Real-Time PCR Curves, Cp Values, and BIOFIRE® Systems

Introduction

The purpose of this technical note is to share information about real-time PCR curves and Cp values on BIOFIRE® Systems and explain the differences of nested multiplex PCR vs non-nested PCR technologies. Additionally, information is provided on how to access Cp values via a cloud-based software solution, BIOFIRE® FIREWORKS®.

Real-Time PCR Background

First described in the early 1990s[1], real-time polymerase chain reaction (PCR) is a method by which fluorescence detection is used to monitor the progression of amplification of specific DNA sequences in 'real-time' throughout the cycles of the PCR reaction. The technique was developed using a DNA intercalating dye as the fluorescent reporter, but the use of fluorescently labeled probes is also common[2, 3]. When using double-stranded DNA dyes, DNA melting analysis is performed at the end of the PCR reaction to identify that the intended product was amplified, while probe-based reactions achieve specificity of the fluorescent signal during the reaction via homology between the sequence of the probe and the amplicon. The ability to monitor the formation of amplicon during the exponential phase of the PCR reaction is the foundation for the transition of PCR from an almost strictly qualitative output to a technique with the potential for quantitative analysis.

For a well-designed and efficient assay, the number of cycles needed to generate fluorescence signal that is distinguishable from background [referred to as the crossing point (Cp), cycle threshold (Ct), and/or quantification cycle (Cq)] reflects the amount of template in the reaction. However, Cp/Ct/Cq values are sensitive to many variables and characteristics of the sample, reaction, instrumentation, and analysis. Hence, for real-time PCR to be quantitative (qPCR or quantitative PCR) the entire process from sample collection to analysis should be properly designed, controlled, and interpreted. qPCR can provide 'absolute' or 'relative' target nucleic acid quantification when comparing Cp/Ct/Cq values of the 'unknown' target to Cp/Ct/Cq values from a calibration curve consisting of known quantities of template or by normalizing real-time data relative to a reference gene or template within the sample.

The positive impact of real-time PCR (both qualitative and quantitative) in research as well as industrial and clinical applications is substantial and undeniable. Notwithstanding its advantages and routine use in most molecular laboratories today, standardization of the principles of real-time PCR design, optimization, validation, and use is still lacking in much of the published research. In 2009, guidelines for quantitative real-time PCR were published to "help ensure the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency" (The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments)[4]. Several other (ISO) standards[5] and (CLSI) guidelines[6, 7, 8, 9] are available for the validation, evaluation, and implementation of quantitative assays such as real-time PCR, particularly for clinical settings. Yet, monitoring of the body of literature referencing real-time PCR technology reveals that decades after the technique was developed, many of the guidelines for reliable use, interpretation, and reporting of data are still not routinely applied[10].

The SARS-CoV-2 pandemic and the flood of assays developed to help diagnose this critical infection, along with studies intended to elucidate infectivity or disease severity and progression based on PCR, further highlighted the

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tremendous variability in real-time PCR data[11, 12, 13]. In the absence of reliable and consistent data and interpretation, the utility of real-time PCR data for clinical applications cannot be appropriately established or implemented without risk to patients and public health initiatives. In response, public health and infectious disease organizations issued statements dissuading the use of Cp/Ct/Cq values (particularly from assays with a qualitative intended use) as an estimate of viral load or for other clinical interpretations for patient care and management [11, 14, 15, 16]. However, the availability of International Standards or reference materials along with the development, validation, and consistent use of accurate and commutable quantitative assays could one day pinpoint clinical use cases for qPCR in SARS-CoV-2 and other infections (similar to the use of qPCR assays for monitoring systemic infections such as HIV [17]).

Real-Time PCR Analysis in the BIOFIRE® FILMARRAY® Pneumonia Panels

The BIOFIRE Pneumonia Panel and BIOFIRE Pneumonia *plus* Panel report melt-based qualitative results for atypical bacteria, viruses, and antimicrobial resistance genes but also provide detection results with semi-quantitative nucleic acid concentration (copies/mL) for many bacterial analytes. These panels were specifically designed with semi-quantitative capabilities to align with standard practice and clinical guidelines of the time[19, 20, 21], which suggested that estimating the abundance, or levels, of bacteria in lower respiratory tract specimens by quantitative and semi-quantitative culture techniques can aid in diagnosis and decisions related to patient care and management.

The bacterial assays were designed and optimized purposefully to obtain copies/mL estimates based on real-time amplification data (Cp values) in relation to an internal control of known quantity called the Quantified Standard Material (QSM). Panel development focused on key principles of quantitative assay design (e.g. single-copy gene targets) and tight requirements for achieving optimal and equivalent efficiency in both the multi-plex PCR1 reaction and the single-plex nested PCR2 reaction(s). Results analysis for the bacterial assays retains the qualitative melt-based detection of amplicon for specificity, followed by Cp-based analysis relative to the QSM (which is introduced during the specimen lysis stage and is subject to all stages of the test process) only for positive assays. The semi-quantitative results reported by the BIOFIRE Pneumonia Panels requires the QSM and panel validation included assessment of characteristics that are not typically required for validation of qualitative assays, including linearity, accuracy, and reportable range.

The BIOFIRE Pneumonia Panels are currently the only IVD multiplex real-time PCR tests in the infectious disease diagnostics market to provide approximate nucleic acid concentrations, which is supported by robust validation in accordance with the product intended use.

Considerations for Analysis of BIOFIRE System Cp Values

Several features of the BIOFIRE system and panels that make the tests both comprehensive and user friendly also influence the real-time amplification data (Cp values) generated by the system and should be kept front-of-mind if interpreting BIOFIRE Cp data for research and other non-clinical uses [NOTE: bioMérieux does not support or recommend use of Cp data from IVD panels for clinical interpretation].

In order to provide comprehensive test results, all current BIOFIRE panels use two-stage or ‘nested’ multiplex PCR. The first stage (PCR1) contains primers for all assays combined into one mix. No real-time fluorescence amplification data are collected during the highly multiplexed PCR1 reaction. At the end of PCR1 cycling, the reaction products are diluted and combined with a dye-containing master mix for second stage (PCR2) reactions with assay-specific primers in each well of the array. In total, approximately 50 cycles of PCR are performed

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between the two stages of nested PCR, but real-time monitoring of fluorescence and Cp values are determined only in PCR2, which is generally fewer than 30 cycles. By contrast, a typical single-stage real-time PCR assay may run as many as 50 cycles and will collect fluorescence data from all cycles. The consequence is that the Cp values generated by the nested reaction in the BIOFIRE system reflect the amount of (diluted) PCR1 amplicon added to PCR2 and will generally be much different (typically lower) than a Cp/Ct/Cq value from a non-nested reaction for the same sample/template concentration collected over more total cycles. Though it is not best practice to compare real-time cycle data between different assays in general, nested PCR values in particular should not be compared to non-nested assays, and especially not to infer or compare possible pathogen titers.

Many real-time PCR assays require multiple manual or automated steps (nucleic acid extraction and PCR reaction set-up) with liquid manipulations using calibrated micropipettes dispensing known volumes. BIOFIRE tests are designed to be very easy to use and do not require precise sample or liquid handling steps or measurements. Consequently, some variation is expected in the amount of sample loaded into the pouch and in the automated liquid movement and reaction dilutions within the pouch. Small differences in volume at any stage of the testing process could be observed as run-to-run variation of Cp values (observable when the same sample is tested repeatedly). Qualitative detection by melt analysis is tolerant of these variations while detection based on a Cp cut-off could decrease the reliability of detection at low concentration (near the cut-off). Potential volume-dependent influences on Cp values are controlled in the BIOFIRE® FILMARRAY® Pneumonia Panels by inclusion of the Quantified Standard Material (QSM) that travels through the pouch with the sample. If using Cp values from BIOFIRE panels (or any assay) for supplemental analyses, results should be interpretated in the context of the characterized run-to-run, sample-to-sample, system-to-system error, or imprecision of the data.

BIOFIRE panels monitor fluorescence in PCR2 with a double-stranded DNA intercalating dye, which will generate signal when bound to any double-stranded DNA fragment, including primer-dimers or other non-specific products. Post-PCR DNA melt analysis is applied to enhance specificity of the fluorescence signal for qualitative detection of only those products with the correct melting characteristics. Though the BIOFIRE system requires a positive melt-based assay result before generating a Cp value, interpretation of a BIOFIRE Cp value should consider the potential contribution of signal generated during the PCR reaction from non-specific products.

Accessing the BIOFIRE System Cp Values

For customers who require access to Cp values, we have made them available through a cloud-based software solution, BIOFIRE® FIREWORKS™.

FIREWORKS offers a wide range of features and functionalities designed to enhance the user experience of our BIOFIRE products. One of these features is the ability to access Cp values for positive test results. However, these values are offered for Research Use Only and should not be used for clinical diagnostic purposes. As part of FIREWORKS implementation, users must agree to specific terms to access Cp values.

FIREWORKS is not available in all countries, please reach out to your local bioMérieux representative for more information.

Summary

Real-time PCR data from the BIOFIRE systems (and other platforms) are not inherently quantitative and can be affected by multiple variables. Though amplification curves and Cp/Ct/Cq values may be provided or accessible in primary or accessory software tools, IVD product literature (Instructions for Use) will provide relevant interpretation guidelines for amplification data (if any) and will disclose whether the product and data are intended and validated for qualitative or quantitative interpretation in the indicated clinical applications.

- Real-time PCR is a powerful analytical technique with qualitative and quantitative applications depending on assay design and controls.
- Most BIOFIRE panels are designed, optimized, and validated for qualitative detection of nucleic acids using DNA melting analysis rather than real-time amplification curves.
- Use and interpretation of Cp values generated by the BIOFIRE systems for qualitative assays/tests has not been validated. Cp values are only provided for assays that are determined positive by melt analysis.
- Semi-quantitative results reported by the BIOFIRE Pneumonia Panels based on real-time PCR data required specific design considerations and rigorous validation testing not typically applied to qualitative assays.
- BIOFIRE panels use nested multiplex PCR with fluorescence detection and monitoring only in the second stage reaction (typically less than 30 cycles). Cycle values from nested reactions are not comparable to single-stage PCR assays.
- BIOFIRE tests do not use precise measurements in any steps from sample handling/loading to microfluidic movements within the pouch. The lack of precise volumes does not affect qualitative detection by DNA melting analysis but could contribute to Cp value variability and affect interpretation.
- Cp values are only accessible through a cloud-based software solution, BIOFIRE FIREWORKS, and are offered to customers for Research Use Only and should not be used for clinical diagnostic purposes. As part of FIREWORKS implementation, users must agree to specific terms to access these values.

bioMérieux does not support or recommend supplemental analysis, including the use of Cp data from BIOFIRE panels, for non-validated clinical applications or interpretation.

Technical Support Contact Information

bioMérieux is dedicated to providing the best customer support available. If you have questions or concerns about this process, please contact your local bioMérieux representative or your authorized distributor.

*All product names, trademarks and registered trademarks are property of their respective owners.

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