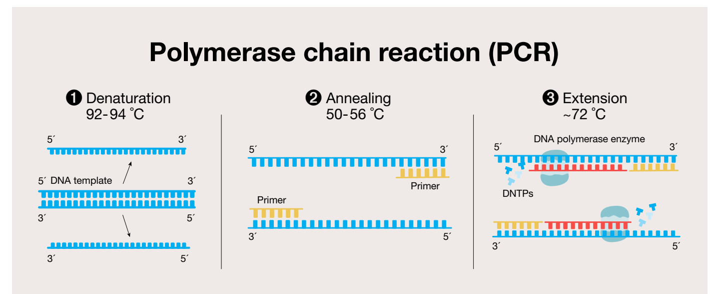
Recalls & PCR Results Interpretation - PCR Software that Protects Your Brand

Real-time polymerase chain reaction (RT-PCR) is commonly used for detection of pathogens in food products as part of a food safety program. RT-PCR works by making multiple copies of the nucleic acid present in the sample, allowing it to be detectable by the equipment.

The principle behind RT-PCR is simple; the amount of DNA present (amplified) is measured after each cycle via fluorescent dyes that yield increasing fluorescent signals in proportion to the number of PCR products generated. The change in fluorescence during the reaction is measured by an instrument that combines thermal cycling with the capability of scanning the fluorescence levels.



There are three major steps that make up each RT-PCR cycle. These are:

1. **Denaturation** – The sample is heated to high temperatures to separate or "melt" double-stranded DNA into single strands and loosen the secondary DNA structure for primer binding.
2. **Annealing** – Short nucleic acid sequences (primers or probes) are allowed to hybridize or bind to specific (complementary) sequences within the target DNA to be used as a starting point for DNA synthesis (elongation).
3. **Extension** – DNA polymerase binds to the double-stranded regions (target + bound primer) and extends the primers creating a duplicate copy of DNA.

By using RT-PCR technology, a number of advantages can be identified:

- The PCR reaction can be monitored as it is occurring (hence, in real time)
- The amount of amplified DNA can be precisely measured at each cycle, allowing for quantification of the amount of starting target DNA
- The dynamic range of detection is expanded
- A single tube can be used for amplification and detection, eliminating post-PCR manipulations

Of importance is understanding the following:

1. **The Baseline fluorescence in the RT-PCR reaction** ("noise") – this is equivalent to the background or 'noise' seen in the reaction, generally established in the earlier cycles of amplification.
2. **The Threshold level** – this is the level of signal that reflects a statistically significant increase over the calculated Baseline signal.
3. **The Ct, Threshold Cycle** – this is the cycle number at which the fluorescent signal of the reaction crosses the threshold. The Ct value is inversely related to the starting amount of target DNA, so can be used to calculate the initial DNA copy number if the PCR is operating at 100% efficiency.

   Note: The BAX® System Q7 Software utilizes multi-variable algorithms to objectively provide reliable Ct values without the need for subjective calls by eye.

To achieve accurate and reproducible expression of target genes using RT-PCR, it is critical to use an internal positive control (INPC) for each reaction. Addition of the INPC ensures every part of the PCR process is accurately performed from sample preparation to software result interpretation.

The INPC can indicate if an error occurred during the processing or analysis, including the following:

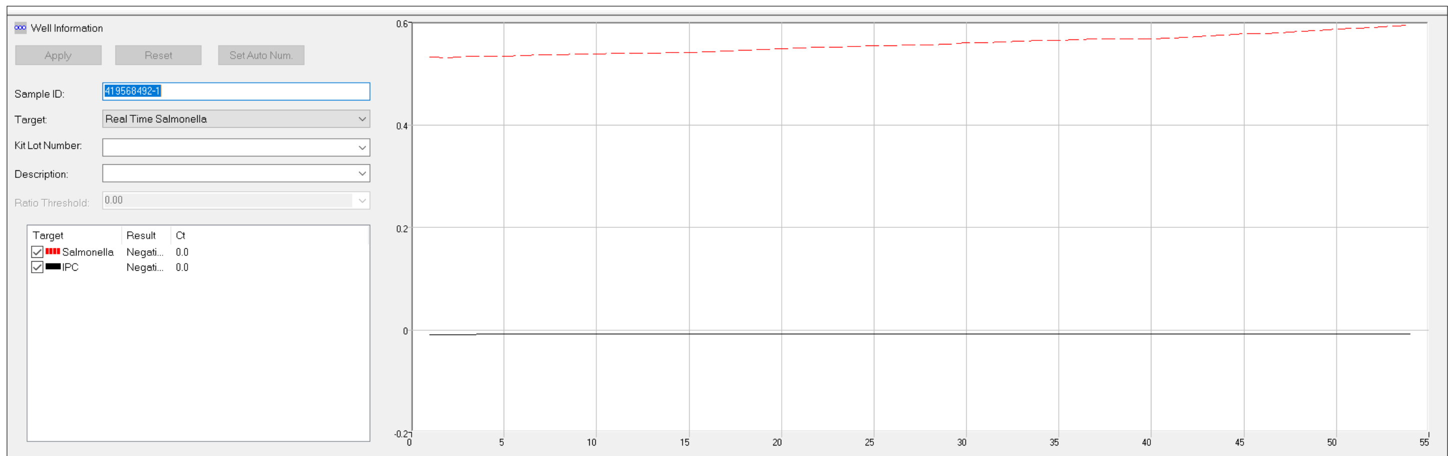
- The lysis reagent wasn't properly inactivated during the lysis step (the INPC won't be detected)
- The sample preparation was done incorrectly (changes in the INPC result)
- The sample contained PCR inhibitors (INPC is sensitive to these inhibitors, so will not be detected)

As a result, the INPC prevents false-negative results from being reported as negative, ensuring food safety results are accurate and in the best interests of the consumer and food safety brand protection.

Note: That is why all BAX System PCR and Real-Time PCR Assays include a INPC in each assay well.

However, even with an INPC, testing can still return some inconclusive results. When this occurs, the INPC may have provided the expected results, but the fluorescence signal from the target DNA cannot be interpreted as clearly positive or negative. This is generally because a higher Ct was observed, a lower fluorescence intensity was detected or the INPC did not generate a positive Ct value. These results are reported as "indeterminate" and additional work must be done to confirm if that specific sample is positive or negative for the target DNA.

Salmonella - BAX System software graph



Many PCR systems shy away from reporting "indeterminate" results for this very reason. They choose to only report two options – either positive or negative. This can result in false-negatives for any of the reasons noted above. This false-negative result can be detrimental to the business if consumers get sick from contaminated product, leading the food producer to generate recalls, handle lawsuits, and incur lost revenue in addition to dealing with FDA and USDA investigations.

What are the next steps when an indeterminate result is obtained? Typically, it means retesting from the already made lysate, recreating a new lysate, or adding in a regrowth step with additional PCR reactions. While this takes additional time and consumables, the advantages are many.

First, and most importantly, retesting an enriched sample ensures the integrity of the food product and the accuracy of the result. Indeterminate results flag that something went wrong and prevents the reporting of a false-negative, risking consumer safety.

Retesting also allows the laboratory to identify potential root causes of the indeterminate result. It could be as simple as a missed well or particulate introduction. That is why is it vital to review all possible steps affecting the results before running the second test. Technicians must be careful to check that all lysis tubes contain the same level of lysis reagent, the proper amounts of enriched sample are added to each lysis tube, any matrix particles from samples are minimized during transfer to the lysis buffer, and proper heating and hydration steps are performed accurately. The technicians can also closely review the indeterminate results and analyze the plots to determine what the potential problem was, asking: Was the INPC negative, was the Ct value for the INPC in the acceptable range, or was there any curve observed for the sample even if short of the Ct?

Some other examples that can lead to atypical results can be seen if straight line signals are observed, i.e., the INPC and target curves appear flat (and Ct value for INPC is zero), it could indicate either a process or reagent issue (*Salmonella* - BAX System software graph). Either the second lysis step was not performed properly (usually not at 95 ˚C), cleaning detergents or amplicon may be interfering with the assay, PCR tablets could be missing from a tube or a tablet remains dry (pipettor has loose tips or is clogged). If exponential decay is seen where the INPC and target curves start high and then drop and remain flat, then the culprit is most likely humidity or tablet issues. Either the PCR tablets were exposed to air for too long before being hydrated with lysate, the tablets were not fully hydrated or were exposed to humidity during storage if improperly stored.

No matter the cause, retesting the samples after an indeterminate result provides an extra fail-safe against false negatives and potential recalls on unsafe products. Not only does this help ensure product integrity, it also allows laboratories to fine tune their procedures to further streamline processes and minimize indeterminate results in the future.