**Chapter 4**

# Results obtained in the process of standardization of a real time PCR for the *Escherichia coli* quantification and *Salmonella* spp detection

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The results will be presented according to the methodology developed; therefore, first will be described the results of concentration and bacterial elution process standardization for *Escherichia coli* DNA extraction from potable water and seawater samples. The second section will aboard the results of the standard curve development process for *Escherichia coli* quantification by real-time PCR and finally the standardization results of the *Salmonella* spp. detection method by real-time PCR in drinking water and sea water.

## CONCENTRATION AND BACTERIAL ELUTION TECHNIQUES STANDARDIZATION FOR DIRECT DNA EXTRACTION FROM HUMAN DRINKING WATER AND SEA WATER

### Concentration methods

About the concentration method, according to the obtained results, it was evidenced that the filtration method is the most adequate for bacterial concentration, since as shown in table 4.1, 82 % of the inoculated bacterial load was recovered, unlike of the centrifugation that under the conditions evaluated only recovered 1 % of the inoculated bacterial load.

**Table 4.1.**
**Concentration methods evaluation**

| Method | Dilution | Count (cfu) | Inoculated volume (µl) | % recovery |
|---|---|---|---|---|
| Filtration | 10-6 | 386 | 500 | 82 % |
| Centrifugation | 10-6 | 9 | 900 | 1.0 % |

### Elution methods

Within evaluated elution strategies, the first one was the type of buffer with different vortexting times, which, according to the obtained results, which are shown in figure 4.1, a greater cell count was obtained with the glycine buffer when used for five minutes, because in three minutes time the number of CFUs is lower. With the PBS buffer at different pH and time there was not a very noticeable difference in cell recovery and was always much lower than the count obtained with the glycine buffer for five minutes.

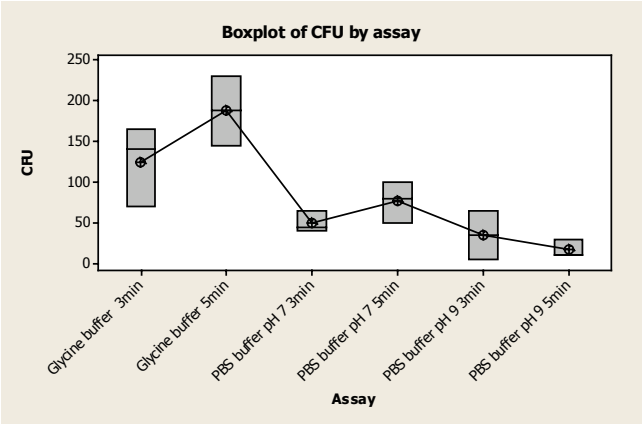


**Figure 4.1.**
**Evaluation of different vortex times and buffers**

In relation to the use of different incubation times evaluation of the filter submerged in PBS buffer at different pH results, an increase in the number of cells with respect to the inoculated was evidenced, which was more noticeable with the use of the PBS buffer at pH 7 as shown in figure 4.2.

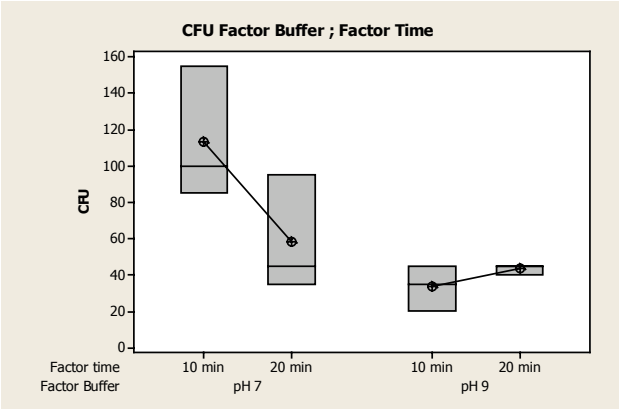


**Figure 4.2.**
**Evaluation of different incubation and buffering times**

## Mechanical elution

Within the strategies of mechanical elution according to the preliminary

tests, with zirconia beads use to separate the cells of the filter was obtained good purity DNA, but in low concentration as evidenced in table 4.2.

**Table 4.2.**
**Mechanical elution with zirconia beads**

| Method | Replicate | Concentration (ng/µl) | Purity (260/280) |
|---|---|---|---|
| Zirconia Beads | 1 | 5.6 | 1.89 |
| | 2 | 5.4 | 2.04 |
| Zirconia Beads Control | 1 | 1.8 | 1.77 |
| | 2 | 2.4 | 1.41 |

On the other hand, filter trituration allowed obtaining a greater DNA amount that was able to be amplified by real time PCR, evidencing that with the use of the glycine buffer better results were obtained as presented in table 4.3. Also the filter cutting allowed obtaining greater quantity of DNA, but nevertheless the obtained Ct value in real time PCR was greater than with the filter trituration; these data are shown in table 4.3.

**Table 4.3.**
**Mechanical elution evaluation by filter trituration and cutting**

| Protocol | Buffer type | Replicate | Purity (260/280) | Ct | Average |
|---|---|---|---|---|---|
| Filter trituration | Glycine | 1 | 2.2 | 35.45 | 36.04 |
| | | 2 | 2.19 | 36.91 | |
| | | 3 | 2.2 | 35.78 | |
| | PBS | 1 | 1.45 | 36.5 | 36.63 |
| | | 2 | 2.26 | 36.77 | |
| | | 3 | - | NO PCR | |
| Filter cutting | Glycine | 1 | 2.11 | 36.04 | 36.89 |
| | | 2 | 2.11 | 36.76 | |
| | | 3 | 2.12 | 37.87 | |
| | PBS | 1 | | NO PCR | NA |
| | | 2 | | | |
| | | 3 | | | |

## Protocols testing and optimization

The assay results with the use of filtration and elution with filter trituration in glycine buffer inoculated with different CFUs showed that there is

no variation in DNA concentrations and obtained Ct values by varying the amount of inoculated CFU as is shown in table 4.4.

The results of the seven replicates inoculated with 56 CFU in drinking water are shown in table 4.5; it is shown that amplification was achieved with a low microbial load with a percentage of variance between Ct values of 12,6 %. The DNA concentrations were in the order of 10 ng/µL and the purity was maintained at a constant value between the replicates.

**Table 4.4.**
**Protocols verification and optimization**

| CFU | Replicate | DNA concentration (ng/µl) | Purity(260/280) | Ct | Average |
|---|---|---|---|---|---|
| 50 | 1 | No | No | No PCR | 34.03 |
| | 2 | 18.2 | 1.87 | 33.49 | |
| | 3 | 12.85 | 1.99 | 34.57 | |
| 100 | 1 | 11.2 | 1.91 | No PCR | 33.4 |
| | 2 | 6.15 | 1.64 | 33.92 | |
| | 3 | 11.45 | 1.92 | 32.88 | |
| 500 | 1 | 15.5 | 1.98 | 33.68 | 33.63 |
| | 2 | 13 | 1.92 | 33.59 | |
| | 3 | 19.3 | 1.82 | No PCR | |
| 1000 | 1 | 12.7 | 1.74 | 33.68 | 32.69 |
| | 2 | 10.75 | 1.64 | No PCR | |
| | 3 | 18.7 | 1.83 | 31.70 | |

**Table. 4.5.**
**Human drinking water verification assay results**

| Replicate | Concentration (ng/µl) | Purity (260/280) |
|---|---|---|
| 1 | 11.7 | 1.74 |
| 2 | 6.2 | 1.70 |
| 3 | 8.35 | 1.56 |
| 4 | 9 | 1.46 |
| 5 | 7.15 | 1.56 |
| 6 | 12.95 | 1.77 |
| 7 | 17.05 | 1.73 |
| AVERAGE | 10.34 | 1.64 |
| Std. Dev. | 3.802 | 0.11 |
| VC % | 36.8 % | 7.2 % |
| Confidence level 95 % | 3.51 | 0.10 |
| Upper limit | 13.85 | 1.75 |
| Lower limit | 6.82 | 1.53 |

On the other hand, the amplifications with sea water were achieved in the seven replicates using a DNA sample of 10 ng/mL with a percentage of variance of 2.7 %; however, when utilizing less diluted samples, in the case of the R2 and R4 replicate, the Ct value decreases without inhibition by any sea water component. The obtained DNA concentrations were higher than in drinking water and the purities above 2.0; results are shown in table 4.6.

**Table. 4.6.**
**Sea water verification assay results**

| Replicate | Concentration (ng/µl) | Purity (260/280) |
|---|---|---|
| 1 | 162.2 | 2.1 |
| 2 | 175.95 | 2.05 |
| 3 | 203.25 | 2.10 |
| 4 | 228 | 2.1 |
| 5 | 328.5 | 2.8 |
| 6 | 311.5 | 2.1 |
| 7 | 177.5 | 2.1 |
| 2 (20 ng/µL) | ---- | ---- |
| 2 (50 ng/µL) | ---- | ---- |
| 4 (20 ng/µL) | ---- | ---- |
| 4/2 (50 ng/µL) | ---- | ---- |
| AVERAGE | 226.70 | 2.19 |
| Std. Dev. | 67.41 | 0.27 |
| VC % | 0.30 | 0.12 |
| Confidence level 95 % | 62.34 | 0.25 |
| Upper limit | 289.04 | 2.44 |
| Lower limit | 164.36 | 1.94 |

## STANDARD CURVE DEVELOPMENT FOR *E. coli* QUANTIFICATION IN WATER SAMPLES

### Preliminaries *E. coli* standard curve results

The first results obtained from the calibration curve were performed using both *Salmonella* spp. and *Escherichia coli*. In the case of *Salmonella* spp. it is shown in table 4.7 that obtained DNA amount does not vary significantly when using different bacterial counts at each dilution.

**Table 4.7.**
**First assay of preliminary *Salmonella* spp standard curve**

| Dilution | CFU | Average CFU | DNA conc. (ng/µl) | DNA aveg conc. (ng/µl) |
|---|---|---|---|---|
| -6 | 636 | 685.3 | 2.5 | 4.2 |
| | 664 | | 5.05 | |
| | 756 | | 5.15 | |
| -6.5 | 356 | 374.0 | 1.7 | 2.6 |
| | 384 | | 2.35 | |
| | 382 | | 3.7 | |
| -7 | 324 | 338.7 | 4.7 | 4.7 |
| | 316 | | 4.35 | |
| | 376 | | 4.9 | |
| -7.5 | 100 | 127.3 | 2.5 | 7.5 |
| | 156 | | 16.9 | |
| | 126 | | 3.2 | |
| -8 | 39 | 41.7 | 3.7 | 3.2 |
| | 47 | | 1.95 | |
| | 39 | | 4 | |
| -8.5 | 18 | 14.3 | 5.85 | 5.1 |
| | 13 | | 4.8 | |
| | 12 | | 4.5 | |
| -9 | 1 | 0.7 | 5.35 | 5.1 |
| | 0 | | 5.35 | |
| | 1 | | 4.65 | |

In the second preliminary assay with *Salmonella* spp. DNA concentration variation in the first dilutions is observed; however, from dilution $10^{-3}$ no difference between the obtained DNA concentrations was perceived, even in some dilutions its detection was not possible, as shown in table 4.8.

**Table 4.8.**
**Second assay of preliminary *Salmonella* spp standard curve**

| Dilution | CFU | Average CFU | DNA conc. (ng/µl) | DNA aveg conc. (ng/µl) |
|---|---|---|---|---|
| -0 | - | - | 590.3 | 584.05 |
| | | | 577.8 | |
| -1 | - | - | 64.2 | 64.55 |
| | | | 64.9 | |
| -2 | - | - | 1.7 | 0.95 |
| | | | 0.2 | |
| -3 | - | - | 2 | 1.65 |
| | | | 1.3 | |
| -4 | - | - | N | 0.02 |
| | | | 0.02 | |
| -5 | - | - | N | N |
| | | | N | |
| -6 | 348 | 358 | 0.4 | 0.3 |
| | 368 | | 0.2 | |
| -7 | 31 | 35 | 2 | 1.85 |
| | 39 | | 1.7 | |
| -8 | 3 | 4 | - | - |
| | 5 | | | |

In the second preliminary assay using *E. coli*, real-time PCR amplifications were performed with the extracted DNA, linearity being observed in the first dilutions even though that DNA concentrations did not vary significantly, but from dilution $10^{-7}$ linearity is lost because of having lower Ct values, which is shown in table 4.9 and figure 4.3.

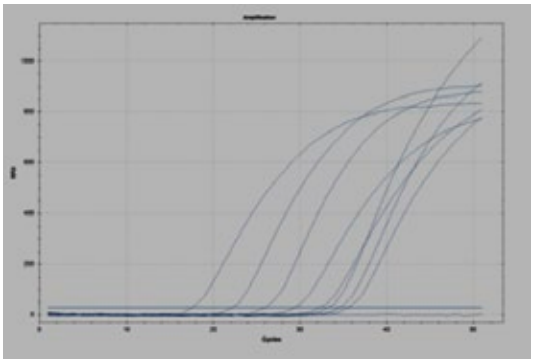

**Figura. 4.3.**
**E. coli second preliminary assay amplification**

**Table 4.9.**
**Standard E. coli curve second assay results**

| Dilution | CFU | Average CFU | DNA conc. (ng/µl) | | Ct* |
|---|---|---|---|---|---|
| -2 | - | - | R1 | 5.5 | 22.20 |
| | | | R2 | 5.25 | |
| | | | R3 | 4.45 | |
| -3 | - | - | R1 | 1.95 | 26.01 |
| | | | R2 | 5.15 | |
| | | | R3 | 5.95 | |
| -4 | - | - | R1 | 4.6 | 29.39 |
| | | | R2 | 2.8 | |
| | | | R3 | 2.8 | |
| -5 | - | - | R1 | 9.8 | 32.27 |
| | | | R2 | 7.8 | |
| | | | R3 | 3 | |
| -6 | 113 | 110 | R1 | 2.9 | 35.49 |
| | 108 | | R2 | 2.75 | |
| | | | R3 | 4.4 | |
| -7 | 10 | 10 | R1 | 3.8 | 34.52 |
| | 11 | | R2 | 4.1 | |
| | | | R3 | 5.7 | |
| -8 | 10 | 6 | R1 | 8.65 | 33.23 |
| | 8 | | R2 | 5.55 | |
| | | | R3 | 4.8 | |

**Correspond to replica number one*

## Definitive *E. coli* standard curve results

*E. coli* final curve analysis was performed with the results of the seven replicates; four curves were elaborated according to the points included in each one of them with the objective of selecting the most adequate one according to $R^2$ value and to the efficiency of the curve. Figure 4.4 shows that the best efficiency was obtained in the six-point curve with a value of 95.71 %, and a $R^2$ of 0.98. It was not possible to use the nine-point curve because it has an $R^2$ inferior to 0.9. In all tests, linearity is lost below a bacterial density of 1000 CFU with an average Ct value of 31.11.

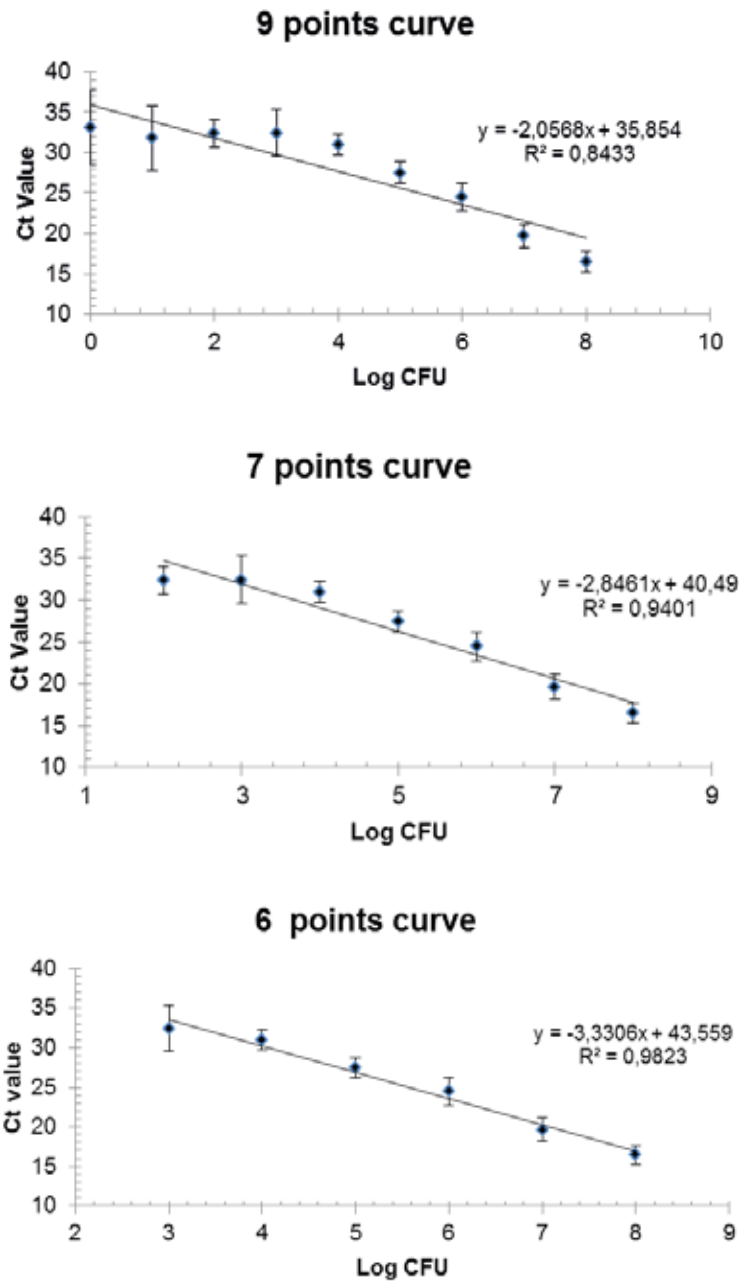


***Figure 4.4 Standard curves for E.coli quantification***

## METHOD DEVELOPMENT FOR SALMONELLA SPP. DETECTION BY REAL TIME PCR IN WATER HUMAN DRINKING WATER AND SEA WATER

### Preliminary assays

The first of the preliminary test results is shown in figure 4.5, where *invA* gene amplifications obtained by conventional PCR from a 24-hour pre-enrichment culture extracted DNA of one inoculated water sample with both 95 CFU and 10 CFU in 1L of water.

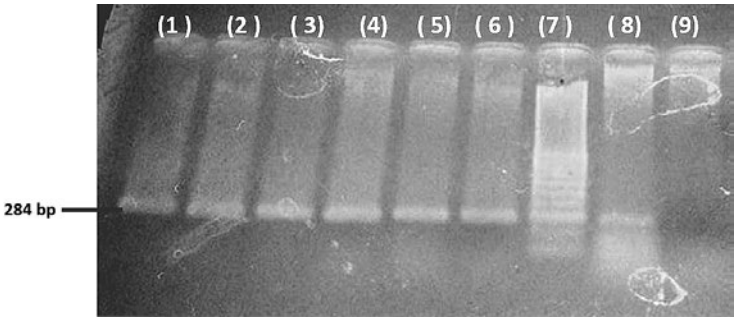


**Figure 4.5.**
**Amplification assay results: Lane 1: Replica 1 with 95 CFU/100 mL. Lane 2. Replica 2 with 95 CFU/100 mL. Lane 3. replica 3 with 95 CFU/100 mL. Lane 4. Replica 1 with 10 CFU/100 mL. Lane 5. Replica 2 with 10 CFU/100 mL. Lane 6. replica 3 with 10 CFU/100 mL. Lane 7. Molecular weigth marker, Lane 8. Positive control. Lane 9. Negative control**

As well, amplification of all tap water samples from the second test was achieved, those inoculated with 327 CFU and those inoculated with 9.5 CFU. The *invA* gene could be amplified also in the group of 9.5 CFU, including those in which DNA extraction was performed with the commercial kit, which are shown in figure 4,6 as lighter bands than those obtained with the DNA extracted by the organic method.

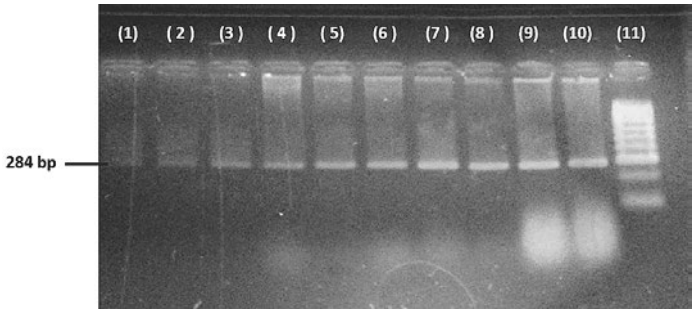


**Figure 4.6.**
**Amplification assay 2 results. Lane 1. 9.5 CFU with kit replica 1. Lane 2. 9.5 CFU with kit replica 2. Lane 3. 9.5 CFU with kit replica 3. Lane 4. 9.5 CFU with organic extraction method replica 1. Lane 5. 9.5 CFU with organic extraction method replica 2. Lane 6. 9.5 CFU with organic extraction method replica 3. Lane 7. 327 CFU with organic extraction method replica 1. Lane 8. 327 CFU with organic extraction method replica 2. Lane 9. 327 CFU with organic extraction method replica 3. Lane 10. Control (+ ). Lane11. Molecular weight marker**

In preliminary assay 3 was achieved the amplification in the first and third replicates of the 100 mL sea water assay and in all replicates in the 1L assay, but in both cases the bands obtained on the electrophoresis were very tenuous. The amplifications of the 100 mL assay are shown in figure 4.7.

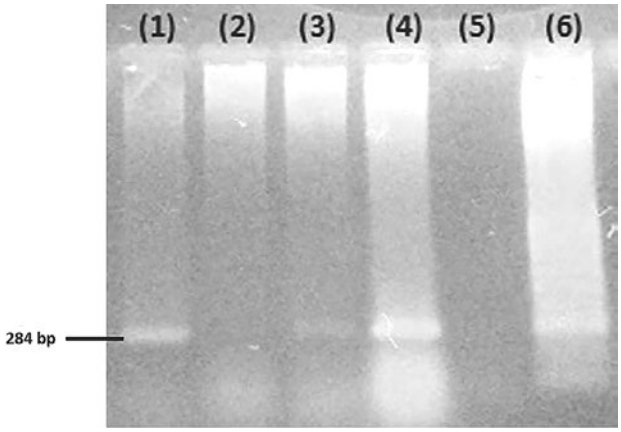


**Figure 4.7.**
**Amplification assay results with 100 mL of sea water. Lane 1. Replica 1. Lane 2. Replica 2, Lane 3. Replica 3. Lane 4. Positive control. Lane 5.**

**Negative control. Lane 6. Molecular weight marker**

## Verification test of the *Salmonella* spp. by real-time PCR detection method

After the preliminary tests confirmed by conventional PCR, the tests were performed with real-time PCR, this way DNA extraction was verified for techniques, the commercial kit and the organic method; greater amounts of DNA were obtained with the use of the organic method; however, DNA purity was better with the commercial kit. This test showed that with this technique it is possible to detect 4 CFU of Salmonella spp. in 1L of drinking water and 10 CFU in 1L of sea water. The amplification curves obtained are shown in figures 4.8, 4.9, 4.10 by type of water and extraction method used.

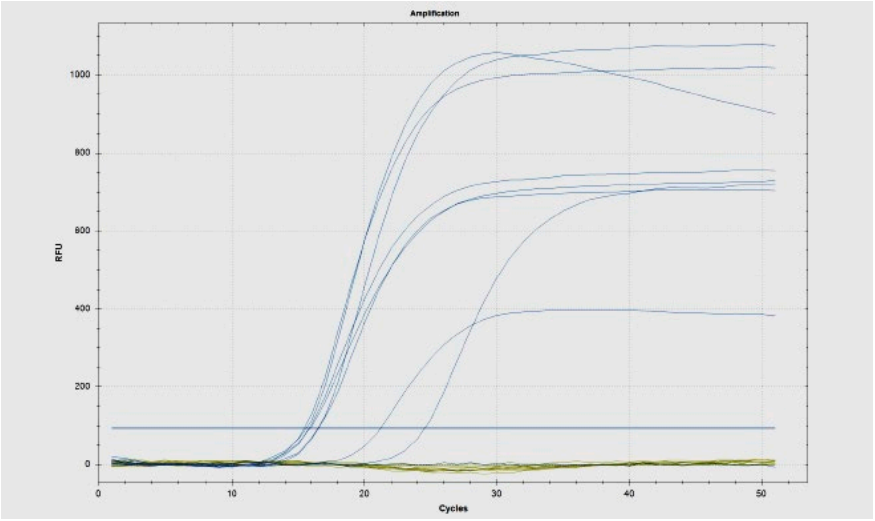


**Figure 4.8.**
**Real time PCR amplifications with commercial kit DNA extraction method from drinking water**

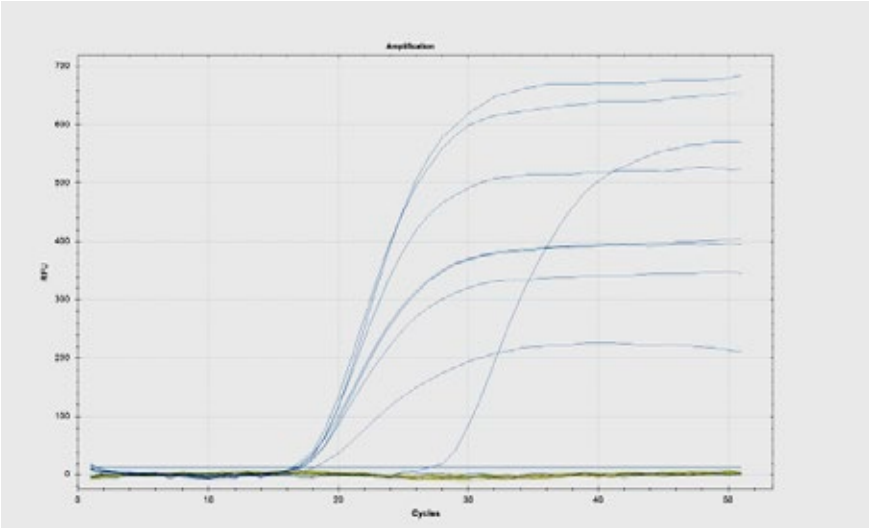


**Figure 4.9.**
**Real time PCR amplifications with organic DNA extraction method from drinking water**

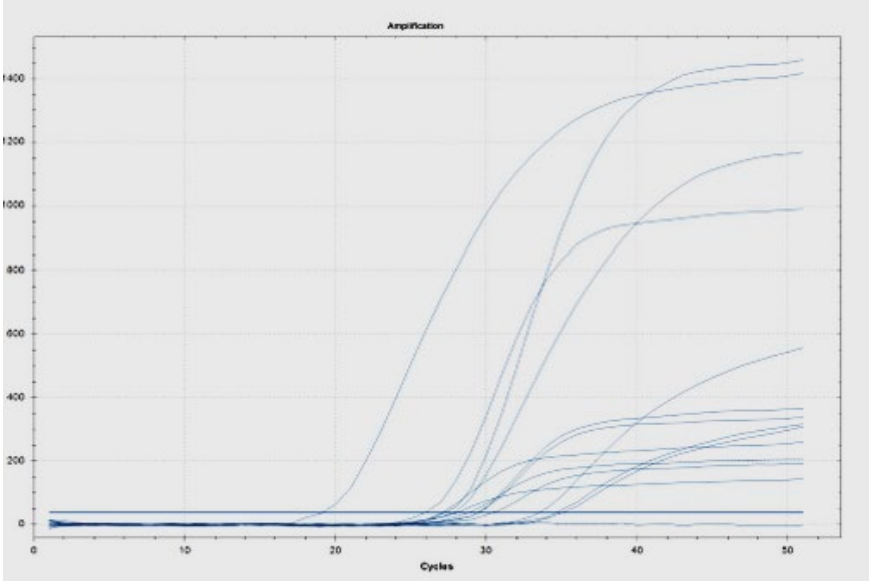


**Figure 4.10.**
**Real time PCR amplifications with commercial kit DNA extraction method from sea water**

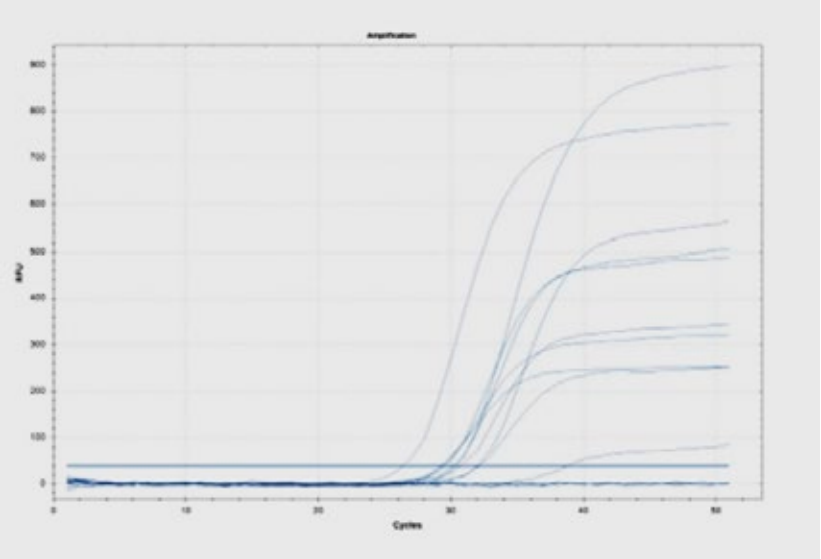


**Figure 4.11.**
**Real time PCR amplifications with organic DNA extraction method from sea water**

## Definition of Ct value to determine the positivity of samples for Salmonella spp.

According to the developed analysis with seven replicates inoculated with 1 CFU/1L showed in table 4.10, 33 was defined as the maximum Ct value to catalog a water sample as positive for *Salmonella* spp. This value was determined according to the mean value obtained, which was 29.23 and this was added the 95 % confidence level, which was 3.8.

***Table 4.10.***
***Salmonella sp. data statistical summary***

| **Cq 1 CFU** | |
|---|---|
| Average | 29.23142857 |
| Typical error | 1.56274092 |
| Standard deviation | 4.134623838 |
| Sample variance | 17.09511429 |
| Minimum | 20.17 |
| Maximum | 32.34 |
| Count | 7 |
| Confidence level (95 %) | 3.823889278 |

**How to quote this chapter:**
Soto Varela Z, Rosado Porto D, Pichón González C, Granado Pantoja B. Results obtained in the process of standardization of a real time PCR for the Escherichia coli quantification and Salmonella spp detection. In Badillo Viloria M, Pérez Lavalle L, editors. Real-time PCR applied to bacterial waterborne pathogens detection and quantification. Barranquilla: Ediciones Universidad Simón Bolívar; 2018. p. 55-70.