AMA Salmonella Genotyping Real – Time qPCR Detection Kit

This product is intended for research use only (RUO)

Made in
SAUDI ARABIA
www.havensci.com

Table of content

A **Introduction and Principles of the Test**

B **Kit Contents and Storage**

C **Performance Evaluation**

Sensitivity
Specificity
Precision
Limit of detection (LoD)
PCR efficiency (singleplex vs multiplex)

D **Protocol**

Protocol for non-bacterial culture samples
Protocol for bacterial culture samples

E **Data Interpretation**

F **Support**

G **Quality Control**

Introduction and Principles of the Test

Salmonella is generally classified as follows:

- **Salmonella enterica**
  - Salmonella enterica arizonae
  - Salmonella enterica diarizonae
  - Salmonella enterica enterica
  - Salmonella enterica houtenae
  - Salmonella enterica indica
  - Salmonella enterica salamae
- **Salmonella bongori**

Each of these subspecies is further divided into multiple serovars. Of these, Salmonella enterica subsp. enterica serovar Enteritidis and Salmonella enterica subsp. enterica serovar Typhimurium are some of the most prevalent serovars that can also infect humans. Our fourplex, probe-based real-time qPCR assay is intended for detection of all known serovars of Salmonella, with the ability of distinguishing the Enteritidis and Typhimurium serovars, in less than 1 hour (with extracted DNA as the starting material).

The assay targets a 130bp-long segment of the bacterial spaQ gene, a highly conserved gene that is present only in Salmonella. It also targets a 155bp and 92bp segments of serovar Enteritidis and serovar Typhimurium, respectively. These two segments are highly conserved and are present only in their respective serovars.

Target	spaQ gene (Pan Salmonella)	Typhimurium (S. typhimurium)	Enteritidis (S. enteritidis)	Internal Control (IC)
Detection Channel	FAM	HEX	Cy5	ROX

Due to the variety of possible input samples (human, poultry, cell culture, etc.) a synthetic DNA spike-in is included in the kit to serve as the extraction internal control. This approach ensures that the extraction and amplification steps are accounted for, thus eliminating the possibility of false negatives due to erroneous extraction and/or amplification.

Kit Contents and Storage

The kit comes in a package of five tubes, as indicated in the following table. All components must be stored at -20°C. The qPCR Master Mix and AMA Oligo Mix should not be exposed to light for extended periods of time.

Component	Volume (uL)
qPCR Master Mix	1,000
AMA Oligo Mix	200
Negative Control	50
Positive Control	50
Extraction Internal Control (IC)	1,000

Sensitivity

As of December 31st, 2022, there were 1,207 full sequences for all Salmonella enterica and bongori species. The Pan Salmonella oligos in our assay have perfect complementarity to 99.23% of these sequences.
Furthermore, the Enteritidis-specific and Typhimurium-specific oligos have perfect complementarity to all published Enteritidis and Typhimurium full genomic sequences (146 and 193 sequences, respectively).

Specificity

In-silico analysis shows that the Enteritidis-specific and Typhimurium-specific oligos have virtually no cross reactivity with any other Salmonella serovar.
Furthermore, our in-silico analysis shows that our assay has no cross-reactivity with any of the organisms listed in the table below.

Organism Name (NCBI TaxID)		
Homo sapiens (taxid:9606)	Gallus gallus domesticus (taxid:9031)	Campylobacter (taxid:194)
Bacillus (taxid:1386)	Clostridium (taxid:1485)	Bacillus/Clostridium group (taxid:1239)
Staphylococcus aureus (taxid:1280)	Escherichia coli (taxid:562)	Escherichia coli O157:H7 (taxid:83334)
Legionella (taxid:445)	Cronobacter (taxid:413496)	Yersinia enterocolitica (taxid:630)
Legionella pneumophila (taxid:446)	Listeria (taxid:1637)	Listeria monocytogenes (taxid:1639)
Shigella (taxid:620)	Shigella flexneri (taxid:623)	Yersinia (taxid:629)

Precision

Positive and negative samples of salmonella (serovars Arizonae, Enteritidis, and Typhimurium) were each run at least 5 different times using our assay. The samples were called correctly 100% of the times.

Limit of detection (LoD)

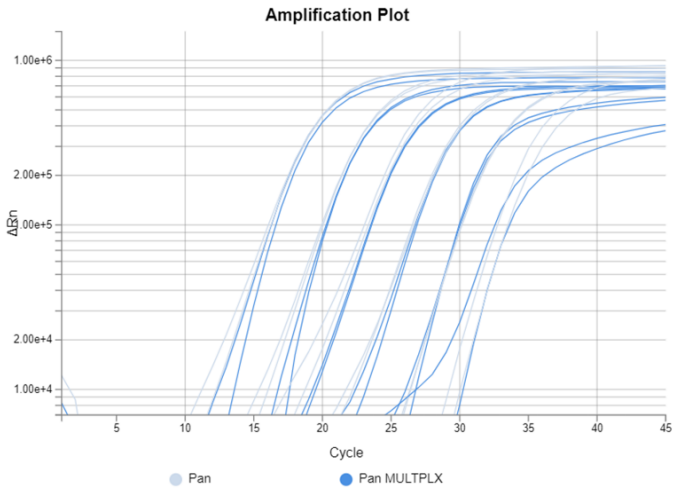
Salmonella samples were diluted to a final concentration of 1,000 copies/µL (Salmonella serovar enteritidis, Salmonella serovar typhimurium, and Salmonella enterica arizonae). From these, further serial dilutions (with equal amounts of the IC spiked in) showed that our assay has the following limit of detection for each target:

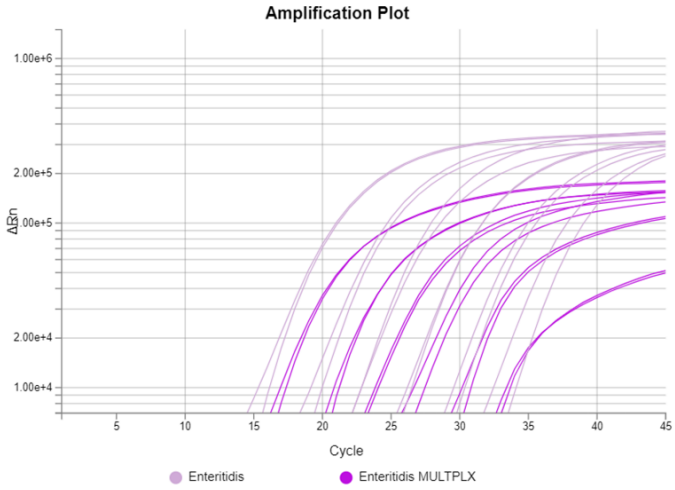
Target (sample used)	Pan-Salmonella (Salmonella enterica arizonae)	Enteritidis-specific (Salmonella serovar enteritidis)	Typhimurium-Specific (Salmonella serovar typhimurium)
Limit of Detection	8 Copies/reaction	40 Copies/reaction	8 Copies/reaction

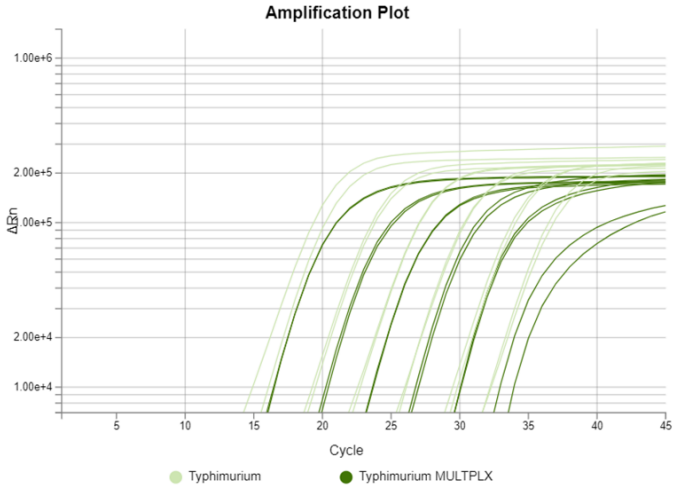
PCR efficiency (singleplex vs multiplex)

To analyze the effect of multiplexing the four targets in our assay, we measured the PCR efficiency for each target before and after multiplexing. Reactions were carried out with a serially diluted mixture of Salmonella serovar enteritidis and Salmonella serovar typhimurium from 5 million copies of each serovar per reaction down to 50 copies/reactions. The dilution factor was 10X (six dilution points). The dilutions contained equal amounts of the synthetic Internal Control to account for the activity of the fourth IC target. The results are listed in the table and figure below.

Assay		R^2	Slope	PCR Efficiency
Pan-Salmonella	Singleplex	0.998	-3.392	97.2%
	Multiplex	0.997	3.268	102.3%
Enteritidis-specific	Singleplex	0.996	3.483	93.7%
	Multiplex	0.997	3.256	102.8%
Typhimurium-Specific	Singleplex	0.997	3.364	98.3%
	Multiplex	0.998	3.351	98.8%


Amplification Plot
1.00e+6
2.00e+5
1.00e+5
2.00e+4
1.00e+4
ΔRn
5
10
15
20
25
30
35
40
45
Cycle
Pan
Pan MULTPLX


Amplification Plot
1.00e+6
2.00e+5
1.00e+5
2.00e+4
1.00e+4
ΔRn
5
10
15
20
25
30
35
40
45
Cycle
Enteritidis
Enteritidis MULTPLX


Amplification Plot
1.00e+6
2.00e+5
1.00e+5
2.00e+4
1.00e+4
ΔRn
5
10
15
20
25
30
35
40
45
Cycle
Typhimurium
Typhimurium MULTPLX

Protocol for non-bacterial culture samples

In this protocol, the Extraction Internal Control will be added to the sample during extraction, serving as a control for both the extraction and amplification steps.

1. Extract DNA or total nucleic acids from each sample as in your laboratory's approved internal protocol. However, during bacterial DNA extraction, mix 10 µL of the Extraction Internal control (IC) provided in the kit with each sample **AFTER** adding the lysis buffer. The extracted nucleic acid should be used immediately after extraction (within 30 minutes) or stored at -20°C immediately after extraction.

2. Thaw all components at room temperature then place them on ice. Mix each tube by gentle flicking followed by a quick spin down to collect the tube's component to the bottom of the tube. **Do NOT exceed 3 freeze-thaw cycles!**

3. Prepare the reaction master mix by mixing the following components (5% overage should be included to account for pipetting errors):
   a. **10 µL of qPCR Master Mix / reaction**
   b. **2 µL of the AMA Oligo Mix / reaction**

*Example

Component	Per 1 reaction	X10 reactions (+5% overage)
qPCR Master Mix	10 µL	105 µL
AMA Oligo Mix	2 µL	21 µL

NOTE: Don't forget to figure in the positive and negative controls in your calculations!

4. Transfer 12 µL of the reaction master mix (prepared in step3) into each designated well on a 96-well optical qPCR plate.

5. Add 8 µL of each sample's extracted nucleic acid to its designated well; add 8 µL of the positive and negative controls to their designated wells.

6. Seal the plate with optical cap strips or an optical adhesive seal.

7. On the Real-Time PCR thermal cycle software, define the detection filters as follows:

Target	Pan Salmonella	Typhimurium	Enteritidis	IC
Detection Channel	FAM	HEX	Cy5	ROX

8. On the software, define your Positive and Negative Controls.

9. Run the Real-Time PCR thermal cycler for 40 cycles as follows:

Step1: 2 minutes - 95°C (initial denaturation)

Step2: 10 seconds - 95°C (denaturation)

Step3: 30 seconds - 60°C (annealing/extension) → DATA COLLECTION

Step4: go to step2 (X39 times)

10. Analyze the results according to your thermal cycler's software. Use your judgement to set/modify the threshold of detection for each filter and/or omit amplification artifacts (e.g. non-sigmoidal amplification curve).

Protocol for bacterial culture samples

Bacterial cultures contain considerable amounts of DNA. Since high amounts of DNA are known to inhibit PCR, samples must be diluted before undergoing the test.
In this protocol, the Extraction Internal Control will be added to the sample after extraction, serving as a control for the amplification step, but not for the extraction step. Alternatively, extraction will be assessed either spectrophotometrically by Nanodrop or fluorescently by Qubit.

1. **Extract DNA or total nucleic acids from each sample as in your laboratory's approved internal protocol. No need to add the Extraction Internal control (IC) provided in the kit. The extracted nucleic acid should be used immediately after extraction (within 30 minutes) or stored at -20°C immediately after extraction.**

2. **Measure the concentration of your DNA with Nanodrop, Qubit, or equivalent. Adjust the sample concentration to ~5-10ng/µL. Dilute your sample 1,000–10,000 fold.**

3. **Thaw all components at room temperature then place them on ice. Mix each tube by gentle flicking followed by a quick spin down to collect the tube's component to the bottom of the tube. Do NOT exceed 3 freeze-thaw cycles!**

4. **Prepare the reaction master mix by mixing the following components (5% overage should be included to account for pipetting errors):**
   a. **10 µL of qPCR Master Mix / reaction**
   b. **2 µL of the AMA Oligo Mix / reaction**
   c. **1µL of the IC/ reaction**

*Example

Component	Per 1 reaction	X10 reactions (+5% overage)
qPCR Master Mix	10 µL	105 µL
AMA Oligo Mix	2 µL	21 µL
IC	1 µL	10.5 µL

NOTE: Don't forget to figure in the positive and negative controls in your calculations!

5. Transfer 13 µL of the reaction master mix (prepared in step3) into each designated well on a 96-well optical qPCR plate.

6. Add 8 µL of each sample's extracted nucleic acid to its designated well; add 8 µL of the positive and negative controls to their designated wells.

7. Seal the plate with optical cap strips or an optical adhesive seal.

8. On the Real-Time PCR thermal cycle software, define the detection filters as follows:

Target	Pan Salmonella	Typhimurium	Enteritidis	IC
Detection Channel	FAM	HEX	Cy5	ROX

9. On the software, define your Positive and Negative Controls.

10. Run the Real-Time PCR thermal cycler for 40 cycles as follows:

- **Step1:** 2 minutes - 95°C (initial denaturation)
- **Step2:** 10 seconds - 95°C (denaturation)
- **Step3:** 30 seconds - 60°C (annealing/extension) → DATA COLLECTION
- **Step4:** go to step2 (X39 times)

11. Analyze the results according to your thermal cycler's software. Use your judgement to set/modify the threshold of detection for each filter and/or omit amplification artifacts (e.g. non-sigmoidal amplification curve).

Sample calling

For the three Salmonella targets, the acceptable cycle threshold of detection is Ct <40. If only the Pan target is detected, Salmonella infection is positive (non-Enteritidis/non-Typhimurium). Enteritidis or Typhimurium infection is positive if the serovar-specific target is detected along with the Pan target. If only the serovar-specific target is detected, serovar-specific infection is presumptive positive, but re-extracting DNA and retesting may be necessary.

If all of the Enteritidis-specific, Typhimurium-specific, and Pan targets are detected, there is co-infection of Enteritidis and Typhimurium. If the serovar-specific targets are detected without the Pan target, co-infection is presumptive, but we recommend re-extracting DNA and retesting.

If none of the three Salmonella targets is detected, the sample is true negative only if the cycle threshold of the Internal Control was ≤35. Otherwise, there may be a problem in the extraction step, or the sample may be contaminated with PCR inhibitors. In this case, re-extracting DNA and retesting is necessary.

The following table summarizes our sample calling recommendations. A positive (+) sign indicates a Ct < 40 whereas a negative (–) sign indicates no amplification.

	PAN (FAM) CT	TYPHIMUR (HEX) CT	ENTERIT (CY5) CT	IC (ROX) CT	INTERPRETATION
CASE 1	+	-	-	Any	Salmonella infection
CASE 2	+	+	-	Any	Typhimurium infection
CASE 3	+	-	+	Any	Enteritidis infection
CASE 4	+	+	+	Any	Enteritidis/Typhimurium co-infection
CASE 5	-	+	-	Any	Presumptive Typhimurium infection; retest
CASE 6	-	-	+	Any	Presumptive Enteritidis infection; retest
CASE 7	-	+	+	Any	Presumptive Enteritidis / Typhimurium co-infection; retest
CASE 8	-	-	-	≤35	No infection
CASE 9	-	-	-	>35	Extraction failed; re-extract the sample

Run validity

For the run to be valid, the Negative Control must return a negative infection result, whereas the Positive Control must return a positive co-infection result (see sample calling above). If not, the whole run is considered invalid.

Support

For questions, suggestions, or technical support, feel free to contact us by email on: support@havensci.com

Quality control

Each lot is tested against predetermined parameters to ensure consistent performance among all lots.

HavenSci

AMA Salmonella Genotyping Real – Time qPCR Detection Kit

Product Manual (PD780)
Version 2.0
www.havensci.com