# Validation of Nala PGx Core® on CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems

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## Abstract

The clinical utility of pharmacogenomics (PGx) has been gaining momentum, due to findings that up to 70% of adverse drug reactions (ADRs) are associated with genetic variations. Nala PGx Core® is a comprehensive multi-gene panel employing qPCR-based methodology. This panel encompasses 18 genetic variants and 2 CYP2D6 Copy Number markers, spanning four pharmacogenes: CYP2C9, CYP2C19, CYP2D6, and SLCO1B1. An evaluation conducted across diverse ethnic groups in Singapore and Indonesia, utilizing the Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use, demonstrated robust and accurate genotyping results. With the introduction of the next-generation real-time PCR technology by Bio-Rad, here we report results from a validation study performed to assess the suitability of the qPCR platform for running Nala PGx Core® on both the CFX 96 Real-Time PCR (RUO) and CFX 96 Dx Real-Time PCR Systems.

## Introduction

A validation study was performed to expand the utility of Nala PGx Core® for use with specific qPCR platforms, as each qPCR platform may differ in its Cq range and Last Cycle Relative Fluorescence Unit (RFU) measurements. Therefore the purpose of this study is to determine the acceptable settings and thresholds for genotyping specimens with Nala PGx Core® on Bio-Rad CFX Opus 96 Real-Time PCR systems (RUO and Dx) and evaluate whether these conditions are similar to Nala PGx Core®'s Minimum Viable Product (MVP) platform, the Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use. The scope of study includes a determination of the Positive Control acceptance ranges and the genotyping thresholds specifically for CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems.

## Materials and Method

### DNA Samples

This study utilized a total of 39 DNA samples acquired from the HapMap Project at the Coriell Institute of Medical Research. 30 HapMap samples were used for the verification runs and to generate the genotyping threshold on the CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems: HG00111, HG00358, HG01085, NA11992, NA12003, NA18861, NA06989, NA07357, NA12006, NA12155, NA12762, NA12872, NA18526, NA18552, NA18564, NA18592, NA18608, NA18959, NA18961, NA18971, NA19143, NA19152, NA19153, NA19393, NA21114, HG00140, HG01094, HG01845, NA11892, and NA11931. 9 HapMap samples were used for genotyping threshold validation on Bio-Rad CFX Opus 96 (RUO and Dx): HG00366, HG00463, HG01097, HG01883, HG02029, HG03686, HG03854, HG04210, and NA11881.

The positive controls used in this study, specifically the Single Nucleotide Polymorphism Positive Control (SNP PC) and the Copy Number Variant Positive Control (CNV PC), are available in the Nala PGx Core® kit. Nuclease-free water is used as the no-template control (NTC).

DNA samples were diluted with Tris-EDTA buffer pH 8.0 to 2 ng/uL before use as template for qPCR reactions.

### qPCR Reagents

qPCR reactions in this study were carried out using reagents provided in Nala PGx Core®. The preparation procedure, plate layout, and qPCR reaction conditions are all provided in the instructions for use (IFU) of the kit for the Nala PGx Core®.

Genotype calls and CNV calculations in this study were performed on Nala CDS Core®, a companion software used in tandem with Nala PGx Core® which provides the genotyping thresholds and CNV calculation methods.

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## Results and Discussion

CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems and the Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use all employ the same method of detection. Thus, the experiments of this study aimed to validate that the settings and thresholds used on Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use would produce reliable results if implemented on the CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems. As such, the settings and thresholds of the Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use were analyzed for application to the CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems to determine whether adjustments were needed. The FAM, HEX, and Cy5 detection channels are used for genotyping in Nala PGx Core®, and each channel requires its own specific settings and thresholds. The FAM channel corresponds to wild-type genotype detection, while the HEX and Cy5 channels correspond to mutant genotype detection.

### Positive Control (PC) Settings and Acceptance Criteria on Opus

Six plates of PC, each containing 3 runs, were run on the CFX Opus 96 Real-Time PCR (RUO) System using the Nala PGx Core® following the instructions provided here and were then analyzed with Baseline Threshold settings from the Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use: Single Threshold = 300.00, Begin = 10, End = 20. Results showed that the SNP9 assay amplification values detected in the Cy5 channel were too low to cross the baseline threshold and generate a Cq value. Therefore, the baseline threshold for SNP9 on Cy5 channel was adjusted so that Cq values can be generated for testing purposes. The preliminary baseline threshold was calculated at 10 times of the standard deviation for the NTC RFU obtained from the SNP9 assay across all plates. The run data from the 6 plates of PC were then reanalyzed using the adjusted settings and were able to generate Cq values (Figure 1), confirming that the Cy5 baseline threshold setting is suitable for use. The runs were then reanalyzed again on all assays and channels with the newly adjusted settings, and were able to generate Cq values that passed the PC acceptance criteria (Figure 2).

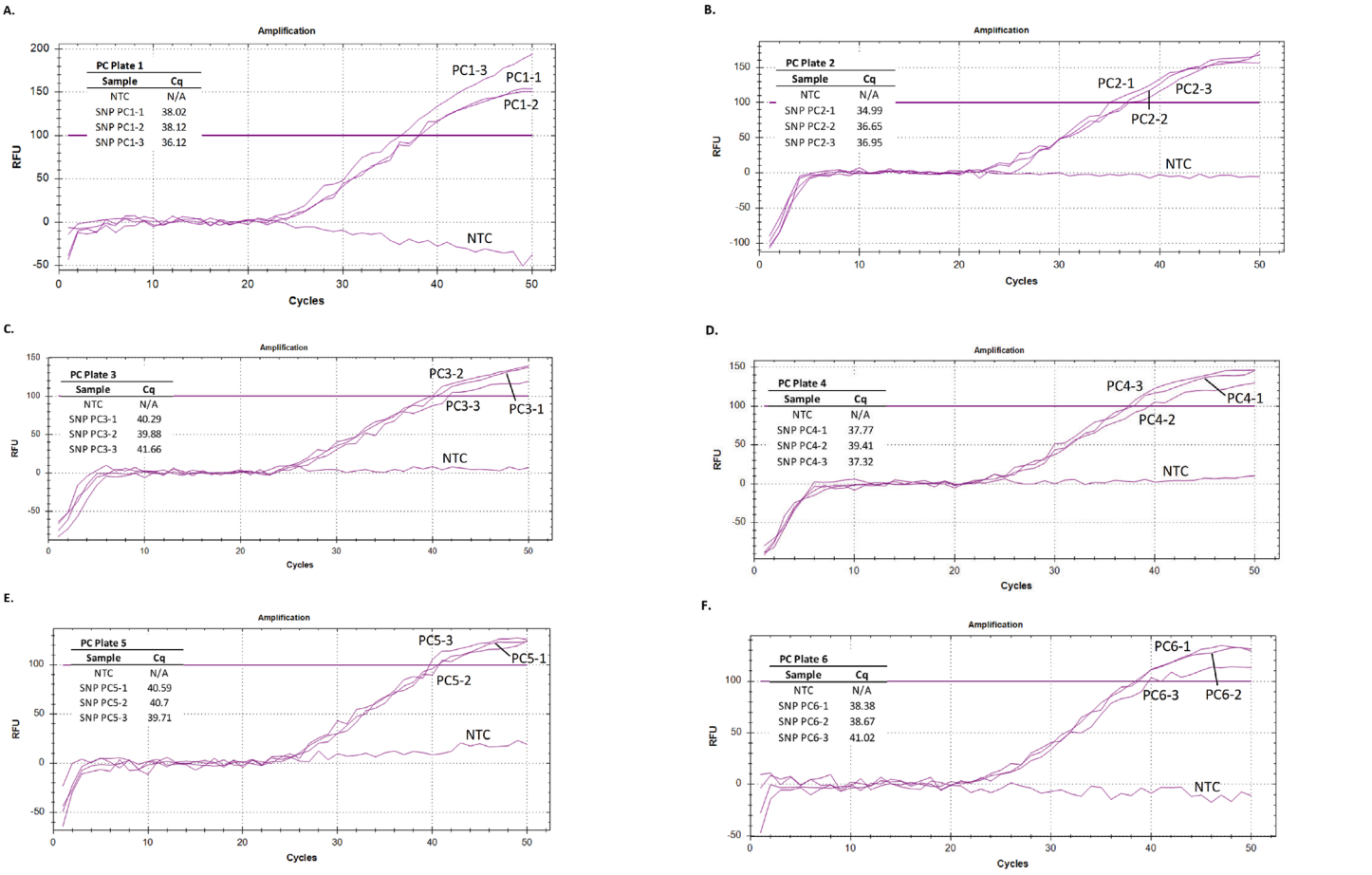



**Fig. 1. Amplification plots for the Cy5 channel of the 6 plates containing the PC run on the Bio-Rad CFX Opus 96 Real-Time PCR (RUO) system with the adjusted baseline threshold.**
Resulting run files from the Cy5 channel from the 6 plates of PC run on CFX Opus 96 Real-Time PCR (RUO) were analyzed with the adjusted baseline threshold settings (**A** – **F**) and were able to generate Cq values. This finding shows that the adjusted setting is suitable for use on CFX Opus 96 Real-Time PCR (RUO).

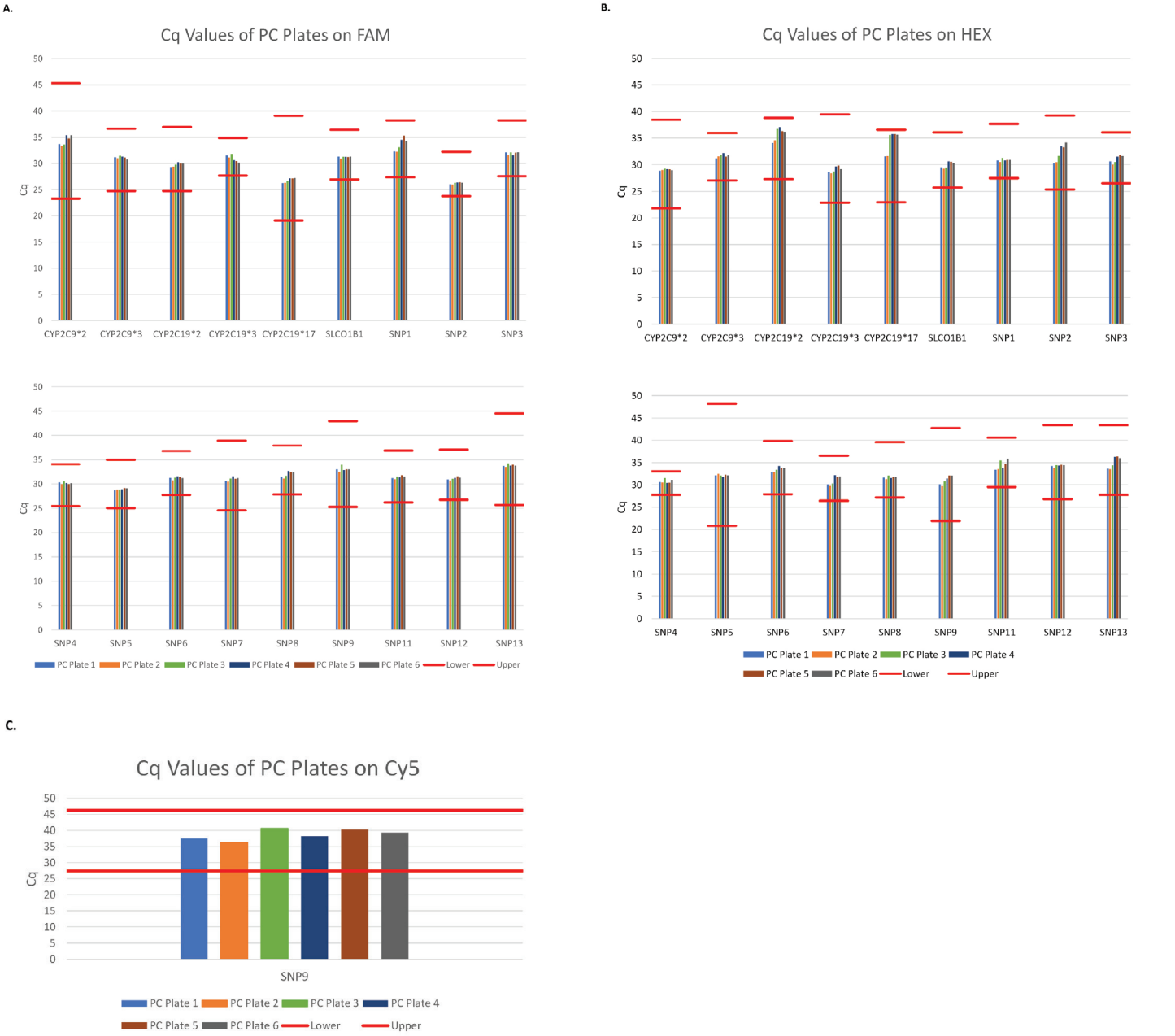



**Fig. 2. Cq values of the 6 plates containing the Positive Control (PC) run on CFX Opus 96 Real-Time PCR (RUO) system on FAM, HEX, and Cy5 channels obtained after analysis using the adjusted baseline threshold settings.**
The 6 plates containing the PC that were run on CFX Opus 96 Real-Time PCR (RUO) system were reanalyzed for all assays and channels (**A**, FAM channel; **B**, HEX channel; **C**, Cy5 channel) using the adjusted baseline threshold settings. All assays across all channels yielded Cq values that were within the ranges of the PC acceptance criteria (red line), showing that the adjusted baseline threshold setting is suitable for use with the CFX Opus 96 Real-Time PCR (RUO) system.

One additional plate of PC was run on Bio-Rad CFX Opus 96 Dx (IVD) Real-Time PCR System and analyzed with the adjusted settings, and was able to pass the acceptance criteria as well (Figure 3).

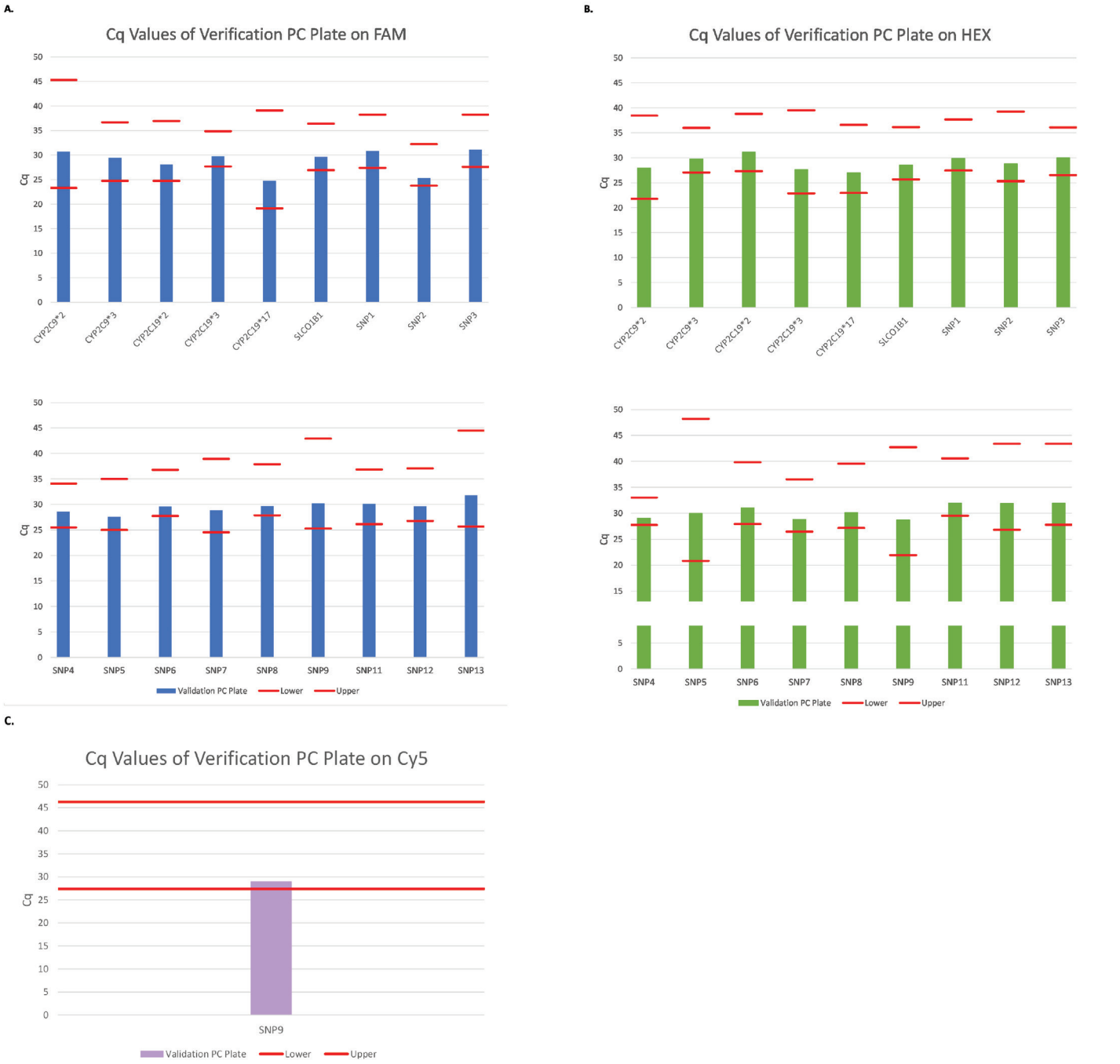



**Fig. 3. Cq values of the validation Positive Control (PC) plate run on CFX Opus 96 Dx (IVD) Real-Time PCR System for the FAM, HEX, and Cy5 channels following analysis using the adjusted baseline threshold settings.**
The plate of PC run on Bio-Rad CFX Opus 96 Dx (IVD) Real-Time PCR System was also analyzed for all assays and channels (**A**, FAM channel; **B**, HEX channel; **C**, Cy5 channel) using the adjusted baseline threshold settings. All assays across all channels yielded Cq values that were within the ranges of the PC acceptance criteria (red line), showing that the adjusted baseline threshold setting is also suitable for use with Bio-Rad CFX Opus 96 Dx (IVD) Real-Time PCR System.

These results showed that the adjusted settings are suitable for use on both the CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems, as all PC runs were able to generate Cq values that passed the acceptance criteria. Thus, the same PC acceptance criteria validated for use of the Nala PGx Core® on the Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use is verified for use on CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems as well.

#### Nala PGx Core® Genotyping Thresholds on CFX Opus 96 (RUO and Dx)

30 HapMap samples of varying genotype calls in all Nala PGx Core® assays were run on both Bio-Rad CFX Opus 96 RUO and Dx. The runs were analyzed using baseline settings generated in the previous section, and genotypes were called using the Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use that is available for use with Nala CDS Core®. Genotype call results showed that all assays were called with concordance rate of >95% except for SNP1 assay (Table 1). Additionally, CNV calculations performed in Nala CDS Core® using the settings of the Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use were also fully concordant. Hence, only SNP1 genotyping threshold required revision to be suitable for use with Bio-Rad CFX Opus 96 (RUO and Dx); genotyping thresholds for all other assays remain the same as Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use.

**Table 1. Genotype Concordance Rate for Calls Determined Using the Genotyping Threshold and Settings of the Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use.** HapMap samples were run on Bio-Rad CFX Opus 96 (RUO and Dx) and their genotypes were called with the genotyping threshold of the Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use. The percentage in red represents assays that are called with concordance rate of <95%.

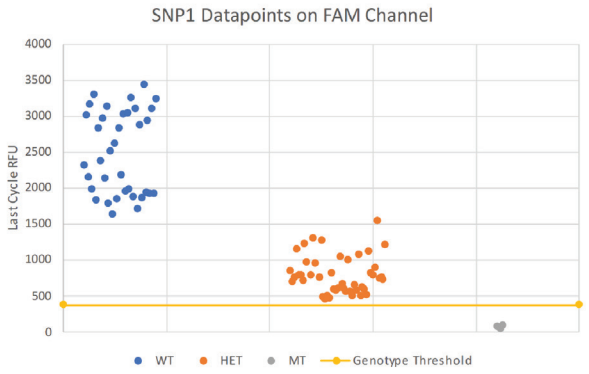
| | 9*2 | 9*3 | 19*2 | 19*3 | 19*17 | SLCO1B1 | SNP1 | SNP2 |
|---|---|---|---|---|---|---|---|---|
| Number of Concordant Samples | 30 | 30 | 30 | 30 | 30 | 29 | 68 | 30 |
| Total Number of Samples | 30 | 30 | 30 | 30 | 30 | 30 | 84 | 30 |
| Concordance Rate Call (%) | 100 | 100 | 100 | 100 | 100 | 96.67 | 80.95 | 100 |
| | SNP3 | SNP4 | SNP5 | SNP6 | SNP7 | SNP8 | SNP9 | SNP11 |
| Number of Concordant Samples | 30 | 30 | 29 | 30 | 29 | 30 | 30 | 30 |
| Total Number of Samples | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 |
| Concordance Rate Call (%) | 100 | 100 | 96.67 | 100 | 96.67 | 100 | 100 | 100 |
| | SNP12 | SNP13 | Intron2 | Exon9 | CNV | | | |
| Number of Concordant Samples | 30 | 29 | 30 | 30 | 30 | | | |
| Total Number of Samples | 30 | 30 | 30 | 30 | 30 | | | |
| Concordance Rate Call (%) | 100 | 96.67 | 100 | 100 | 100 | | | |

To adjust the genotyping threshold for SNP1, the last cycle RFU values for each genotype, homozygous wild-type (WT), heterozygous (HET), and homozygous mutant (MT), in the SNP1 assay across all 30 HapMap samples were compiled and analyzed using our proprietary statistical method. The results provide a candidate threshold to determine amplification detected in each channel, and thus can be used to call genotypes.

The last cycle RFU values of each sample in SNP1 assay were then graphed on a scatterplot to confirm that the candidate thresholds in both FAM and HEX channels were lower than the lowest HET datapoint, and were able to correctly call all the corresponding samples, while remaining higher than the highest suppressed genotype datapoints in order to avoid mistaken calls of those suppressed genotypes (Figure 4). The thresholds set for both FAM and HEX channels fulfill these requirements and were then used as the final genotyping threshold for SNP1 assay on Bio-Rad CFX Opus 96 (RUO and Dx).

After adjusting the genotyping threshold for SNP 1 assay, the concordance rate became 100% as all samples were called correctly to their actual genotype. All other assays will use the same genotyping threshold as Bio-Rad CFX96 Touch IVD as all 30 HapMap samples were called with >95% concordance in those assays (Table 1).

**A.**




**B.**

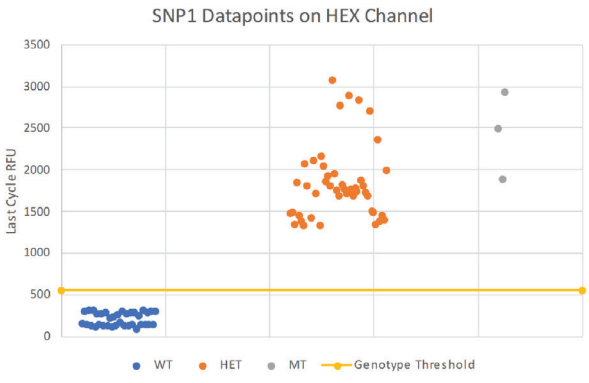



**Fig. 4. Scatterplots of the last cycle Relative Fluorescent Unit (RFU) of HapMap samples using in the SNP1 assay.**
Last cycle RFU values of each sample in the SNP1 assay were plotted as a scatterplot (WT samples in blue, HET in orange, MT in gray) and were compared to the adjusted SNP1 genotyping threshold (yellow line) to confirm the threshold setting provided correct calls of the samples. **A**, scatterplot confirms that the SNP1 genotyping threshold on FAM channel was able to correctly call samples, since it is lower than the lowest HET datapoint and higher than the highest MT datapoint; **B**, similarly, the SNP1 genotyping threshold on HEX channel was also able to correctly call samples, since it is lower than the lowest HET datapoint and higher than the highest WT datapoint.
WT - wild type; HET - heterozygous; MT - homozygous mutant.

### Validating Genotyping Threshold for CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems

A total of 9 HapMap samples were run, analyzed, and genotyped using the final baseline settings and genotyping thresholds discussed in sections above. The resulting analysis showed that all 9 samples were called correctly across all assays, and CNV calculations were concordant (Table 2).

**Table 2. Validation of the genotyping concordance rate for samples called using the genotyping threshold of the CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems.** Nine HapMap samples were run, analyzed and genotyped using the CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems with newly adjusted thresholds, resulting in correct calls for all 9 samples across all assays.

| | 9*2 | 9*3 | 19*2 | 19*3 | 19*17 | SLCO1B1 | SNP1 | SNP2 |
|---|---|---|---|---|---|---|---|---|
| Number of Concordant Samples | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| Total Number of Samples | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| Concordance Rate Call (%) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | SNP3 | SNP4 | SNP5 | SNP6 | SNP7 | SNP8 | SNP9 | SNP11 |
| Number of Concordant Samples | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| Total Number of Samples | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| Concordance Rate Call (%) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | SNP12 | SNP13 | Intron2 | Exon9 | CNV | | | |
| Number of Concordant Samples | 9 | 9 | 9 | 9 | 30 | | | |
| Total Number of Samples | 9 | 9 | 9 | 9 | 30 | | | |
| Concordance Rate Call (%) | 100 | 100 | 100 | 100 | 100 | | | |

## Conclusion

Nala PGx Core® demonstrated similar performance on CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems as the Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use. Nala PGx Core® exhibited similar performance on both the CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems when compared to the Bio-Rad CFX96 Touch™ Real-Time PCR System registered for IVD use. Only 2 adjustments were made to the initial settings and thresholds for the CFX96 Touch™ Real-Time PCR System registered for IVD use: the Cy5 baseline settings for running the PC and the SNP1 threshold for genotype call samples. The rest of the settings and thresholds in CFX96 Touch™ Real-Time PCR System registered for IVD use were already suitable and applicable to CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems. With the new settings and thresholds, reference samples with known genotype calls across all assays and CYP2D6 CNV values were analyzed, resulting in 100% concordance with their true genotype calls and CNV. This finding validates that Nala PGx Core® is suitable for use on CFX Opus 96 Real-Time PCR (RUO) and the CFX Opus 96 Dx (IVD) Real-Time PCR Systems.

## References

Kothary AS et al. (2021). Validation of a multi-gene qPCR-based pharmacogenomics panel across major ethnic groups in Singapore and Indonesia. Future Medicine Pharmacogenetics Vol. 22, No. 16. https://www.futuremedicine.com/doi/10.2217/pgs-2021-0071, assessed September 8, 2023.

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