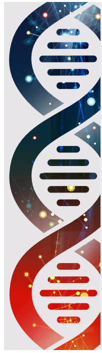

A Model qPCR Instrument Comparability Study: CFX96 Touch and CFX Opus 96 Real-Time PCR Systems

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Abstract

A comparability study is a requirement in the pharmaceutical and biotechnology industries whenever a change is made to existing protocols and/or equipment. The goal of such a study, also called a bridging study, is to provide analytical evidence that the change will not adversely affect the previously approved process. Here, we present a model bridging study to provide evidence that the new CFX Opus 96 Real-Time PCR System is comparable to the CFX96 Touch Real-Time PCR System in terms of specificity, linearity, range, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), robustness, and system suitability.

Introduction

Bio-Rad recently commercialized its CFX Opus Real-Time PCR Systems. The Opus platform is the next evolution of Bio-Rad CFX Real-Time PCR Systems. Our previous generation, the family of CFX Touch Real-Time PCR Systems, has been robust and well-adopted for over a decade. As scientists look to adopt the new Opus platform, work must be undertaken to ensure the systems perform as expected in established workflows.

Labs working under current good manufacturing practices (cGMP) must document and validate all changes to their existing protocols and equipment. With this in mind, we designed the following bridging study to serve as proof data and as an example for others wishing to set up their own comparability or bridging studies with the CFX Opus platform. For example, whereas this study compares the CFX Opus 96 and CFX96 Touch Systems, a similar study design could be used to compare the CFX Opus 384 and CFX384 Touch Systems.

We referred to the guidelines set out in the U.S. Pharmacopeia (USP) (2005, updated 2017) and from the International Conference on Harmonisation (ICH) (2005) as we designed our comparability study. Although there is other guidance available, we believe that basing a comparability or bridging study on these will provide an appropriate framework. The data presented highlight a specific application of the CFX Opus 96 System. They do not represent the complete capabilities of the CFX Opus platform. Additional specifications for the CFX Opus platform can be found in the relevant product information sheets: bulletin 7299 (CFX Opus 96 System) and bulletin 7300 (CFX Opus 384 System).

Experimental Design

This comparability study was designed to compare the following characteristics that must be tested by labs transitioning from one real-time PCR system to another: specificity, linearity (R^2), range, accuracy, precision, limit of detection, limit of quantification, robustness, and system suitability. Detailed definitions for each of these characteristics are provided in the Appendix.

Using the Zika, Dengue, and Chikungunya (ZDC) Real-Time PCR Assays with Reliance One-Step Multiplex RT-qPCR Supermix (as reported by Ma et al. 2019), we compared three CFX96 Touch Systems and three CFX Opus 96 Systems in this study. A total of nine independent standard curves were made over 3 days; each standard curve was used to prepare replicate plates to be run on one CFX96 Touch System and one CFX Opus 96 System. Testing was carried out such that each CFX96 Touch System was paired with a different CFX Opus 96 System on each day. All of our data analysis was carried out using CFX Maestro 2.0 Software. We elected to use CFX Maestro Software because it is the upgrade to CFX Manager Software, and has been the preferred software for use with these systems since it was launched.

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Our predefined data acceptance criteria were as follows:

- Efficiency between 90% and 110%, and linearity (R^2) ≥ 0.99. This is even more stringent than Kibbey (2017), where linearity and slope may be slightly relaxed to R^2 ≥ 0.98 and between –3.1 and –3.8, respectively. (The corresponding efficiency range is roughly 83% to 110% when relaxed as such.) For the last dilution to be included in linearity and efficiency calculations, all 3 replicates must cross the threshold with a standard deviation (SD) less than 0.6
- No template control (NTC) quantification cycle (Cq) > 40 and detection above the LOD in 95% replicates (3 of 3 replicates). Additionally, if any NTCs are positive, the Cq must not be less than the Cq of the lowest standard concentration. The Cq of the lowest standard must not be more than 39

Combined, these acceptance criteria also define system suitability for the ZDC Assay.

Materials and Methods

Individual primers and probes (proprietary sequences) for use in Droplet Digital PCR (ddPCR) were purchased from Integrated DNA Technologies (IDT). Human genomic DNA (gDNA) was purchased from Takara and used at a final concentration of 10 ng per 20 µl reaction. The ZDC Multiplex RT-PCR Assay (Bio-Rad Laboratories, Inc., catalog #12003818) was used with the Reliance One-Step Multiplex RT-qPCR Supermix (Bio-Rad, #12010220).

Fivefold serial dilutions of ZDC Control RNA were made in Tris EDTA (TE) containing 5 ng/ml Yeast tRNA (Thermo Fisher Scientific, #AM7119). A constant input of internal positive control synthetic RNA template and human gDNA was added to each reaction. All reverse transcription quantitative PCR (RT-qPCR) reactions were 20 µl, prepared with Reliance One-Step Multiplex Supermix, and used the 12.5x ZDC Multiplex PCR Assay Mix at 1x final concentration. We added the optional RNase P Assay (Cy5.5) (Bio-Rad, #12004601) to detect gDNA. Reactions were run in triplicate on white-well Hard-Shell 96-Well PCR Plates (Bio-Rad, #HSP9655) and sealed with Microseal 'B' PCR Plate Sealing Film (Bio-Rad, #MSB1001).

Unknowns were prepared by mixing purified Zika virus (ZKV) and chikungunya virus (CHK) RNA (gift from Steve Okino and Michelle Kong) with dengue virus (DV) synthetic DNA template (gBlocks gene fragment purchased from IDT).

The following RT-qPCR protocol was run for all experiments: reverse transcription at 50°C for 10 min; reverse transcriptase inactivation and polymerase activation at 95°C for 10 min; and 45 cycles of 95°C for 10 sec and 60°C for 30 sec with data capture at this step.

All data analysis was performed using CFX Maestro 2.0 Software (Bio-Rad). Efficiency and Cq values were obtained by setting the threshold for each fluorophore at 10% of the maximum relative fluorescence units (RFU) in that channel. Data were exported to Excel 2013 (Microsoft) and analyzed with the Analyse-It Method Validation Edition version 5.66 (Analyse-It).

Droplet Digital PCR was used to determine copy number. Droplets were prepared using the Automated Droplet Generator (AutoDG) (Bio-Rad, #1864101), and DNA was amplified using a 96-well Thermal Cycler (Bio-Rad, #1861096). Droplets were read with the QX200 Droplet Digital PCR System (Bio-Rad, #1864001). Twenty microliter ddPCR reactions used the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, #1864022) with 900 nM primers and 250 nM probes. ZKV (FAM) and CHK (HEX) were assayed together in the same wells. DV (HEX) was assayed individually in separate wells.

Results and Discussion

As an initial test, we prepared serial dilutions of the positive control RNA that comes with the ZDC Assay and ran replicate plates on both qPCR platforms. We also included a fixed concentration of internal positive control RNA and human gDNA in each reaction. Figure 1 shows the results of this experiment and serves as a visual guide for the assay that formed the basis of this comparability study. The three positive control RNAs were present at somewhat different starting amounts, resulting in two targets (ZKV and CHK) with 6-point standard curves and the third target (DV) with a 5-point standard curve.

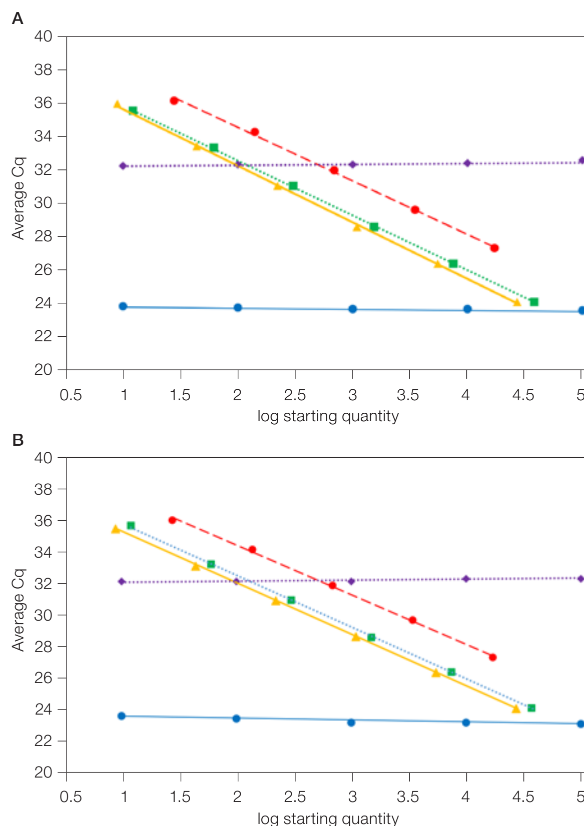

Fig. 1. Demonstration of five-target multiplexing with the (A) CFX96 Touch and (B) CFX Opus 96 Systems. The ZDC Multiplex RT-PCR Assay was used with Reliance One-Step Multiplex Supermix to demonstrate linearity of ZKV (■ FAM), CHK (▲ HEX), and DV (● Texas Red) RNAs, while the internal positive control (IPC) synthetic RNA template (● Cy5) and gDNA (◆ Cy5.5, RNase P) remained constant. The results here represent one run on each platform; they are shown for the purpose of demonstrating the experimental design. Cq, quantification cycle.

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Prior to the present comparability study, we investigated intra-system equivalence to confirm that all three systems within each platform were performing comparably. The Bland-Altman test was used to demonstrate equivalence between systems of the same platform. Sample results are shown in Figure 2 with the predicted 95% limits of agreement (LOA) between systems. Table 1 includes intra-system repeatability correlation coefficients for three variable and two fixed analytes, demonstrating intra-system equivalence in all five detection channels.

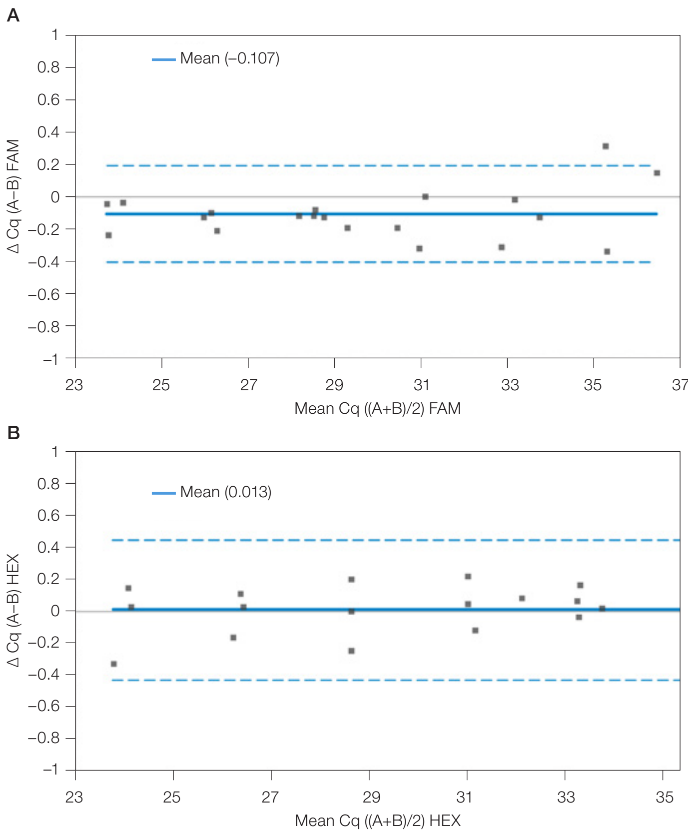

Fig. 2. Demonstration of platform equivalence between (A) CFX96 Touch (FAM) and (B) CFX Opus 96 (HEX) Systems using Bland-Altman LOA plots. Dashed blue lines represent the 95% LOA between systems, and the solid line represents the mean. The mean is an estimate of the average bias. Cq, quantification cycle.

Table 1. Equivalence of three systems for each platform was demonstrated by Deming regression analysis of data in all five channels.

Real-Time PCR System	Intraplatform Comparison	Correlation Coefficient	Slope	95% CI	Intercept
CFX Opus 96	1 vs. 3	1.000	1.007	0.9902 to 1.023	0.1395
	1 vs. 2	1.000	1.015	0.9890 to 1.042	–0.1287
	2 vs. 3	1.000	0.992	0.9782 to 1.005	0.2680
CFX96 Touch	1 vs. 3	1.000	1.011	0.9845 to 1.037	–0.4630
	1 vs. 2	1.000	1.011	0.9886 to 1.034	–0.1566
	2 vs. 3	1.000	0.999	0.9880 to 1.011	–0.3065

Our criteria for specificity were that NTCs must not cross the threshold with a Cq less than or equal to 40. This was achieved on both the CFX96 Touch (0/27) and CFX Opus 96 (0/27) Systems in our experiments. Robustness was demonstrated by including a small amount of human gDNA in all reactions.

We report our findings on linearity, efficiency, range, LOQ, and LOD in Table 2 for the ZKV, CHK, and DV detection assays. Notably, the range, LOQ, and LOD were determined to be equivalent on both platforms. The linearities and efficiencies of the three assays were well within accepted limits and almost identical between platforms.

Table 2. Summary of linearity, efficiency, LOQ, and LOD on CFX96 Touch and CFX Opus 96 Systems.

Parameter	Analyte	CFX96 Touch System	CFX Opus 96 System
LOD	ZKV	2.17 copies	2.17 copies
	CHK	7.6 copies	7.6 copies
	DV	28.2 copies	28.2 copies
LOQ	ZKV	10.8 copies	10.8 copies
	CHK	7.6 copies	7.6 copies
	DV	141 copies	141 copies
Linear range	ZKV	33,880 to 10.8 copies	33,880 to 10.8 copies
	CHK	23,840 to 7.6 copies	23,840 to 7.6 copies
	DV	17,635 to 141 copies	17,635 to 141 copies
Linear range, Cq	ZKV	23.86 to 35.31	23.90 to 35.31
	CHK	24.06 to 35.75	23.97 to 35.68
	DV	27.12 to 36.38	27.23 to 36.29
Linearity, R^2	ZKV	0.999	0.999
	CHK	0.999	0.999
	DV	0.996	0.998
Efficiency, %	ZKV	101.60	101.73
	CHK	103.08	101.54
	DV	100.60	102.10

A note about linearity for the ZDC Assay: we used fivefold serial dilutions of control RNAs (premixed) and determined that our LOQ was six dilution points for ZKV and CHK, and our LOD was seven dilution points for ZKV. However, the DV template was a bit more dilute than the other two templates, such that our LOQ was only four dilution points and our LOD was five dilution points. It is important to note that ICH guidelines recommend that five dilution points be used to establish linearity. The LOD we present for CHK remains the same as the LOQ because our last dilution point yielded positive results in only 84% and 88% of total wells on each platform. As this last dilution did not result in the required 95% detection, we cannot claim this as the LOD, and additional

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testing of different dilutions would be necessary to identify the true LOD. The results presented in Table 2 are virtually indistinguishable between platforms.

We investigated precision by carrying out our testing over several days and repeatability by testing three systems of each platform on each day. The results are presented in Table 3. Intermediate precision was incorporated into this study by including testing over multiple days, preparing three independent standard curves each day (to simulate user-user variability), and using all three of each of the tested systems. We did not calculate the intermediate precision of the IPC because its concentration varied across days. The goal of the IPC is to confirm presence/absence calls, which does not require a highly precise Cq value. The variability we observed here does not affect the precision of the other analytes.

The accuracy of both CFX platforms, expressed as relative bias, was determined by measuring unknowns of known copy number (by Droplet Digital PCR) against the standard curve. The mean bias over the tested range was 6.3% for the CFX Opus 96 Systems and 11.3% for the CFX96 Touch Systems.

The regression analysis of all measurements over the course of this investigation in the FAM, HEX, and Texas Red channels is shown in Figure 3. The resulting slopes with 95% CI indicate that we can accept the hypothesis that the platforms are equivalent.

When viewed as a whole, the data presented here also support the suitability of this assay on both platforms.

Table 3. Precision of CFX Opus 96 and CFX96 Touch Systems, as calculated from one input. For each CFX Real-Time PCR System, N=27 (3 individual units x 3 days x 3 observations).

			Inter-run		Interday		Intermediate
Platform	**Fluorophore**	**Analyte**	**SD**	**95% CI**	**SD**	**95% CI**	**SD**
CFX Opus 96	FAM	ZKV	0.045	0.034 to 0.067	0.107	0.072 to 0.208	0.189
	HEX	CHK	0.020	0.015 to 0.030	0.105	0.068 to 0.225	0.131
	Texas Red	DV	0.057	0.043 to 0.085	0.166	0.110 to 0.336	0.166
	Cy5	IPC	0.246	0.223 to 0.275	0.305	0.249 to 0.392	N/A
	Cy5.5	gDNA	0.152	0.137 to 0.169	0.156	0.141 to 0.175	0.173
CFX96 Touch	FAM	ZKV	0.039	0.029 to 0.057	0.111	0.073 to 0.224	0.165
	HEX	CHK	0.041	0.031 to 0.060	0.224	0.145 to 0.481	0.224
	Texas Red	DV	0.042	0.032 to 0.062	0.093	0.063 to 0.179	0.149
	Cy5	IPC	0.237	0.215 to 0.264	0.404	0.293 to 0.649	N/A
	Cy5.5	gDNA	0.171	0.155 to 0.190	0.222	0.177 to 0.296	0.222

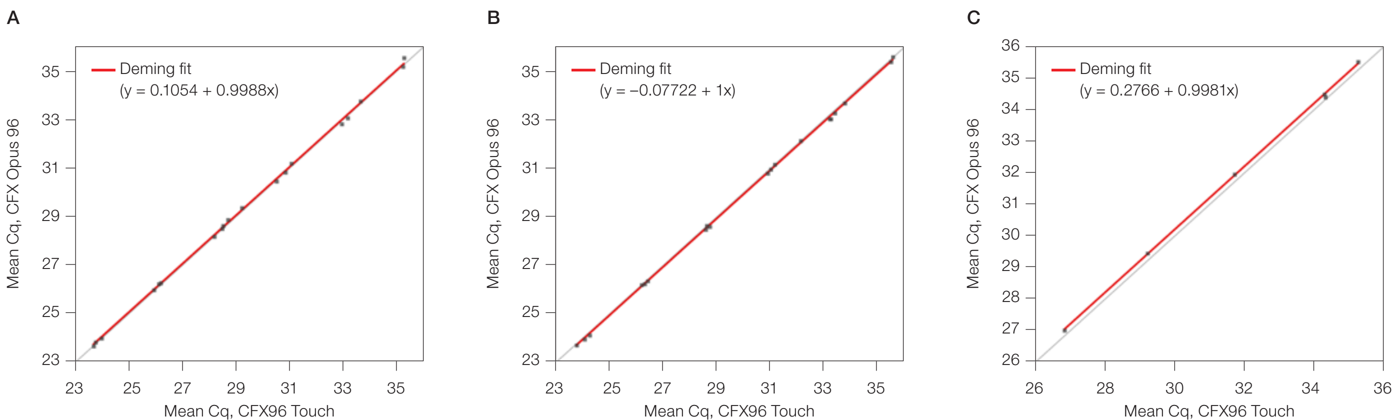

Fig. 3. Deming regression analysis of measurements in all channels. A, FAM channel: slope = 0.9988 (95% CI: 0.9800 to 1.018); **B**, HEX channel: slope = 1.000 (95% CI: 0.9923 to 1.008); and **C**, Texas Red channel: slope = 0.9981 (95% CI: 0.9699 to 1.026). Triplicate measurements of nine independent runs over 3 days on three systems of each platform were analyzed. Cq, quantification cycle.

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Conclusion

We conducted a comparability study as a guide for labs that wish to replace or supplement their CFX96 Touch System with a CFX Opus 96 System. We addressed instrument comparability by assessing performance characteristics described in the USP and ICH guidelines. The results of our model bridging study demonstrate equivalence between the two qPCR platforms.

Our study may also serve as a model study that can be modified to address additional characteristics as necessary.

References

Food and Drug Administration (FDA) Office of Regulatory Affairs (2020). Document ORA-LAB.5.4.5: Methods, Method Verification and Validation, Laboratory Manual Volume II. fda.gov/media/73920/download, accessed February 5, 2021.

Gurtler C et al. (2018). Transferring a quantitative molecular diagnostic test to multiple real-time quantitative PCR platforms. J Mol Diagn 20, 398-414.

International Conference on Harmonisation (2005). Q2(R1) Validation of Analytical Procedures: Text and Methodology.

Kibbey MC (2017). USP General Chapters and Reference Standards that Support the Development and Characterization of Biologics. BioPharm Intl. cdn.sanity.io/files/0vv8moc6/biopharn/567a2675cb84cf642712d04e99d5ddf20eb55334.pdf, accessed February 5, 2021.

Ma J et al. (2019). Sensitive codetection of viral-pathogen RNA and DNA using one-step multiplex RT-qPCR. Bio-Rad Bulletin 7196.

United States Pharmacopeial Convention. (2005, updated 2017). U.S. Pharmacopeia <1225> Validation of Compendial Methods. uspbpep.com/usp29/v29240/usp29nf24s0_c1225.html, accessed February 5, 2021.

Appendix: Definition of Characteristics

The following definitions were compiled from ICH (2005), USP (2017), and U.S. FDA (2020) guidelines.

Accuracy — the nearness of a result or the mean of a set of measurements to the true value.

Limit of detection (LOD) — the lowest concentration level that can be determined as statistically different from a blank at a specified level of confidence. It is determined from the analysis of sample blanks.

Limit of quantification (LOQ) — the level above which quantifiable results may be determined with acceptable accuracy and precision.

Linearity — the ability of a method to elicit results that are directly proportional to analyte concentration within a given range.

Precision — agreement between a set of replicate measurements. Precision does not necessarily refer to the true value. The precision of test results is described by statistical methods such as a standard deviation or confidence limit. Repeatability expresses precision under the same operating conditions over a short period of time. Intermediate precision expresses precision across within-laboratory variations, such as different days, different analysts, and different equipment. Reproducibility expresses the precision between laboratories.

Range — the interval between the upper and lower concentration of analyte in a sample for which it has been demonstrated that the analytical procedure has an acceptable level of accuracy, precision, and linearity.

Robustness — an analytical procedure's capacity to remain unaffected by small but deliberate variations in method parameters. It provides an indication of the procedure's reliability during normal usage.

Specificity — the ability to assess unequivocally an analyte in the presence of impurities, degradation products, or other components that may be expected to be present.

System suitability — system suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. See pharmacopoeias for additional information.

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