Automated, high-throughput quantitative PCR for accurate, economical and precise copy number determination

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Introduction

Gene copy number variation (CNV) in the form of duplications and deletions represents a large portion of the overall genetic variation and research continues to highlight the importance of CNV in a variety of research areas. For this reason, a variety of techniques have been developed to identify these changes in copy number, with each method having its own advantages and disadvantages. Quantitative PCR (qPCR) is a popular choice due to its high sensitivity, relatively low cost, flexibility in assay design, and short time to result. The hydrolysis probe formats offered by LGC Biosearch Technologies are well suited for CNV analysis. BHQplus® probes incorporate duplex-stabilisers allowing enhanced binding stability, enabling compact probe sequences for improved target specificity. The novel BHQnova™ probe introduces an internal quencher, improving quenching efficiency for longer probe sequences. This leads to improved signal to noise and provides flexibility in assay design for regions with challenging base composition. Pairing BHQ® probes with the IntelliQube® real-time qPCR instrument* provides an effective solution to CNV analysis. Utilising Array Tape® technology, the IntelliQube integrates liquid handling with qPCR analysis in miniaturised reaction volumes.

In this study we investigated the performance of qPCR assays incorporating BHQ probes run on the IntelliQube for determination of cytochrome P450 2D6 (CYP2D6) gene copy number. Deletions and duplications of CYP2D6 can impact the metabolism of many clinically relevant drugs; therefore, making it a popular target for continued research into human health. Accuracy and reproducibility were assessed using purified gDNA samples from cell lines obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research. Copy number results were compared to previously published literature and to data obtained using a competitor's assay designs. The results demonstrate that the IntelliQube, when used in conjunction with BHQ probes, provides a powerful solution for accurate, automated, high-throughput CNV research and analysis.

Materials and methods

Fifty-six purified genomic DNA samples were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research. DNA samples were diluted to 6.25 ng/µL with TE buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA) before use. CYP2D6 copy number variation was assessed using a duplexed reaction: one assay targets a region of the CYP2D6 gene, and the other is an endogenous reference gene. A BHQplus assay targeting intron 2 and BHQnova assays targeting exon 9 and hTERT (endogenous reference) were used. The assays consisted of a final concentration of 200 nM probe and 600 nM of each primer in the reaction. For comparison of assay performance, a competitor's commercially available BHQ Probe Master Mix and assays targeting the same CYP2D6 regions were used with RNase P as an endogenous reference. Two qPCR BHQ Probe Master Mix, one commercially available were used in these experiments.

CYP2D6 (Intron 2)

Forward Primer: GCTGTCCCCACTCGCTG
Reverse Primer: GGACCCGGGTGGGTG
BHQplus Probe: FAM-TGCCCAGCTCGGAC-BHQ-1

CYP2D6 (Exon 9)

Forward Primer: GCTTCTCGGTGCCCACT
Reverse Primer: GGCACAGCACAAAGCTCA
BHQnova Probe: FAM-CCAGCCACC-Nova-ATGGTGTCTTTGC-BHQ-1

hTERT (Endogenous Reference)

Forward Primer: CCTGCGCGTCATCTCTGA
Reverse Primer: TCCCTGCGTTCTTGGCTTTC
BHQnova Probe: CAL Fluor Orange 560-ACGGCCTCC-Nova-CTCTGCTACTCCA-BHQ-1

Instrumentation and Analysis:

The IntelliQube was used for all sample and reagent dispensing, thermal cycling, and real-time fluorescence detection and analysis. DNA samples (800 nL) were dispensed into 768-well Array Tape with the multi-channel, CyBio dispense pipette. BHQ Probe Master Mix (800 nL) containing 2X assay was dispensed with the non-

contact dispense jet to create a total reaction volume of 1.6 µL. Thermal cycling was performed in the IntelliQube according to the manufacturer's instructions. Copy number was determined using IntelliScore® Software, using the comparative Cq method (ΔΔCq). For our analysis, Coriell DNA sample NA17280 was selected as the calibrator sample containing two copies per genome. Each sample was run in quadruplicate.

Results

One of the common challenges for accurate CNV analysis using qPCR is the need for near equivalent PCR efficiencies for both the target and reference assays. The comparative Cq method relies on ΔCq values ($Cq_{target} - Cq_{reference}$), which can be directly impacted by differences in PCR efficiencies. This challenge is of particular importance when using a wide range of DNA input concentrations (1). To address these concerns, the BHQ assay designs were optimised to achieve efficiencies near 100 % with minimal difference between the target and reference assays. These efficiencies were assessed by creating a standard curve (1.56-50 ng/µL) of representative Coriell samples having variable CYP2D6 copy number (1, 2, and 3 genome copies). The standard curve data for the sample with 2 genome copies is shown in Figure 1A and 1B. The target and reference assays had an average difference in efficiency of 0.54% for Intron 2 and 1.29% for Exon 9. To further evaluate the CNV assay performance over a range of DNA inputs, ΔCq was determined at each concentration in the standard curve. The ΔCq values stayed consistent across the range of DNA inputs tested for both CYP2D6 targets assays (Figure 1C and 1D).

The optimised assays were then used to evaluate 56 Coriell hgDNA samples for gene copy number using both CYP2D6 targets. Results from the BHQ assay designs were compared to a competitor's commercially available CYP2D6 copy number assays. The copy number bar graphs for each sample are displayed in Figure 2. The BHQ assays had 100 % concordance with previously published results for both assays (2). The commercially available CYP2D6 Intron 2 assay had a single discrepancy for sample NA17039, identifying it as having 1 copy when the expected copy number is 2. It is possible that this discrepancy is due to differences in primer/probe design. Copy number results determined for all assays were consistent across three runs with two different BHQ Probe Master Mix (data shown for only one BHQ Probe Master Mix).

The exon 9 assay is traditionally run in conjunction with other CYP2D6 targets in order to identify the presence of potential CYP2D6-CYP2D7 hybrid alleles. When a CYP2D6-CYP2D7 hybrid is present, it is typically amplified by the intron 2 assay, which can lead to an incorrect interpretation of functional gene copies. The exon 9 assay is able to discriminate between a true CYP2D6 target and the hybrid allele. In our Coriell sample set we identified this phenomenon in six samples, NA17109, NA17209, NA17226, NA17058, NA17084, and NA17248, three of which were previously unreported. Using the BHQ assay, sample NA17058 appears to have 2 hybrid alleles, leading to a calculated copy number of 4 using the Intron 2 assay and a copy number of 2 for the Exon 9 assay. Using this sample as a putative 4 copy sample,

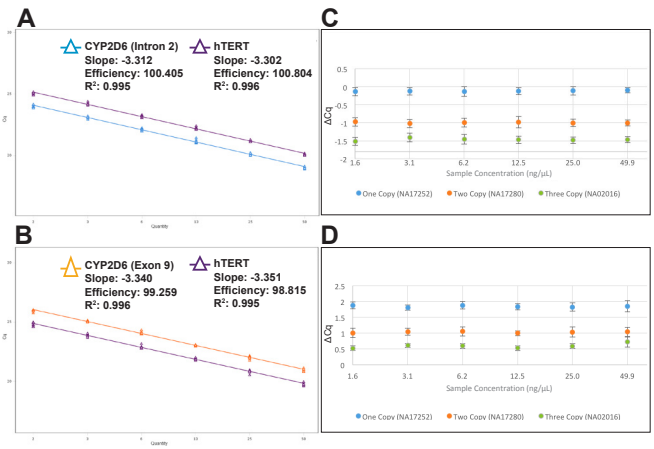

Figure 1. A) The standard curve data including slope, PCR efficiency, and R^2 values are shown for the CYP2D6 Intron 2 assay duplexed with the hTERT reference assay. The standards consist of a 2-fold dilution series from 50 ng/µL to 1.56 ng/µL. B) The ΔCq values ($Cq_{target} - Cq_{reference}$) for the Intron 2/hTERT duplex were plotted for the range of concentrations tested in the standard curve. Error bars indicate the 95 % confidence interval based on eight replicates per sample. C) The standard curve data including slope, PCR efficiency, and R^2 values are shown for the CYP2D6 Exon 9 assay duplexed with the hTERT reference assay. The same standard curve was used as described above. D) The ΔCq values for the Exon 9/hTERT duplex were plotted for the range of concentrations tested in the standard curve. Error bars indicate the 95% confidence interval based on eight replicates per sample.

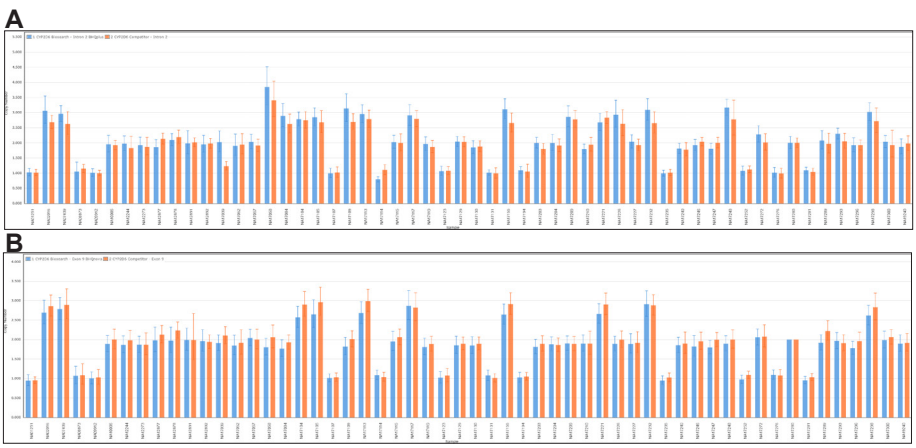

Figure 2. Copy number bar graphs were plotted for Intron 2 (A) and Exon 9 (B) using the IntelliScore software. The copy number results for all 56 Coriell samples are shown in the plots for both the BHQ CNV assay designs (blue) as well as the commercially available competitor assays (orange). Error bars indicate the 95% confidence interval based on four replicates per sample.

the Intron 2 assay was used in subsequent testing with the IntelliQube to assess the precision of the copy number results in relation to the ability to distinguish between different copy numbers. To determine how many replicates are needed to statistically distinguish a four copy sample from a three copy sample, the 95% confidence intervals were calculated with a variable replicate numbers of representative 1, 2, and 3 copy samples and the putative 4 copy sample. It was determined that with as little as three replicates, the three and four copy number samples

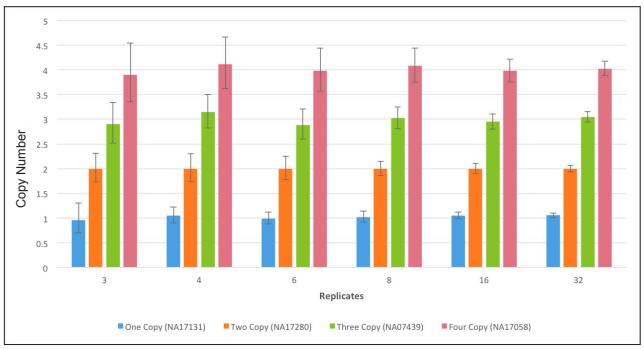

Figure 3. Copy number analysis using a variable number of replicates. Results are shown for samples with one (NA17131), two (NA17280), three (NA07439), and four copies (NA17058) of the CYP2D6 Intron 2 target. The test consisted of 32 replicates of each sample. Replicates were systematically removed from the analysis using a random number generator. Copy number and the associated 95 % confidence intervals were calculated based on the number of replicates remaining. The results showed that for this particular experimental setup, the 3 copy sample could be distinguished from the 4 copy sample with 95 % confidence with as few as three replicates.

could be distinguished from each other assuming a 95% confidence (Figure 3).

Conclusions

BHQnova and BHQplus probes partner well with the IntelliQube for accurate copy number determination. The availability of different probe formats allows for greater flexibility in assay design when needed for particularly difficult nucleotide sequences. Additionally, the integration of liquid handling, thermal cycling, detection, and analysis within a single instrument enables researchers to benefit from more efficient qPCR workflows. The data presented here suggests that IntelliQube users can experience the economic benefits of miniaturised reactions volumes, without compromising the data quality necessary for CNV analysis. The ability to resolve 3 versus 4 copies (a 1.33-fold difference) with as little as three replicates in the experiment with the Intron 2 assay, highlights the very low standard deviation (σ) of the system as a whole. According to statistical models described by Weaver et al. (3), a PCR system with a σ of 0.16 would require 11 replicates to distinguish 3 from 4 copies with 95% confidence. The results achieved on the IntelliQube are promising for future applications where even finer resolution (i.e., 4 copies vs 5 copies or 5 copies vs 6 copies) may be desired. It is important to note that the overall system σ may vary based on inputs to the system such as assay, sample, or BHQ Probe Master Mix and should be assessed on a case by case basis. Regardless, the excellent resolution of the system paired with the ability to easily create numerous PCR replicates through the automated dispensing capabilities, may make this a powerful tool for additional CNV applications.

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