20  $\mu\text{l}$ , 20-200  $\mu\text{l}$ , and 200-1000  $\mu\text{l}$ ) and tips (aerosol resistant)
6. Vortex mixer
7. Heat block capable of maintaining  $100 \pm 1^\circ\text{C}$

### B. Media and Reagents

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

LAMP primers (**Table 1**) are synthesized commercially with standard desalting purification. Prepare stock solutions of each primer (100  $\mu\text{M}$ ) by rehydrating the primer with appropriate amount of sterile molecular grade water. Mix well by vortexing for 10 s and store at  $-20^\circ\text{C}$  ( $-80^\circ\text{C}$  for long-term storage).

Prepare the primer mix (10 $\times$ ) 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  $\mu\text{l}$  per microcentrifuge tube and store at  $-20^\circ\text{C}$ .

**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 any *Salmonella* reference strains, 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  $24 \pm 2$  h at  $35 \pm 2^\circ\text{C}$ . Transfer several single colonies to 5 ml of [trypticase soy broth \(TSB\)](#) or [brain heart infusion \(BHI\) broth](#) and incubate  $16 \pm 2$  h at  $35 \pm 2^\circ\text{C}$  to reach *ca.*  $10^9$  CFU/ml. Serially dilute the overnight culture in 0.1% peptone water to obtain *ca.*  $10^7$  CFU/ml. Transfer

500 µl of this dilution to a microcentrifuge tube and heat 10 min at  $100 \pm 1^\circ\text{C}$  in a dry heat block. Cool to room temperature and store PC DNA at  $-20^\circ\text{C}$ .

b. Sterile molecular grade water is used as the NTC.

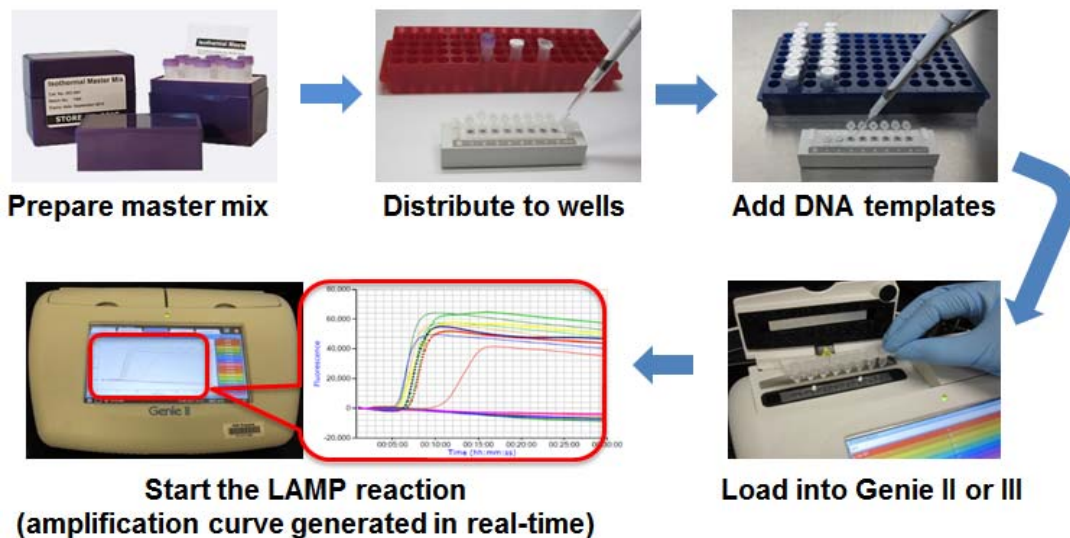
### C. DNA Extraction from Presumptive *Salmonella* Isolates

1. Inoculate presumptive *Salmonella* isolates on a nonselective agar plate and incubate  $24 \pm 2$  h at  $35 \pm 2^\circ\text{C}$ .
2. Transfer several single colonies to 5 ml of [TSB](#) or [BHI broth](#) and incubate  $16 \pm 2$  h at  $35 \pm 2^\circ\text{C}$ .
3. Transfer 500 µl of the overnight culture to a microcentrifuge tube and heat 10 min at  $100 \pm 1^\circ\text{C}$  in a dry heat block.
4. Cool to room temperature and store isolate DNA extracts at  $-20^\circ\text{C}$ .

### D. LAMP Assembly and Data Analysis

#### 1. Overview



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





#### 2. Preparation and run setup

**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.

- a. Clean bench with isopropanol and a DNA- and DNase-degrading solution such as DNA AWAY and clean pipettes and Genie strip holders with DNA AWAY.
- b. Thaw LAMP ISO-001 isothermal master mix, primer mix (10×), molecular grade water, PC *Salmonella* DNA, and isolate DNA extracts at room temperature.
- c. Turn on the Genie II or Genie III instrument and tap the opening screen to access the home screen.

Create a run by tapping [LAMP+Anneal] and selecting [Edit] to enter sample information. The default LAMP 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. Genie II has 2 blocks (A and B) with 8 samples in each block. Genie III has a single block that accommodates 8 samples. Tap each sample row to activate the cursor and enter relevant sample information, using the  icon to switch between the two Genie II blocks. Tap the  icon when all sample information has been entered.

NOTE: You may save the run setup (termed “profile,” which contains sample information along with the default LAMP run profile) for later use. Tap the  icon and give the profile a unique name. The next time you want to test this same set of samples, you may initiate a new run using this saved profile. Tap the  icon at the bottom left of the home screen and select [Profile] to load saved profiles.

### 3. LAMP reaction assembly

NOTE: A LAMP reaction mix contains DNA polymerase, buffer, MgSO<sub>4</sub>, 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, a total of 16 samples), 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 is recommended.

- a. 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 18 samples	Volume (μl) in master mix for 10 samples
ISO-001 isothermal master mix	1.67×	1×	15	270	150
Primer mix	10×	1×	2.5	45	25
Molecular grade water	N/A	N/A	5.5	99	55
Master mix subtotal	N/A	N/A	23	414	230
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**).

- b. Place the Genie strip in the Genie strip holder and distribute 23  $\mu\text{l}$  of the LAMP master mix to each well.
  - c. Vortex all DNA templates and centrifuge briefly. Add 2  $\mu\text{l}$  of DNA template to the appropriate well and cap tightly.
  - d. Remove the Genie strip from the holder and flick wrist to ensure all reagents have pooled at the bottom of the tube.
  - e. 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 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  $T_m$  ( $^{\circ}\text{C}$ ) is the melting/annealing temperature of the final amplified product.

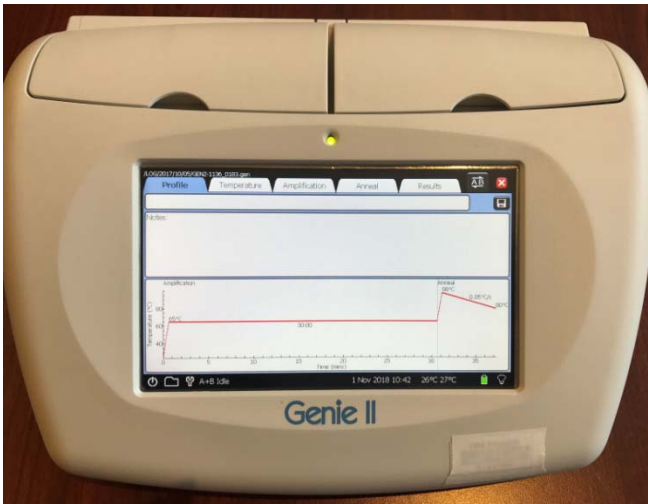
- a. Click on the  icon at the upper right of the screen and select the block(s) containing Genie strip(s) to start the LAMP run.
  - b. OPTIONAL: While the reaction is in progress, tap the [Temperature], [Amplification], and [Anneal] tabs to see dynamic changes of various parameters during the LAMP 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. OPTIONAL: 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 of Genie II or Genie III
- a. Tap the  icon at the bottom left of the home screen and select [log] to navigate to the file location to load 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 three columns (Wells, 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 ( $T_m$ ;  $^{\circ}\text{C}$ ) for any amplified product in that well.
  - d. Interpret the LAMP results as follows: The control wells are examined first. The NTC well should have blank  $T_{max}$  while  $T_m$  can be either blank (both Genie II and Genie III)

- or  $< 83^{\circ}\text{C}$  (Genie II only). The PC well should have  $T_{max}$  between 5 and 10 min and  $T_m$  around  $90^{\circ}\text{C}$ . All samples with the correct  $T_m$  (approximately  $90^{\circ}\text{C}$ ) and  $T_{max}$  (between 5-30 min) are considered positive for *Salmonella*.
- e. If the duplicate runs have consistent results, a 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.
6. Interpretation of LAMP results using the Genie Explorer software (version 2.0.6.3 or later)
- a. Click on the  icon on the left panel and navigate to the file location to load the LAMP run of interest.  
  
NOTE: The computer with the software installed does not need to be connected to the instrument to analyze LAMP results, i.e., remote access is available. The 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**). Click on the  icon at the top right of the screen and adjust the “Peak Detection Threshold Ratio” from 0.020 to 0.010 to ensure that all valid peaks are identified, and the results obtained using the software match with those displayed on the instrument panel.
  - 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 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” ( $T_m$ ;  $^{\circ}\text{C}$ ) for any amplified product in that well.
  - e. Interpret the LAMP results and decide when to repeat samples following the same rules used for the Genie II instrument panel (see **section D.5**) with the exception that the NTC well and other negative samples should have blank  $T_m$  as the Genie Explorer software settings eliminate those  $T_m < 83^{\circ}\text{C}$  results.
  - f. OPTIONAL: Generate a Genie experimental 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.

**Figure 1. Representative LAMP results displayed on the Genie II instrument panel.** In this LAMP run, samples S1 to S6 are 10-fold serial dilutions of *S. enterica* serovar Infantis ATCC 51741 ranging from  $1.1 \times 10^6$  CFU to 11 CFU per reaction. PC is *S. enterica* serovar Typhimurium ATCC 19585 (LT2) at  $1.7 \times 10^4$  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

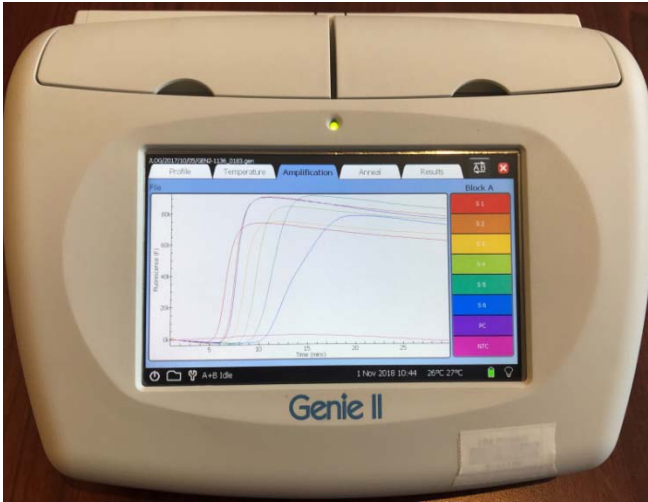


b. The [Temperature] tab

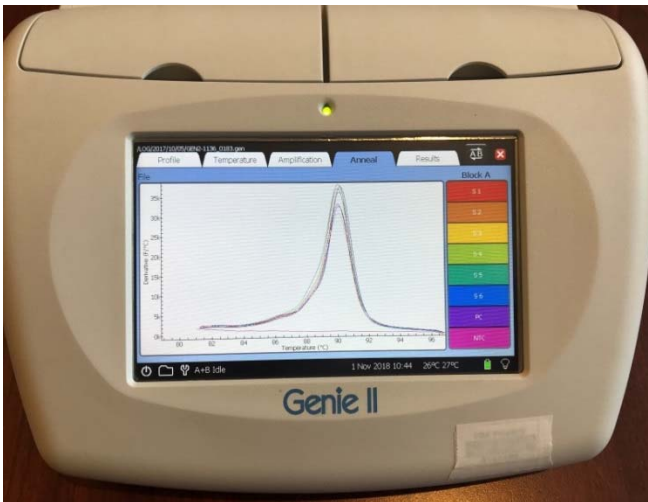




c. The [Amplification] tab



d. The [Anneal] tab



e. The [Results] tab

The screen displays the 'Results' tab, showing a table with the following data:

Well	Amplification (min:sec)	Anneal (°C)
A1 S.1	6:30	90.09
A2 S.2	7:30	90.09
A3 S.3	8:30	90.09
A4 S.4	9:30	90.19
A5 S.5	11:00	90.09
A6 S.6	12:15	90.14
A7 PC	7:45	90.23
A8 NTC		81.35

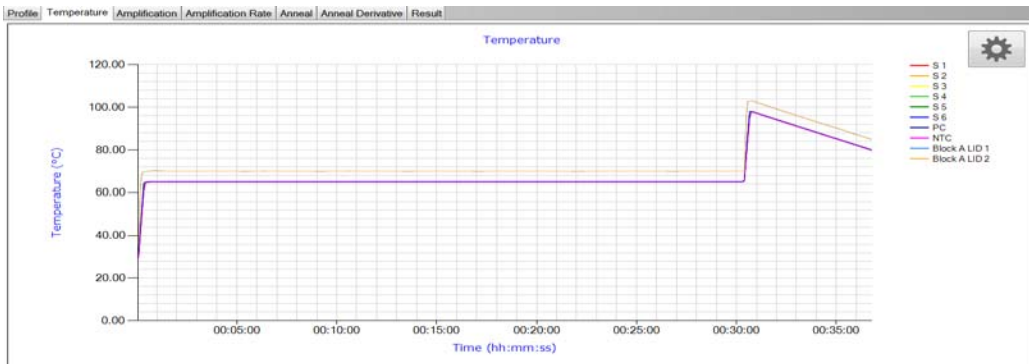


**Figure 2: Representative LAMP results viewed in the Genie Explorer software (version 2.0.6.3).** This is the same run as in **Figure 1**. (a) The [Profile] tab shows the programmed temperature profile. (b) The [Temperature] tab shows actual temperatures in the sample wells as LAMP reaction proceeds. (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.

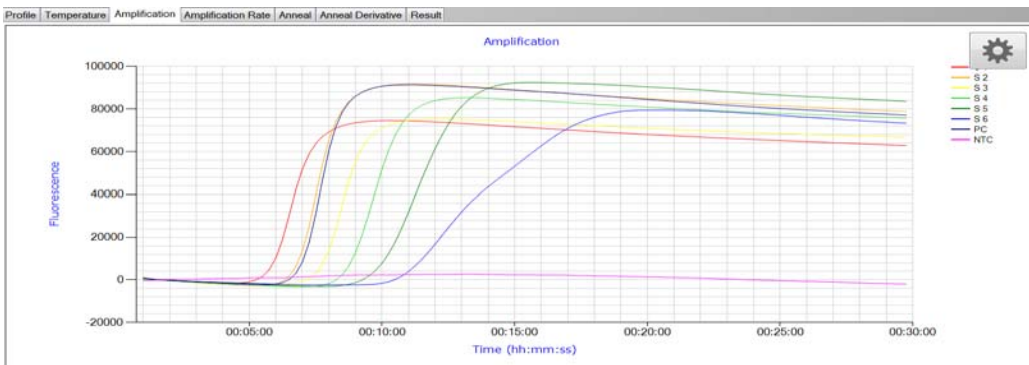
a. The [Profile] tab



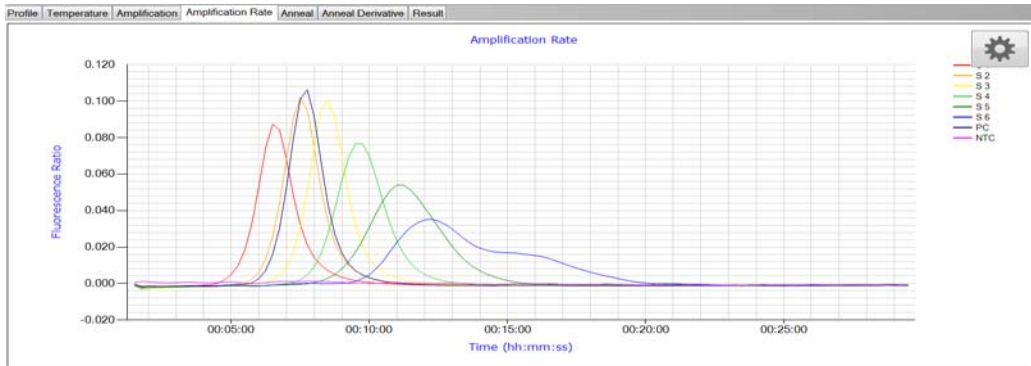
b. The [Temperature] tab



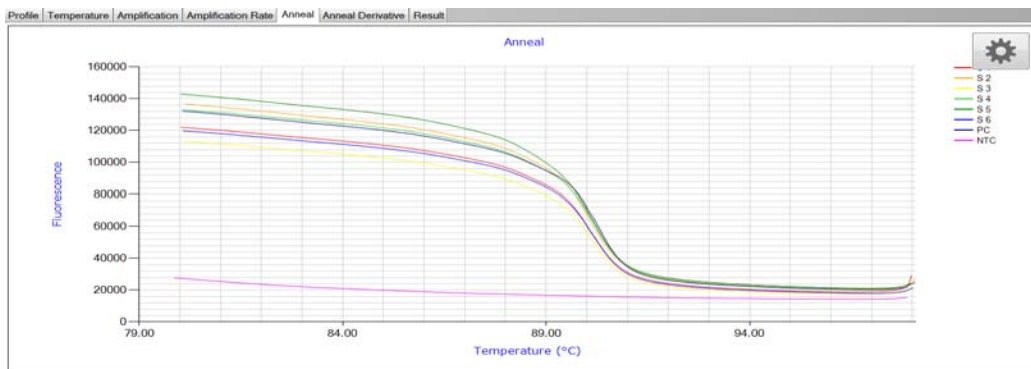
c. The [Amplification] tab



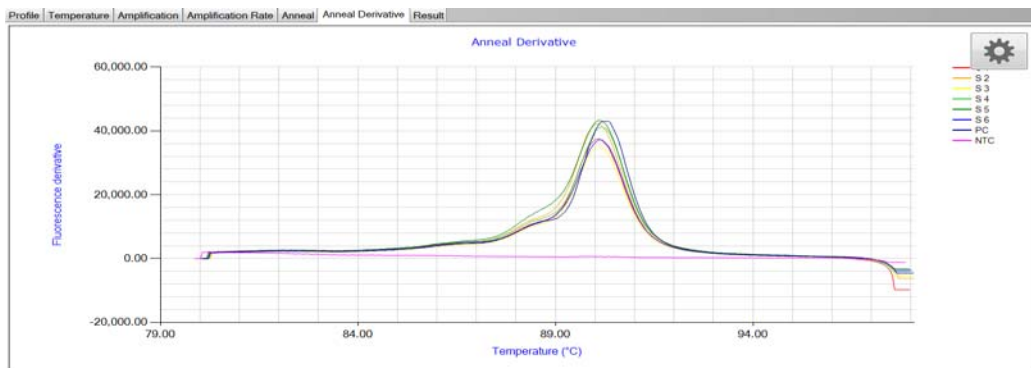
d. The [Amplification Rate] tab



e. The [Anneal] tab



f. The [Anneal Derivative] tab



g. The [Result] tab

Graph Name	Well Number	Well Name	Peak Value min:ss
Amp Time	1	S1	06:30
Amp Time	2	S2	07:30
Amp Time	3	S3	08:30
Amp Time	4	S4	09:30
Amp Time	5	S5	11:00
Amp Time	6	S6	12:15
Amp Time	7	PC	07:45
Amp Time	8	NTC	
Anneal Derivative	1	S1	90.1
Anneal Derivative	2	S2	90.1
Anneal Derivative	3	S3	90.1
Anneal Derivative	4	S4	90.2
Anneal Derivative	5	S5	90.1
Anneal Derivative	6	S6	90.1
Anneal Derivative	7	PC	90.3
Anneal Derivative	8	NTC	

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