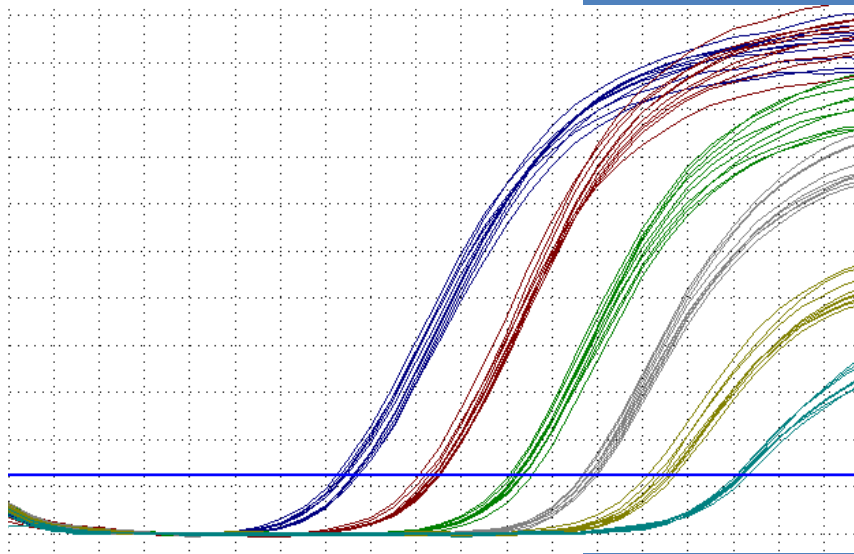# PATHOGEN DETECTION SYSTEMS BY REAL TIME PCR

Microbial

Results Interpretation Guide

Microbial offers real time PCR-based systems for the detection of pathogenic bacteria in food and environmental samples. This technique is fast and robust, allowing the user to obtain confident and unequivocal results in less than two hours. Despite the operative simplicity of real time PCR, an accurate interpretation of the results might be complex without previous experience. Results are graphically expressed by the software accompanying all thermocyclers. A correct interpretation of the amplification curves will help the Laboratory Head to deliver reliable results.

With this guide Microbial provides some basic guidelines to interpret the results obtained by real time PCR when using Microbial pathogen detection systems.

(Revised on September 2009)

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1. REAL TIME PCR FUNDAMENTALS

Real time PCR is based on the exponential amplification of a specific DNA fragment, used as a template to generate millions of copies. Real time PCR differs from conventional PCR because it allows the continuous monitoring of the DNA produced in each cycle by using fluorophore dyes. Fluorescence is released every time a new DNA copy is synthesized, and therefore, the amount of fluorescence is proportional to the quantity of DNA produced. Furthermore, real time PCR systems provide higher sensitivity and robustness, with the additional possibility to quantify the initial amount of target DNA present in a sample.

Real time PCR results are visualized in an amplification plot. Fluorescence is represented on the Y axis, whereas the number of PCR cycles is plotted in the X axis. The amplification curve consists of an initial phase where the produced fluorescence is below the detection level of the thermocycler, a second phase in which the fluorescence increases (being this increment exponential at the beginning of the phase), and a third phase (plateau) where the reaction ends and the fluorescence is stabilized (Figure 1). Within the second phase, it is possible to set a threshold value, which indicates the area of exponential increase. This value is represented in the graph by a horizontal line.
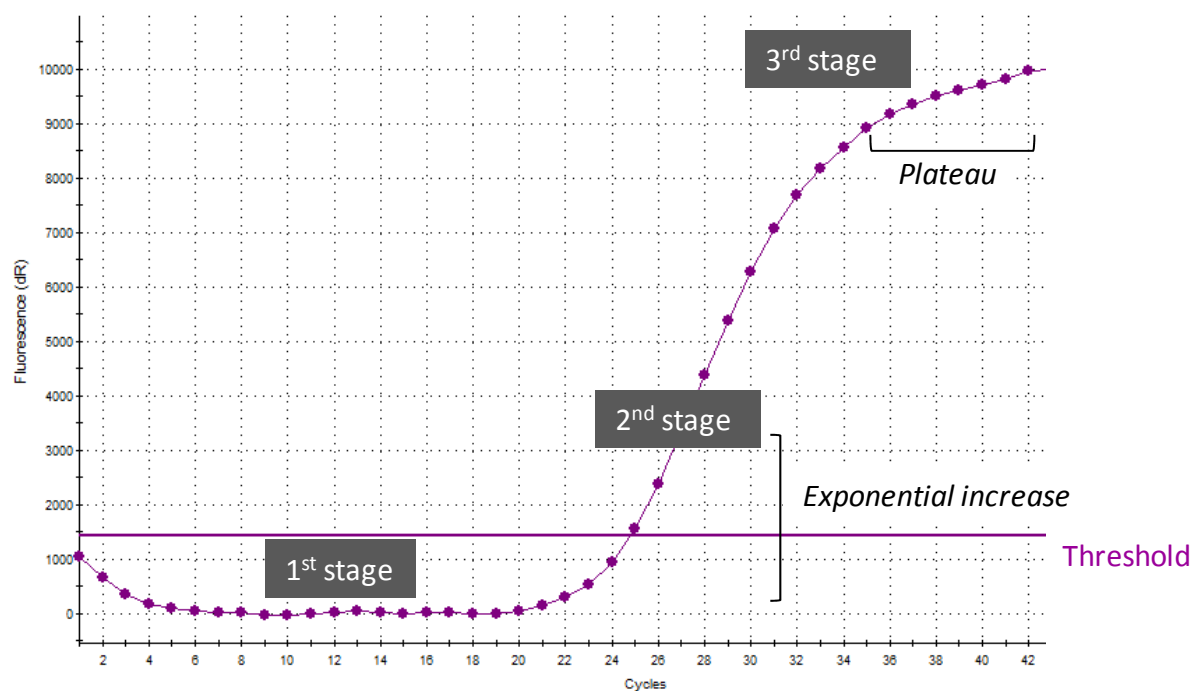

Figure 1. Real time PCR amplification plot showing the three phases.

The intersection point between the amplification curve and the threshold line is called Ct (*Threshold Cycle*). This point indicates the cycle in which the fluorescence reaches the threshold value. The higher the initial DNA amount, the lesser number of cycles are needed (low Ct values) to reach the threshold (Figure 2). As a general rule, considering optimal reaction efficiency, every time a sample is 10-fold diluted, the Ct value will increase in c.a. 3.3 cycles.
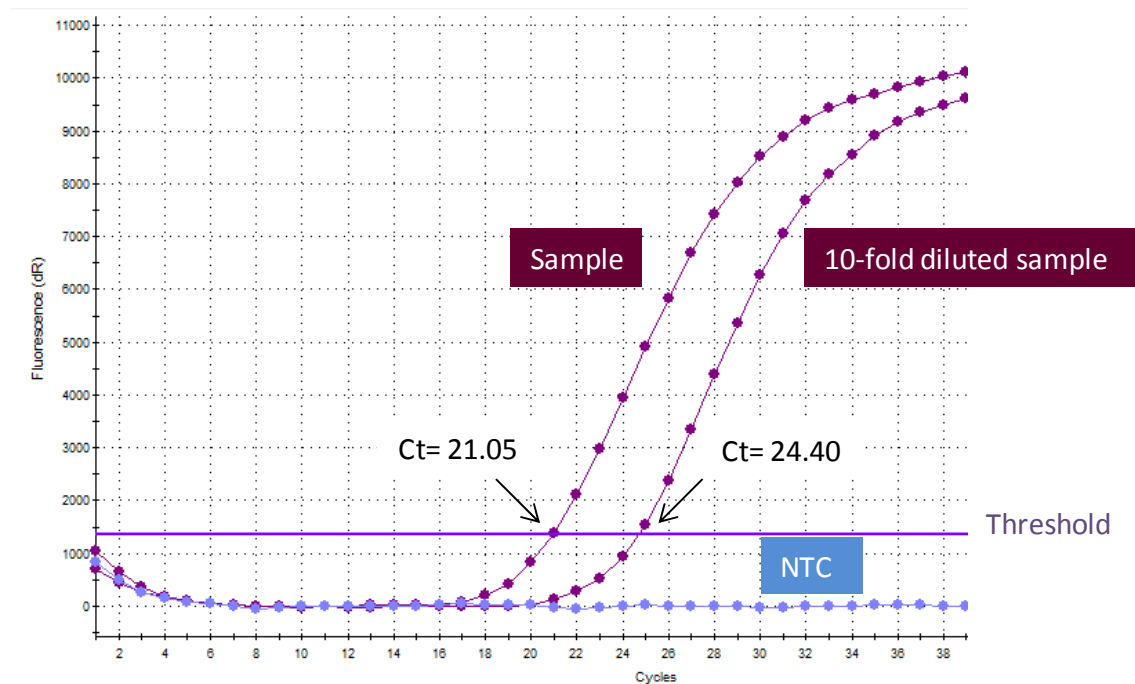
Figure 2. Amplification plot in which a diluted sample crosses the threshold line later than the non-diluted sample. Note that the NTC (non template control) does not reach the threshold because it contains no initial template DNA to amplify.

Amplification plots can be visualized either with the two axes in lineal scale (Figure 3A), or with the Y axis in logarithmic scale (Figure 3B), being both presentations suitable to interpret the results. The semi logarithmic graph may be useful to manually adjust the threshold value within the exponential fluorescence increase in the second phase, since this increase appears as a straight line using this visualization mode (Figure 3B). Several mathematical methods may be used to calculate the best position of the threshold but, in any case, it should be located within the exponential area of the second phase. Today, all thermocyclers include in their software a system to automatically calculate the position of the threshold, although it is also possible to manually adjust it within the recommended parameters.
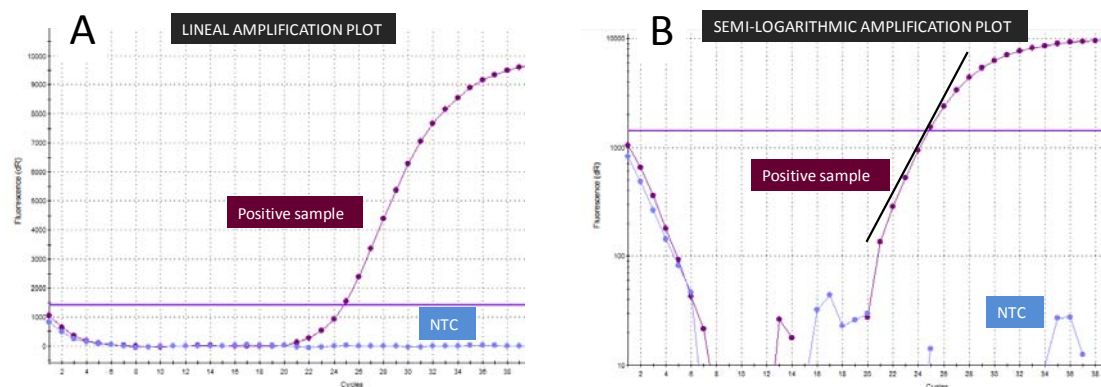
Figure 3. Amplification plot in lineal scale (A) and in semi logarithmic scale (3B). Note that although the amplification curves display different shape the threshold value is the same in both cases.

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2. THE INTERNAL AMPLIFICATION CONTROL

The reliability of the detection kits developed by Microbial is guaranteed by means of an internal amplification control (IAC) included in the Reaction Mix. The IAC system has been specifically designed to validate the accuracy of the test, enabling the distinction of true negative results from false negative results caused by PCR malfunction (due to inhibition, spoilage of the PCR reagents, etc.).

The IAC system consists of the independent amplification of an artificial DNA sequence, which is co-amplified with the pathogen target DNA during the PCR run. The IAC is incorporated into the Reaction Mix to a concentration that has been carefully adjusted. Thus, the low concentration of the IAC ensures that the specificity and sensitivity of the test are not affected by competitive amplification of both DNAs. Consequently, the IAC amplification signal may disappear in positive samples where the pathogen DNA is present in high amounts. Nevertheless, the IAC signal should always be detected in negative samples (absence of the pathogen). When no IAC or pathogen signal is produced, the cause of the PCR failure should be elucidated by checking the integrity of the PCR reagents or applying alternative solutions to overcome inhibitory problems.

Most inhibition problems are due to the presence of particles or substances that remain in DNA extractions from complex samples. These problems may be solved by simply repeating the analysis using a 1/10 or 1/100 dilution of the extracted DNA with nucleic acid free sterile distilled water, as while diluting the extracted DNA of a sample, inhibitors will become also diluted. Higher dilution factors are not recommended since they could lead to an excessive dilution of the target DNA under the detection limit. When inhibitions cannot be solved by diluting, comprehensive DNA extraction methods including several purification steps (i.e. silica gel column-based methods) should be used.

3. DETECTION SYSTEMS WITH 2 FLUOROPHORES

Microbial pathogen detection kits based on the use of 2 fluorophores include real time PCR systems for the detection of *Salmonella* spp. (Salmofast®), *Listeria monocytogenes* (Listerfast®), thermophilic *Campylobacter* (Campylofast), and *Legionella pneumophila* (Legiofast® ENVIRON). These are based on two probes stained with two different fluorophore dyes, FAM and JOE. The probe stained with FAM indicates the presence of the pathogen in the sample, whereas the JOE-stained probe indicates a correct amplification reaction through the use of the IAC, which allows the detection of PCR inhibitors, avoiding false negative results.

The typical result of a real time PCR analysis with a detection system based on two dyes is an amplification plot with a curve for each detector (Figure 4). Since the level of fluorescence signal is variable depending on the dye, a threshold value will be independently set for each curve. The amplification signal for each dye will be considered positive whenever the detector curve crosses its threshold value. Therefore, samples will be considered positive (presence of the pathogen) whenever they display a FAM positive signal. By contrast, samples will be considered negative (absence of the pathogen) only when the FAM signal is negative but the JOE signal is positive.
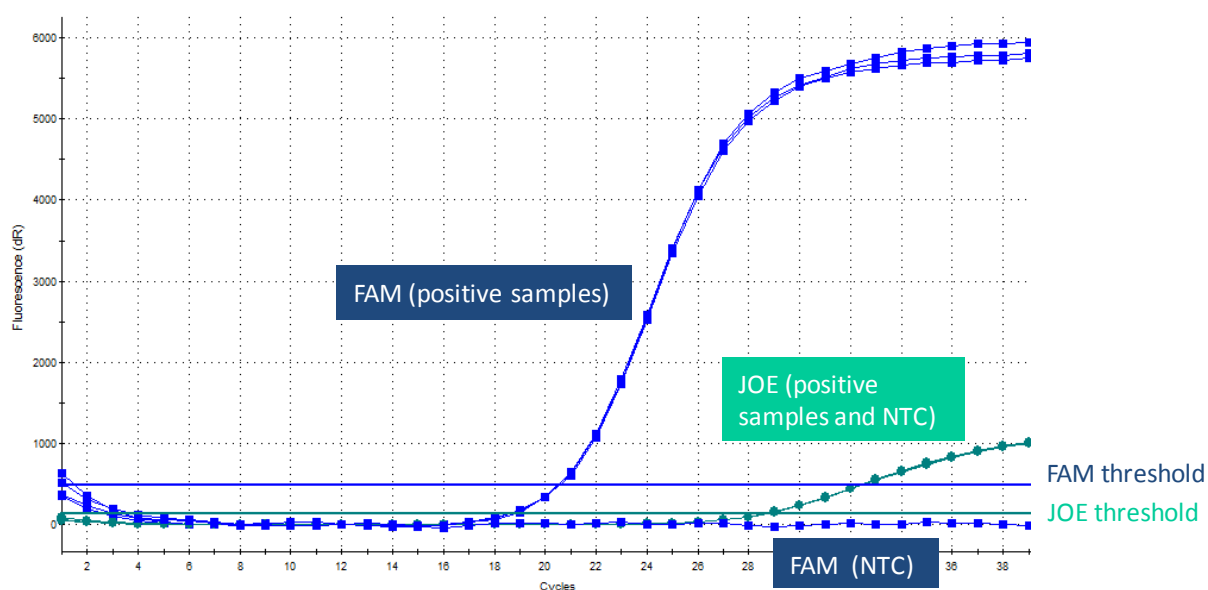
Figura 4. Amplification plot obtained with 2 fluorophores: FAM for the pathogen and JOE for the IAC (internal amplification control). Note that the scale of the two fluorophores is very different, since due to optical reasons FAM fluorescence level is higher than that of JOE.

The recommended strategy to easily analyze the results consists of a first visualization of FAM curves. All samples with a FAM positive signal are positive for the pathogen regardless of the JOE curve behavior. In samples with a negative FAM signal the presence of inhibitions should be ruled out by checking the JOE curve, which should show a positive signal and exhibit Ct values similar to those of the non template control (NTC). In samples where neither FAM nor JOE signals are positive, or if only the JOE signal is detected but the Ct is sensibly higher than in NTC, the PCR reaction will be considered to be inhibited (Figure 5).
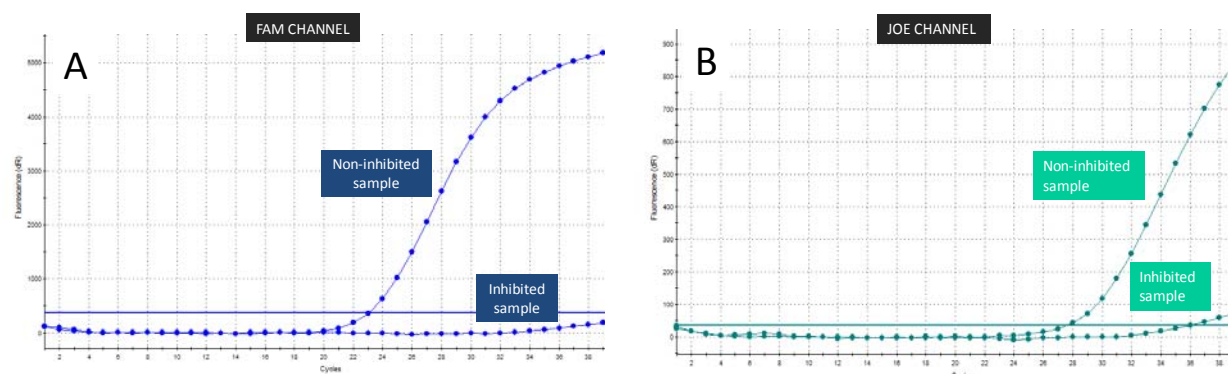
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Figure 5. PCR inhibition. A. Amplification plot for the FAM channel where a positive non-inhibited sample and an inhibited sample are shown. B. Amplification plot for the JOE channel where the signal of the inhibited sample appears significantly later (if the inhibition problem was greater, it would not even cross the threshold).

It must be taken into account that high Ct values or negative signals for JOE may also be due to the presence of high initial amounts of DNA from the pathogen in positive samples, which might cause a bias in the PCR reaction favoring the amplification of the FAM detector (Figure 6).
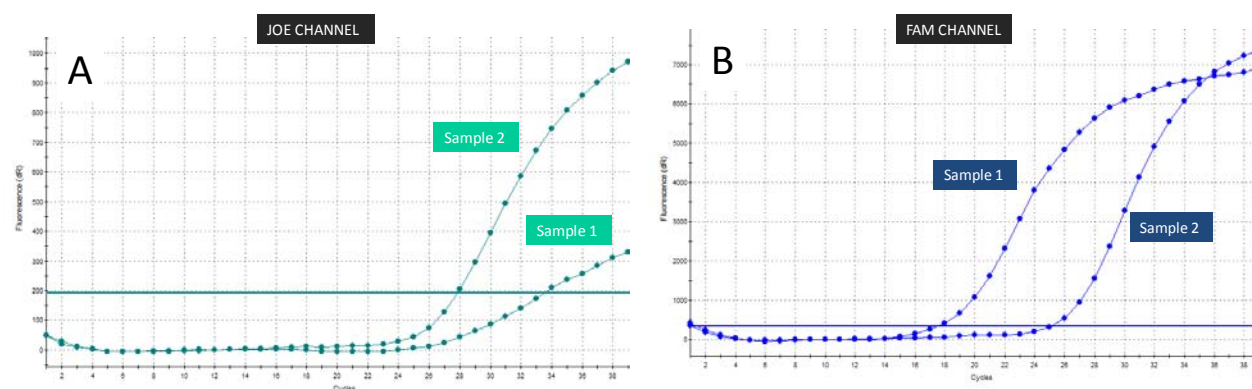
Figure 6. Bias of the PCR amplification in samples containing high initial amounts of pathogen DNA. A. Amplification plot for the JOE channel where the signal in Sample 1 appears much later than in Sample 2, similar to what would be expected in case of inhibition. B. Amplification plot for the FAM channel where the signal in Sample 1 appears considerably sooner than in sample 2, indicating that the high initial abundance of pathogen DNA has caused a bias in the PCR reaction favoring its amplification over the IAC (JOE).

The interpretation of the results for determining the presence/absence of a pathogen in a sample can be summarized in Table 1.

Table 1. Results interpretation for 2 fluorophore-based Microbial detection systems

| Pathogen detector (FAM)	IAC detector (JOE)	Interpretation
Positive signal	Irrelevant	Positive sample (presence of the pathogen)
Negative signal	Positive signal, Ct not delayed compared to NTC	Negative sample (absence of the pathogen)
Negative signal	Negative signal / Ct delayed compared to NTC	Inhibited sample

4. DETECTION SYSTEMS WITH 3 FLUOROPHORES

Bactoplex is the Microbial multiplex system for the simultaneous detection of *Salmonella* spp. and *Listeria monocytogenes* based on the use of three dyes: FAM for the detection of *Listeria monocytogenes*, JOE for the detection of *Salmonella* spp., and CY5 for the detection of the IAC. This system allows the differential and simultaneous detection of two pathogens in a single reaction, with a third channel dedicated to the IAC (Figure 7).
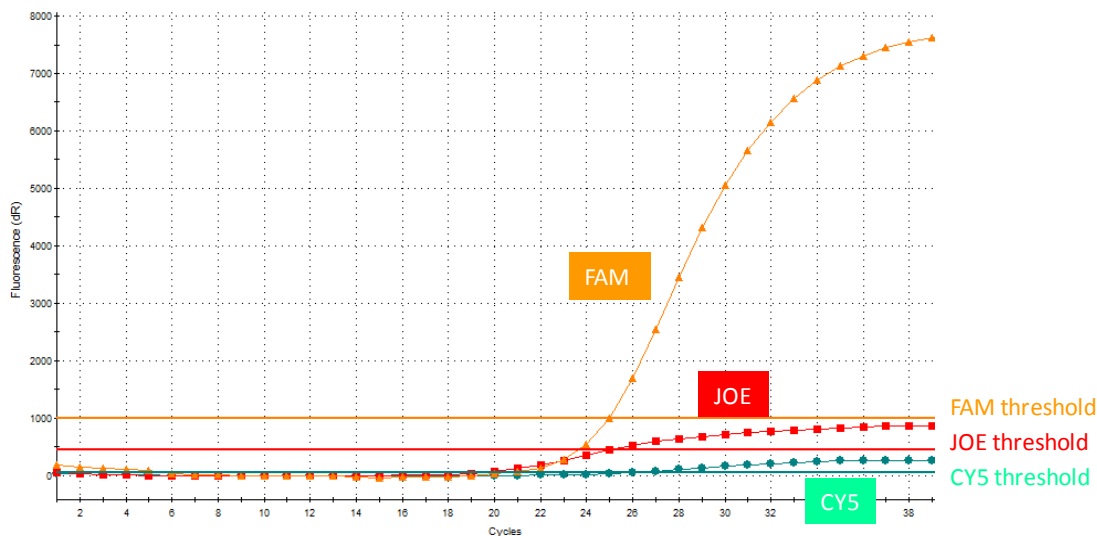
Figure 7. Amplification plot obtained with a detection system based on the use of three fluorophores: FAM for the detection of pathogen 1, JOE for the detection of pathogen 2, and CY5 for IAC. Note that the scale for each of the three dyes is very different. In this example the sample is positive for both pathogens and for the IAC.

As in the case of detection systems with two fluorophores, FAM fluorescence signal may be higher than that of JOE, which, in turn, can be higher than CY5 fluorescence. Therefore, analyzing every detector separately can help interpret the results (Figure 8).
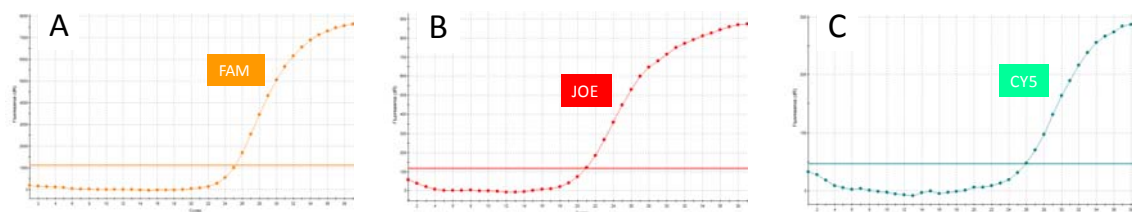
**Figure 8.** Amplification plot for the fluorophores FAM (A), JOE (B) and CY5 (C).

The interpretation of the results to determine the presence/absence of two distinct pathogens in the same sample when using a three-fluorophore-based system is summarized in Table 2. As commented above, high initial amounts of target DNA (of one or both pathogens) in positive samples may cause a bias in the amplification reaction favoring the pathogen detection against the amplification of the IAC. Thus, a sample will be considered negative (absence of both pathogens) only when FAM and JOE signals are negative but the CY5 signal is positive and not delayed with respect to the NTC. Samples without a positive signal of FAM, JOE and CY5 (or with a CY5 signal delayed with respect to the NTC) will be considered to be inhibited.

Table 2. Results interpretation for 3 fluorophore-based Microbial detection systems

Pathogen 1 detector (FAM)	Pathogen 2 detector (JOE)	IAC detector (CY5)	Interpretation
Positive	Positive	Irrelevant	Positive for both pathogens
Positive	Negative	Irrelevant	Positive for pathogen 1
Negative	Positive	Irrelevant	Positive for pathogen 2
Negative	Negative	Positive, Ct not delayed compared to NTC	Negative for both pathogens
Negative	Negative	Negative/ Ct delayed compared to NTC	Inhibition

5. DETECTION SYSTEMS WITH SYBRGREEN

Microbial's *Legionella* spp. detection system (Legiofast) is based on the use of the SYBRGreen dye, which emits fluorescence when it is attached to double stranded DNA. Using Legiofast® SPECIES is a single amplification curve that integrates both the IAC and the pathogen DNA signals (Figure 9).
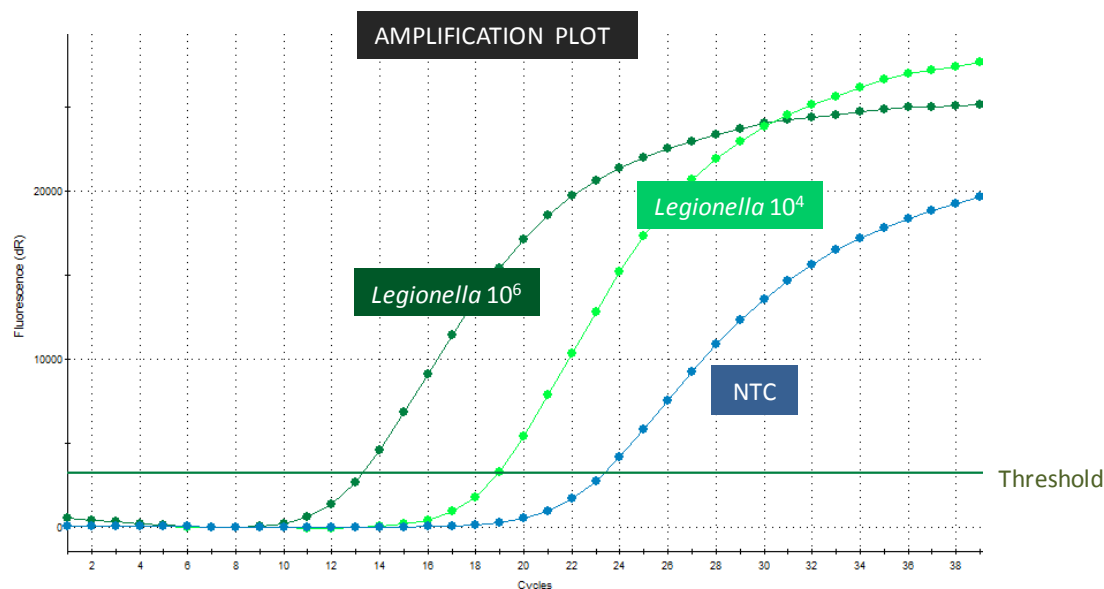

Figure 9. Amplification plot with SYBRGreen, where the amplification of the NTC appears later than the amplification of samples with 10^6 and 10^4 copies of the pathogen.

In this case, target DNA amplification is distinguished from IAC DNA amplification by means of a dissociation curve, performed by continuous reading of the fluorescence while gradually increasing the temperature until complete DNA denaturation is achieved. As a result, the dissociation plot will contain as many peaks as different DNA fragments are produced during the PCR. This process also allows the analysis of the Tm (melting temperature) of each of the originated DNA fragments, which is greater for the pathogen than for the IAC peak since its amplification product is longer (Figure 10). Reference Tm values for the IAC and the pathogen should be determined in NTC and positive control reactions respectively.

Summarizing, the dissociation curve reveals whether the obtained signal corresponds to the pathogen, the IAC, or both. In negative samples, only the peak corresponding to IAC is expected, whereas in positive samples both peaks (pathogen and IAC) should appear, although the IAC peak could disappear when high initial amounts of pathogen template DNA are present. Inhibition of the PCR reaction will be considered to occur when no signal is detected in the amplification plot or when it shows a Ct clearly delayed compared to the NTC.

If none of the peaks corresponding to the IAC or the pathogen appear in the dissociation plot, the analysis will be considered not conclusive, even if a positive signal is detected in the amplification plot. Peaks other than those of the pathogen or the IAC in the dissociation plot will not be taken into account in the interpretation of the results.
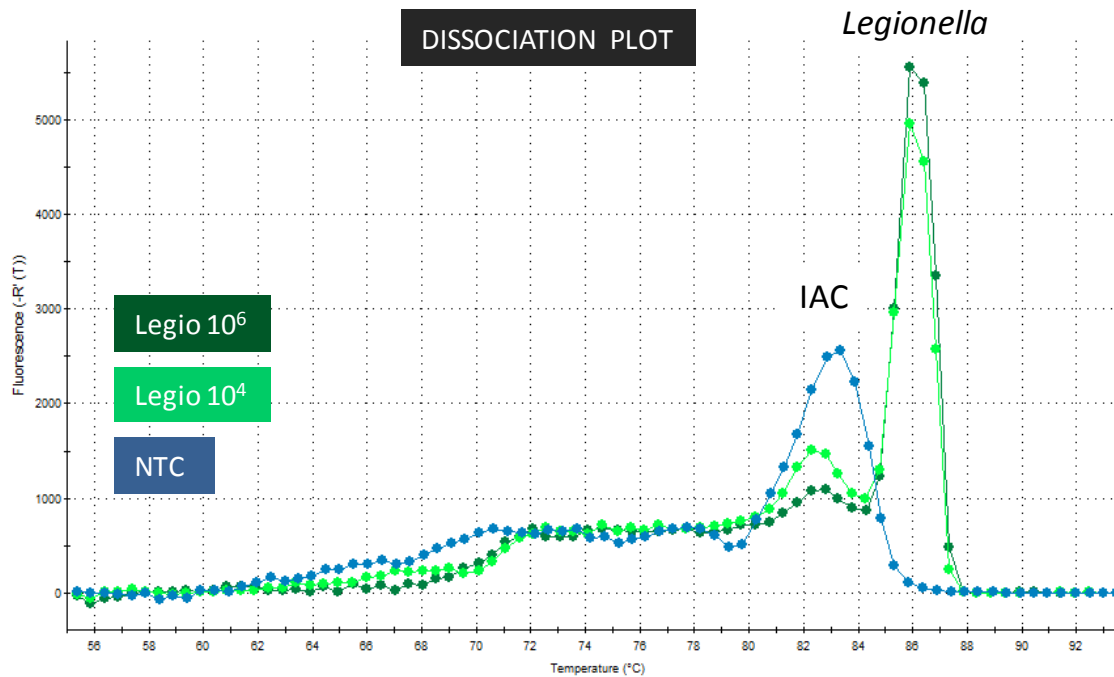
Figure 10. Dissociation plot of samples with 10^6 and 10^4 copies of pathogen target gene. Two peaks are shown: the peak at greater temperature corresponds to the pathogen, and the other at lower temperature corresponds to the IAC. On the contrary, the NTC displays only one peak corresponding to the IAC.

The interpretation of the results to determine the presence/absence of the pathogen is summarized in Table 3.

Table 3. Results interpretation for SYBRGreen-based Microbial detection systems

| Amplification Curve	Dissociation Curve	Interpretation
Positive	Pathogen + IAC peaks	Positive sample
Positive	Pathogen peak	Positive sample (biased PCR)
Positive, CT not delayed compared to NTC	IAC peak	Negative sample
Negative/ Ct delayed compared to NTC	Irrelevant	Inhibited sample

6. QUANTIFICATION BY REAL TIME PCR

Real time PCR allows the quantification of samples, which means to determine the number of copies of the target gene present in a given sample. To use this application of the technique a standard curve is needed, which can be simply built with serial 10-fold dilutions of a sample of known concentration of pathogen DNA (i.e. using the Microbial PCR Quantification Standard or a purified, quantified solution of genomic DNA from the pathogen). In the amplification plot of the serial dilution of a DNA solution, the Ct value is inversely proportional to the initial template DNA concentration (Figure 11).
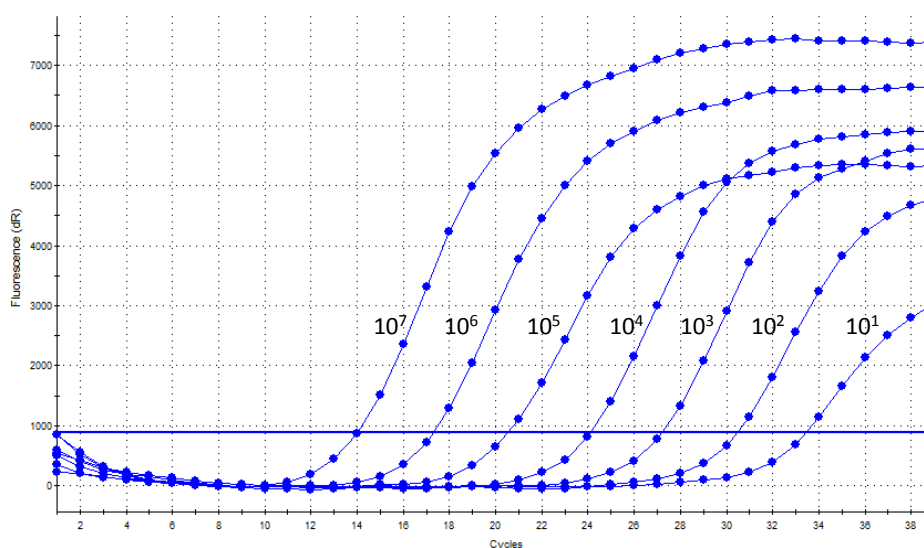
Figure 11. Amplification plot of a 10-fold serial dilution with a range from 10^7 to 10^1 copies of the target gene. Note that final fluorescence is not always proportional to the initial amount of template DNA. In contrast, the Ct values are always inversely proportional to the initial number of target genes in the sample.

The Ct values can be plotted against the DNA concentration (usually as gene copies/µl) in logarithmic scale (Figure 12). Then, the initial number of copies of the target gene in an unknown sample can be obtained by interpolating its Ct value to the standard curve equation. It should be taken into account that, according to the quantification system used, the number of gene copies is equivalent to the number of pathogenic bacteria only when single copy genes are used as targets (all Microbial kits are based on single copy genes except Legiofast® SPECIES). It should be noticed that the result of the quantification of an unknown sample might also depend on the efficiency of the DNA extraction.
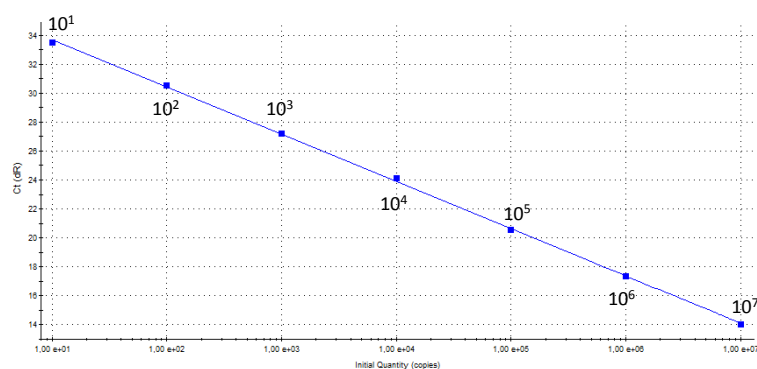

Figure 12. Standard curve showing the Ct values in relation to the concentration of initial target gene copies, obtained by a serial 10-fold dilution of a sample containing 10^7 copies/µl of the target gene. By the interpolation in the standard curve of the Ct of an unknown sample it is possible to calculate the number of copies of the target gene it contains.

7. CONTAMINATION OF REAL TIME PCR

Contamination of real time PCR is detected when the pathogen signal crosses the threshold line in NTC or, in the case of detection systems based on SYBRGreen, when the peak corresponding to the pathogen appears in the dissociation plot in NTC. The presence of contamination invalidates the obtained results, since it makes impossible to distinguish a true positive result from a positive result caused by contamination. Then, the analysis should be repeated taking all the possible measures to avoid new contamination incidences.

Because of the great sensitivity of the PCR technique, it is not uncommon that some negative samples or NTC cross the threshold line beyond cycle 35 (Figure 13). This weak signal may correspond to a small contamination caused by target DNA aerosols produced while preparing the PCR plate, or also to a minimum presence of the pathogen in the sample. If it is considered that this weak signal is due to a contamination effect of little importance, the head of the laboratory, always on its own responsibility, may decide that this does not affect the results and therefore, consider the analysis valid. Nevertheless, in case of doubt, the analysis should be repeated improving, if possible, the preparation of the sample and the loading of the PCR with the extracted DNA.
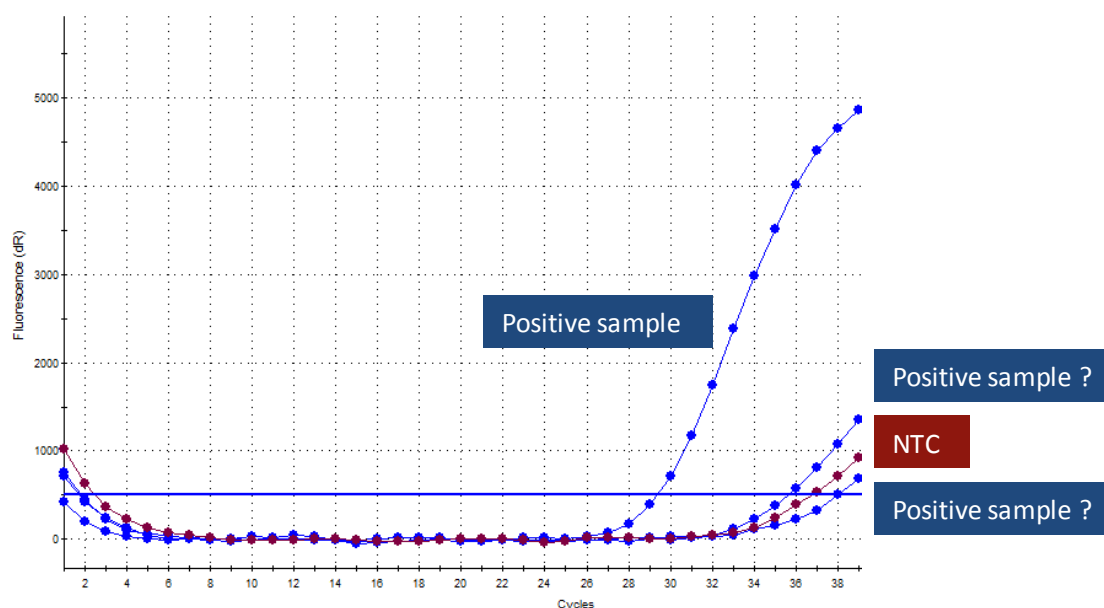

Figure 13. Amplification plot showing a clear positive sample and two doubtful samples which, together with the NTC, cross the threshold between cycles 35 and 38. It is not possible to ascertain whether these samples are really positive or if they are caused by a weak aerosol contamination. In the case of NTC it is obvious that the positive signal is caused by aerosol contamination, since it is not expected to contain target DNA. It is important to be aware of the fact that the effect of this weak contamination is negligible in clearly positive samples.

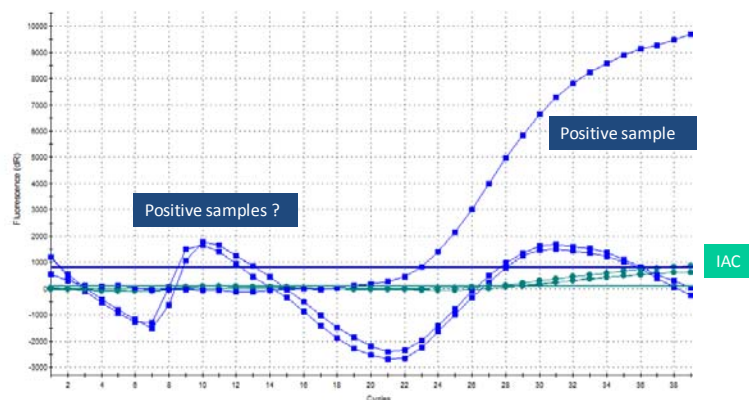## 8. ATYPICAL AMPLIFICATION CURVES

Occasionally, atypical amplification curves may be obtained. This might happen if the extracted DNA contains substances that alter the fluorescence emission by the fluorophore dyes or the fluorescence detection by the thermocycler. Some atypical curves may also be caused by a wrong configuration of the reading system of the thermocycler. Any curve that does not present the three phases previously described in Figure 1 must be discarded and the sample should be analyzed again. Figure 14 shows two examples of atypical amplification curves.

A
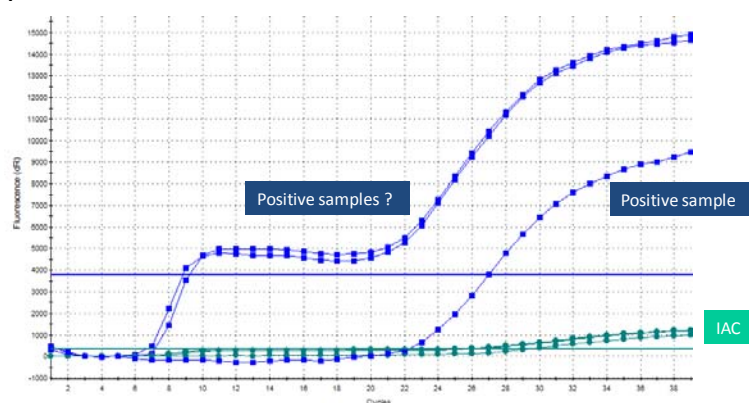
Figura 14. Curve with a double plateau shape (A) and curve with a roller coaster shape which crosses the threshold several times (B).

B

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