

Probing Copy Number Variations Using Bio-Rad's QX100™ Droplet Digital™ PCR System

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Introduction

The Role of Copy Number Variation in Phenotype and Disease

Cytogenetic studies over the past 50 years have hinted at the impact that copy number variations (CNVs) can have on phenotypic traits and disease susceptibility (Beckmann et al. 2008). The advent of holistic discovery methods afforded by DNA microarrays (Redon et al. 2006) and more recently by next-generation DNA sequencing (Sudmant et al. 2010) have allowed us to better understand the extent of genetic variation in genomes. Using single nucleotide polymorphism (SNP) DNA microarrays, Redon et al. (2006) estimated that upwards of 12% of the human genome consists of copy number variable regions greater than 1 kb, harboring approximately 1,000 genes. Next-generation sequencing analyses from the 1000 Genomes Project indicated that the majority of CNVs exist as five or fewer copies per cell, but the 56 most variable gene families have median CNV values between 5 and 368. Moreover, some gene families show significant copy number differences among the three HapMap populations analyzed (Sudmant et al. 2010).

The most recent comparative genome-wide characterizations estimate that over 29% of the reference human genome harbors more than 38,000 CNVs greater than 100 bp (Vissers and Stankiewicz 2012). These variants have the potential to influence phenotype through both gene dosage and structure and underlie hundreds of inherited microdeletion and microduplication syndromes, including early-onset Alzheimer's disease (duplication of the *APP* gene), familial Parkinson's disease (duplication and triplication of the *SNCA* gene), retinoblastoma (deletion of the *RB1* gene), X-linked mental retardation (deletion of *IL1RAPL1* or *CASK*, or deletion and duplication of *SOX3*), spinal muscular atrophy (deletion and duplication of the *SMN1* and *SMN2* genes), and drug metabolism and transporter abnormalities such as deletion and duplication of *CYP2D6* (Vissers and Stankiewicz 2012, Beckmann et al. 2008). Besides these Mendelian traits, deletions and amplifications have been found to be associated with complex traits and somatic copy number alterations, or CNAs (Beckmann et al. 2008, Speleman et al. 2008).

Need for Improved Methods to Validate, Diagnose, and Monitor Copy Number Variations

Given the high incidence and clinical impact of CNVs, a precise, rapid, and cost-effective method is needed for high-throughput validation of candidate CNV associations and for subsequent routine deployment in diagnostic settings. The predominant method used to validate CNVs in larger populations is real-time or quantitative PCR (qPCR), which measures the relative rates of fluorescence increases during the exponential amplification of target and single-copy reference genes. The accuracy and precision of these measurements can be impacted by multiple factors including differences in amplification rates between the target and reference genes, variations in their amplification rates during qPCR, sampling error due to DNA concentration, and analysis errors (Karlen et al. 2007). Weaver et al. (2010) rigorously characterized these factors and found that systemic errors can be addressed by increasing the number of replicates to achieve the desired precision. However, the required number of replicates increases rapidly as finer discrimination is desired, with four replicates required to distinguish a twofold difference (for example, a CNV of 1 vs. 2) and up to 18 replicates to distinguish a 1.25-fold difference (for example, a CNV of 4 vs. 5).

Droplet Digital PCR (ddPCR™) is a DNA molecule counting technique that directly counts the absolute number of target and reference DNA molecules in a sample, enabling small fold differences to be determined for higher-order CNV states (Hindson et al. 2011). In ddPCR, targets of interest are partitioned into droplets and amplified to endpoint with TaqMan probes as in qPCR, then their concentrations are determined based on the number of fluorescently positive and negative droplets in a sample well. Thus, the fluorescence signal in qPCR is converted from an analog signal into a digital one, thereby removing the requirement for standard curves and allowing the determination of target copies on an absolute scale with high precision (Hindson et al. 2011). Our studies using ddPCR to analyze CNV status focused on several clinically important loci including the *CCL3L1* locus, which is thought to influence susceptibility to HIV-1 infection and has been the subject of conflicting results among numerous studies using qPCR (Gonzalez et al. 2005, Liu et al. 2010). Our results demonstrate that Bio-Rad's QX100 Droplet Digital PCR system enables excellent discrimination of copy number states.

Materials and Methods

Assays and DNA samples

All TaqMan hydrolysis probe assays were either predesigned assays obtained from Life Technologies Corporation or were designed at Bio-Rad Laboratories, Inc. and synthesized by Integrated DNA Technologies (BHQ) or Life Technologies (NFQ-MGB). The *MRGPRX1* assay sequences were from Hosono et al. (2008): forward primer, 5'-TTAAGCTTCATCAGTATCCCCA-3'; reverse primer, 5'-CAAAGTAGGAAAACATCATCACAGGA-3'; probe, 5'-FAM-ACCATCTCTAAAATCCT-MGB-3'. *CYP2D6*_FAM-MGB was obtained as a predesigned assay, Hs00010001_cn (Life Technologies). *QL_Eichler_CCL3_LG_FAM*-BHQ sequences were adapted from Sudmant et al. (2010): fwd primer, 5'-GGGTCCAGAAATACGTCAGT-3'; rev primer, 5'-CATGTTCCCAAGGCTCAG-3'; probe, 5'-FAM-TTCGAGGCCAGCGACCTCA-BHQ-3'. *QL_RPP30_1_VIC*-MGB reference assay: fwd primer, 5'-GATTTGGACCTGCGAGCG-3'; rev primer, 5'-GCGGCTGTCTCCACAAGT-3'; probe, 5'-VIC-CTGACCTGAAGGCTCT-MGB-3'. *QL_uc473_chX_VIC*-MGB ultra-conserved reference assay: fwd primer, 5'-GGGAACAGAACAAGCTGGTCTT-3'; rev primer, 5'-GCAAGACCCTGTGTAAGAACTTTGA-3'; probe, 5'-VIC-CTATCACATGTGGATCGTA-MGB-3'. DNA HapMap samples were all obtained as purified DNA from the Coriell Institute for Medical Research cell repositories and diluted to 100 ng/ μ l in 1x TE, pH 8, before use.

DNA Sample Digestion

DNA samples were digested with restriction endonucleases at a concentration of 1 μ g DNA/40 μ l reaction using the vendor's (New England BioLabs Inc.) recommended conditions except that 5 U of enzyme was added per 1 μ g DNA unless otherwise indicated. Restriction digests were diluted a minimum of 7.5-fold in the ddPCR reaction mix to obtain the desired final DNA concentration. When higher DNA concentrations (>1 copy per droplet, CPD) were used in the ddPCR reaction (for example, for the screening of HapMap DNA populations), DNA was digested at the concentration of 1 μ g/20 μ l per reaction. After restriction digestion, the reactions were incubated at 65°C for 20 min to deactivate the enzyme but not treated at a higher temperature to avoid denaturing the DNA target fragments. For restriction endonuclease titration experiments, the enzyme was first diluted into the supplied dilution buffer, and the appropriate enzyme dilution was added to each DNA being digested. For these titration experiments, 33 ng of digested DNA (0.5 CPD of the *RPP30* reference gene) was added per 20 μ l ddPCR reaction.

For experiments with *CCL3L1*, sample DNAs were double-digested with 5 U each of *RsaI* and *MseI* per μ g of DNA and then added to a concentration of 16 ng/20 μ l ddPCR reaction (0.25 CPD of the *RPP30* reference gene or 33 ng/20 μ l

(0.5 CPD of the reference genes). For HapMap screening experiments, 100 ng (1.5 CPD) of digested genomic DNA was run in each ddPCR reaction.

ddPCR Experiments

Genomic DNA samples, either digested or undigested as indicated, were added to the Bio-Rad 2x ddPCR supermix at concentrations ranging from 16.5 ng (0.25 CPD) to 100 ng (1.5 CPD) DNA per 20 μ l ddPCR reaction. TaqMan assay primers and probes were present at final concentrations of 900 nM and 250 nM, respectively. Reaction mixes were briefly mixed by vortexing while avoiding the formation of bubbles, microcentrifuged for 20 sec, then kept on ice until droplet generation.

Samples were converted into droplets, eight samples at a time, by adding 20 μ l of sample to each sample well of the DG8™ cartridge followed by addition of 70 μ l of droplet generation oil to each of the corresponding oil wells. The cartridge was then placed into the QX100 droplet generator within 2 min after oil addition for droplet generation. Droplets were transferred from the droplet wells in the cartridge with an 8-channel P50 pipettor to a 96-well PCR plate (twin.tec real-time PCR plates, Eppendorf). As many as 12 sets of samples were successively prepared in cartridges and transferred to the PCR plate before it was heat sealed with a pierceable foil seal. The plate was transferred to a thermal cycler and, unless otherwise specified, reactions were run under the following standard cycling conditions: 95°C for 10 min followed by 40 cycles of 94°C for 30 sec; 60°C for 60 sec, 98°C for 10 min, and 12°C infinitely; ramp rate = 50% (3°C/sec). For gradient experiments, the annealing/extension temperature was varied from 60°C, as specified in Figure 3. After PCR, the plate was loaded onto the QX100 Droplet Digital reader, which automatically reads the droplets from each well of the plate (~36 wells/hr). Data analysis was performed using Quantasoft™ software (Bio-Rad). Negative droplets, lacking target and/or reference gene DNA, and positive droplets, containing either or both DNAs, were counted to give the fraction of positive droplets. Using Poisson statistics, the concentrations of both DNA species were determined, and confidence intervals (CI) were computed, which were in turn used to compute the CNV value of the sample.

DNA Loading Considerations

When potentially high copy number samples are tested, it is important to reduce the input DNA concentration to avoid exceeding ~5 or 6 CPD for the unknown target gene. The amount of genomic DNA to introduce into a single reaction is governed by the number of droplets per reaction (~20,000), the concentration of the two genes being measured (the unknown target and the known reference genes), and the Poisson statistics for the partitioning of these gene copies into the droplets. We previously calculated (Hindson et al. 2011) that the DNA concentration yielding the most precise concentration measurements for 20,000

partitions is 1.6 CPD. This concentration corresponds to ~100 ng of human genomic DNA in a 20 μ l ddPCR reaction for a single-copy gene. If the target gene is present at more than two copies per diploid genome, for example, four copies per diploid genome, the same 100 ng of DNA will contain 3 CPD of the target gene. If the target gene copy number is more than approximately eight copies per diploid genome, it is necessary to reduce the total input DNA per well in order to maintain a sufficient number of negative droplets so that a reliable concentration and CNV estimate can be obtained. If higher precision is desired, multiple ddPCR wells can be run.

Results and Discussion

Separating Linked Gene Copies

Alterations in the copy number of a locus are commonly due to tandem duplications. It is necessary to physically separate linked copies to achieve an accurate count of all copies in Droplet Digital PCR, as illustrated in Figure 1.

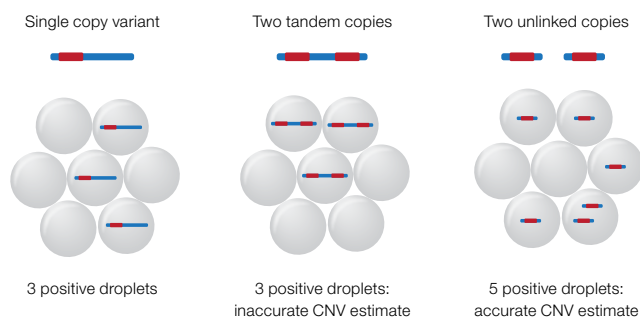


Fig. 1. Tandem gene copies require separation before partitioning. If tandem gene copies (red boxes) remain linked along a DNA fragment (blue bar), they will be partitioned into the same droplet and will result in an underestimated copy number. Their separation onto smaller DNA fragments prior to partitioning into droplets (right panel) allows them to be properly counted.

The most robust and reliable approach for separating linked gene copies is the use of restriction enzymes. We have extensively investigated the use of endonucleases for fragmentation and found that a wide range of enzymes with 4-base and 6-base recognition sites perform satisfactorily for this purpose. The benefit of predigestion for CNV measurement is typically achieved by a wide range of enzyme concentrations. Three considerations should be taken into account in the choice of enzyme for a particular locus: (1) the enzyme should not cut within the PCR amplicon itself; (2) it is best to use an enzyme that is insensitive to methylation to avoid incomplete fragmentation due to methylation of the target DNA; and (3) in some instances, it is optimal to digest the target copy to the smallest size fragment that fully contains the amplicon footprint — preferably under a few hundred base pairs.

The importance of proper target fragmentation for the tandem copies of *MRGPRX1* (Mas-related G-protein coupled receptor member X1), is illustrated in Figure 2 for the Yoruban HapMap sample NA19205. In the mock digestion, where the *MseI* enzyme is absent, the CNV estimate is ~3.5 copies; however, when the genomic DNA is incubated with as little as 0.078 U of enzyme at 37°C for 60 min, the proper CNV value of 5 (Weaver et al. 2010) is obtained when compared with the single-copy reference gene *RPP30* (ribonuclease P protein subunit p30). The measured copy number (and concentration) of the *MRGPRX1* gene increases with the amount of restriction enzyme used as the separation of the linked copies becomes more complete. After complete digestion is achieved, the CNV value is unaffected by the quantity of enzyme used. Similar results are found with the *MRGPRX1/RPP30* duplexed assays when the DNA is digested by *XhoI* or *CviQI* (data not shown).

Our results show that the single-copy *RPP30* reference gene is unaffected by the DNA digestion status. Likewise, *CYP2D6*, which is also a single-copy gene, shows no difference in concentration and CNV value (= 2) with or without digestion by *MseI* (Figure 2) or *XhoI* and *CviQI* (data not shown) when duplexed with the *RPP30* assay. A third locus, *CCL3L1*, was examined and shows five copies per cell, whether digested by *MseI* or not, indicating that the copies are unlinked. Similar results were obtained with *XhoI* (data not shown).

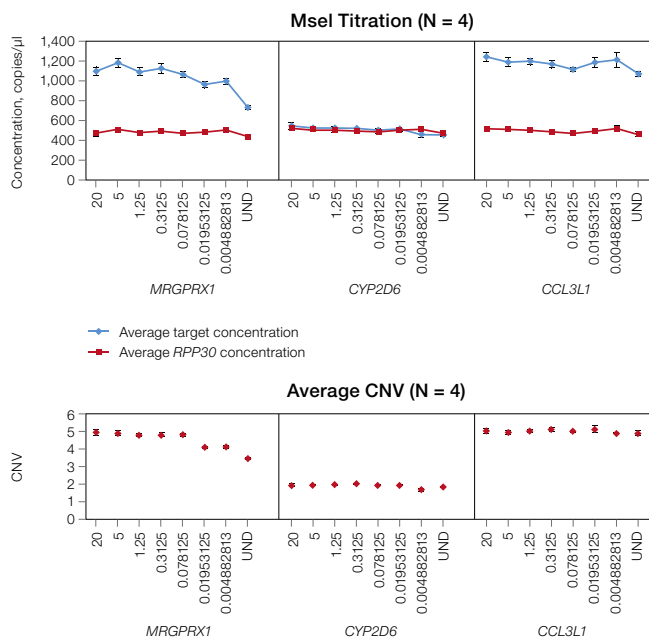


Fig. 2. Effect of restriction endonuclease digestion of genomic DNA on CNV assessment. HapMap sample NA19205 was digested with the indicated concentrations of *MseI* enzyme per 1 μ g DNA. Thirty-three ng of digested DNA (0.5 CPD of the *RPP30* reference gene) was added per 20 μ l ddPCR reaction. ddPCR reactions contained one of three target assays indicated — *MRGPRX1_FAM*, *CYP2D6_FAM*, or *CCL3L1_FAM* — each duplexed with the *RPP30_VIC* reference assay. Each concentration and CNV value shown represents the average of four replicates per condition with 95% CI. CNV, copy number variation; UND, undigested (buffer only).

Optimization of Annealing Temperature of the Duplexed Assays

We recommend establishing the optimal annealing/extension temperature range for newly designed duplex assays by running a thermal gradient PCR experiment. Figure 3 shows the behavior of the *CCL3L1* assay (QL_Eich_CCL3_LG_FAM-BHQ) duplexed with the *RPP30* reference assay (QL_RPP30_1_VIC-MGB) over the annealing temperature range of 57–64.8°C. At lower temperatures, the two assays exhibit the maximal separation of fluorescence amplitudes between positive and negative droplets (Figure 3). At higher temperatures across the tested range, the fluorescence amplitude of negative droplets is only minimally affected; however, the amplitude for positive droplets begins to decrease as the temperature is increased above 59.2°C for *RPP30* and 60.2°C for *CCL3L1*. For *RPP30*, there is still satisfactory separation of fluorescence signals at 64.8°C, and the measured concentration is constant across the entire temperature range (Figure 3B, green square markers). However, the separation for *CCL3L1* is satisfactory only at or below 62.3°C (Figure 3A), as reflected by a measured CNV value of 5 below 62.3°C (Figure 3B). These results indicate that the optimal annealing temperature is between 59 and 61°C in these single-well ddPCR measurements.

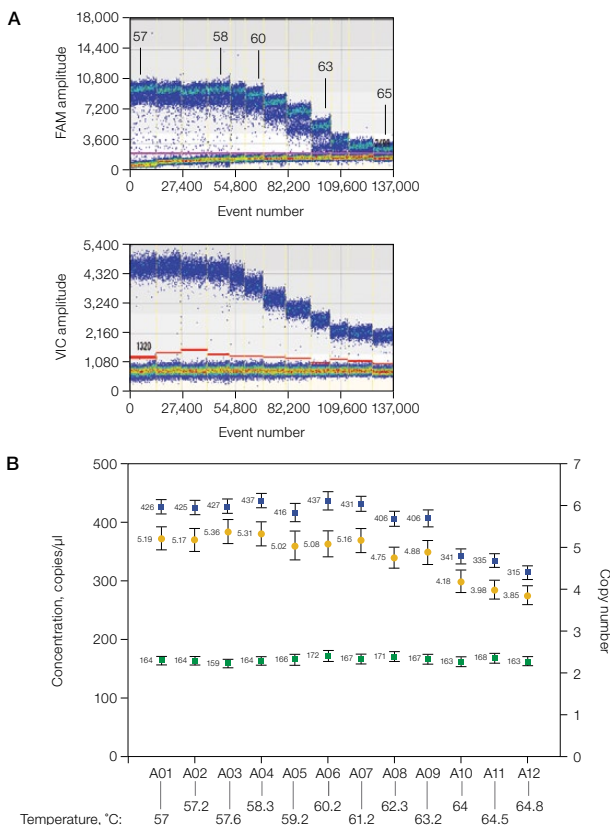


Fig. 3. Annealing temperature optimization of the *CCL3L1/RPP30* duplexed assay for *CCL3L1* copy number determination in HapMap sample NA19108. A ddPCR reaction mix containing the QL_Eich_CCL3_LG_FAM-BHQ and QL_RPP30_1_VIC-MGB assays and 16.5 ng of digested DNA was converted into droplets, and PCR was performed at the designated annealing/extension temperatures before droplet reading. **A**, temporal plots for *CCL3L1* (FAM) and *RPP30* (VIC) fluorescent droplet amplitudes at various annealing temperatures; **B**, concentration values for *CCL3L1* (■) and *RPP30* (■); CNV values (●).

To test the robustness of the optimized *CCL3L1/RPP30* duplexed assay, 11 samples from the Coriell Institute were assessed by ddPCR for the number of *CCL3L1* copies present. The CNV values presented in Figure 4 represent duplicate wells run at 59°C and include the 95% CIs, showing robust determination across a diverse sample set where as few as 1 and as many as 13 copies are present. Similar results were obtained for the same assay run at annealing temperatures of 60°C and 61°C, demonstrating the robustness of the CNV measurement across multiple temperatures (data not shown). The assay appears to provide discrimination between 10 and 11 copies with high confidence, although the actual CNV values for these samples have not been independently confirmed.

For another HapMap sample, NA18507 (not shown here), the CNV value for *CCL3L1* was previously determined by ddPCR to be 6 and was independently estimated to be 5.7 by next-generation sequencing (Hindson et al. 2011). The minor difference in these values likely reflects the many fewer counts generated by sequencing compared with ddPCR, which can achieve read depths of 20,000x for each of two genes per single well. Higher precision can be obtained by combining measurements from additional ddPCR wells.

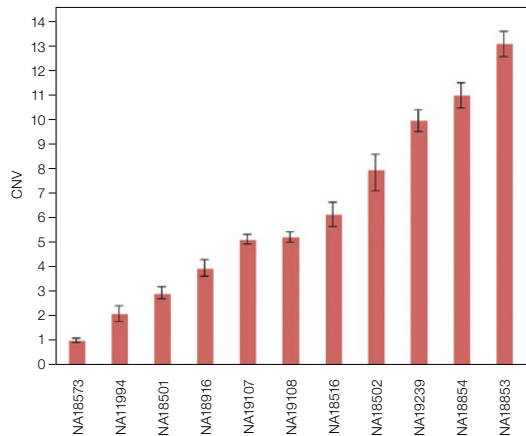


Fig. 4. Robustness of CNV estimates for *CCL3L1* among 11 cell lines. Each DNA was double-digested with *RsaI* and *MseI* and assessed for copy number via ddPCR as described in Figure 3 using the same *CCL3L1/RPP30* duplexed assays. Duplicate reactions of each of the samples were tested at an annealing/extension temperature of 59°C. CNV values shown are from the merged wells with 95% CIs. CNV, copy number variation.

Robustness of CNV Value to Reference Assay Used

In a separate experiment testing different reference assays, *CCL3L1* was duplexed with either the *RPP30* (QL_RPP30_1_VIC-MGB) reference gene (Chr 10) or an ultraconserved Chr X marker reference (QL_uc473_chX_VIC-MGB). Figure 5 shows that both duplexes gave nearly identical CNV values for the cell lines NA18507, NA11994, and NA19221. The slightly higher value seen for NA18502 with the Chr X reference (8.36) than with *RPP30* (8.03) may be due to aneuploidy in this EBV-immortalized line, to which the X chromosome is susceptible

(Redon et al. 2006). Note that the red markers for *CCL3L1*/Chr X-derived CNV values denote male samples which have only a single copy of the X chromosome. Their CNV values were suitably adjusted in the Quantasoft software to reflect the correct copies per diploid genome.

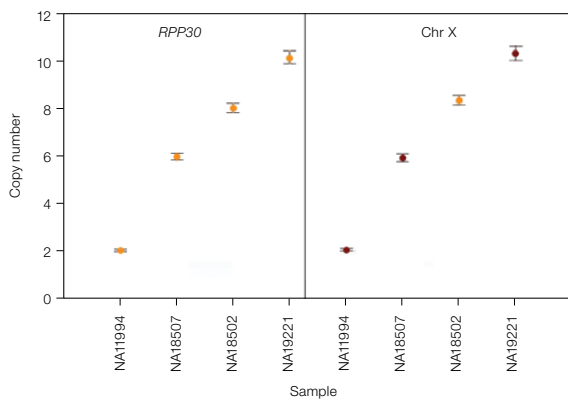


Fig. 5. *CCL3L1* CNV estimates using duplex assays with either *RPP30* or Chr X_u.c. as VIC-labeled reference genes. Each DNA was double-digested and processed in ddPCR as described in Figure 3 using an annealing/extension temperature of 59°C. Samples were assayed with either the *CCL3L1/RPP30* (*RPP30*) duplex or the *CCL3L1/QL_uc473_chX_VIC-MGB* (Chr X) duplex. Samples with two copies of reference gene per cell (●); samples with one copy of reference gene per cell (●). The CNV value and 95% CI for each sample are derived from two merged wells.

High-Throughput Profiling of CNV Status in HapMap Populations

Large populations of individuals can be profiled with ddPCR for copy number status of a gene of interest. Figure 6 presents the results for the *CYP2D6* gene measured in nearly 300 HapMap samples from the 1000 Genomes Project, including 96 British, 120 Kenyan, and 72 Mexican samples (1000 Genomes Project Consortium 2010). Examples of clearly distinguishable CNV values of 1, 2, 3, and 4 are found among them, although the most frequent copy number state in each of the populations is a CNV of 2 (British, 96%; Kenyan, 87.5%; Mexican, 86%). Distinctive features of the tested populations, such as allele distributions, are readily discernible from these data. For example, the Mexican population shows a substantially higher frequency of individuals with a CNV of 3 (~12.5% vs. 1% for British and 5% for Kenyans, including one Kenyan with CNV of 4), a genotype that confers a higher rate of metabolism for a broad range of drugs and other xenobiotics, often with significant pharmacological consequences for these ultra-metabolizing individuals (Johansson and Ingelman-Sundberg 2008). Additionally, one British and one Kenyan individual each had no copies of the *CYP2D6* gene, characteristic of poor metabolizers (Table 1). The ability to obtain high-precision CNV values from single-well measurements — combined with the 96-well format of the QX100 Droplet Digital PCR system and the reader's throughput of >32 samples/hr — enables rapid characterization of 3 x 96 samples in a single day with approximately 1 hr of hands-on time per plate.

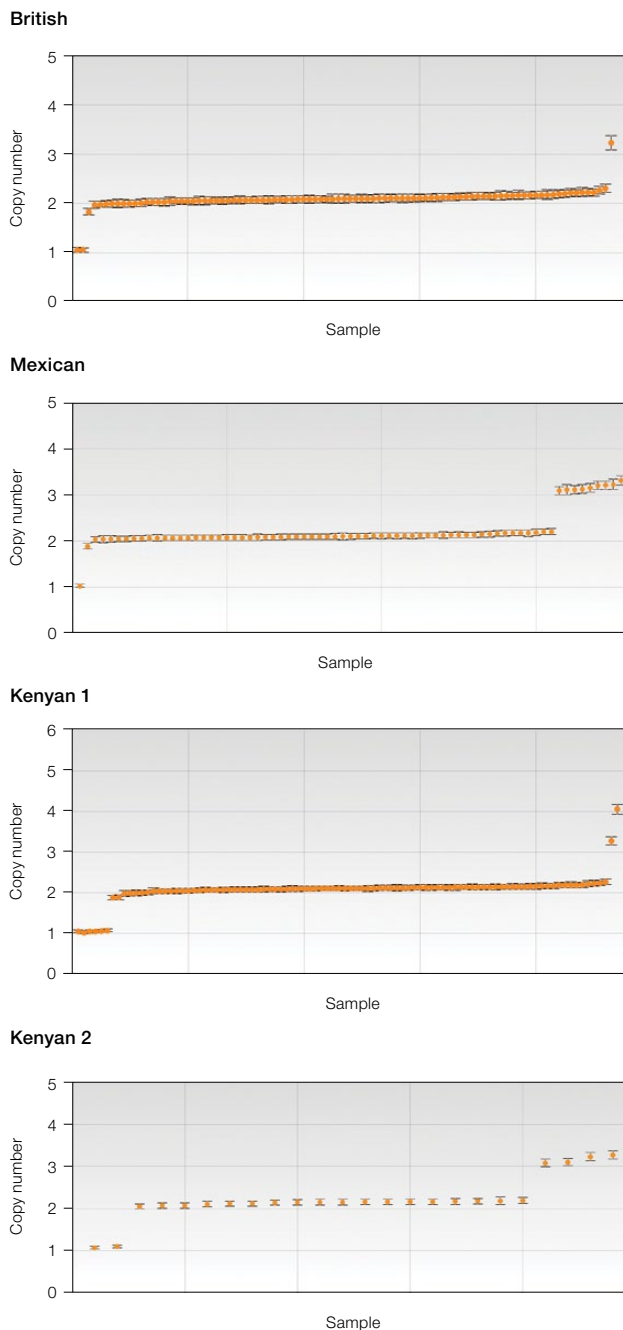


Fig. 6. High-throughput profiling of the *CYP2D6* CNV state in three populations using single-well ddPCR measurements. 100 ng of *Mse*I-digested DNA (1.5 CPD of the single-copy *RPP30* reference gene) of three HapMap populations was analyzed with the *CYP2D6/RPP30* duplexed TaqMan assays.

Table 1. 1000 Genomes Project samples with CNV not equal to 2.

Population	Sample Name	CNV Value	95% CI
British	HG00156*A1	0	0–0
British	HG00099*A1	1.06	1.01–1.12
British	HG00260*A1	1.07	1.02–1.11
British	HG00149*A1	3.23	3.10–3.37
Mexican	NA19783*2	1.03	1.00–1.07
Mexican	NA19719*1	3.1	3.01–3.19
Mexican	NA19660*1	3.12	3.01–3.23
Mexican	NA19761*1	3.12	3.03–3.21
Mexican	NA19723*1	3.13	3.04–3.23
Mexican	NA19685*1	3.16	3.07–3.26
Mexican	NA19752*1	3.21	3.12–3.31
Mexican	NA19788*1	3.22	3.13–3.31
Mexican	NA19649*1	3.23	3.12–3.35
Mexican	NA19785*1	3.32	3.22–3.42
Kenyan 1	NA19317*A1	0	0–0
Kenyan 1	NA19035*A1	1.02	0.98–1.05
Kenyan 1	NA19392*1	1.05	1.02–1.09
Kenyan 1	NA19338*A1	1.05	1.01–1.08
Kenyan 1	NA19036*1	1.05	1.02–1.09
Kenyan 1	NA19430*1	1.06	1.03–1.1
Kenyan 1	NA19359*1	1.08	1.04–1.11
Kenyan 1	NA19308*1	3.27	3.18–3.37
Kenyan 1	NA19378*B1	3.98	3.86–4.1
Kenyan 2	NA19471*1	1.07	1.03–1.10
Kenyan 2	NA19461*1	1.10	1.07–1.14
Kenyan 2	NA19332*1	3.08	2.99–3.17
Kenyan 2	NA19469*1	3.1	3.01–3.19
Kenyan 2	NA19452*1	3.23	3.14–3.33
Kenyan 2	NA19393*1	3.27	3.17–3.37

Summary

Droplet Digital PCR is a highly precise counting technology for simultaneously measuring the concentrations of target and reference genes in DNA samples and for providing precise and reproducible CNV values. It is easy to perform using conventional TaqMan hydrolysis probe assays, and because it directly counts the gene copies in a sample, it does not require the use of a standard curve and is not plagued by the PCR amplification irregularities that can contribute to measurement error in real-time PCR. Precise measurements can be made from only a single ddPCR well if sufficient target DNA is available. Running experiments in a convenient 96-well format makes ddPCR well-suited for high-throughput studies profiling CNV frequencies in populations of interest (for example, for marker associations).

References

- 1000 Genomes Project Consortium (2010). A map of human genome variation from population-scale sequencing. *Nature* 467, 1061–1073.
- Beckmann JS et al. (2008). CNVs and genetic medicine (excitement and consequences of a rediscovery). *Cytogenet Genome Res* 123, 7–16.
- Gonzalez E et al. (2005). The influence of *CCL3L1* gene-containing segmental duplications on HIV-1/AIDS susceptibility. *Science* 307, 1434–1440.
- Hindson BJ et al. (2011). High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal Chem* 83, 8604–8610.
- Hosono N et al. (2008). Multiplex PCR-based real-time invader assay (mPCR-RETINA): A novel SNP-based method for detecting allelic asymmetries within copy number variation regions. *Hum Mutat* 29, 182–189.
- Johansson I and Ingelman-Sundberg M (2008). CNVs of human genes and their implication in pharmacogenetics. *Cytogenet Genome Res* 123, 195–204.
- Karlen Y et al. (2007). Statistical significance of quantitative PCR. *BMC Bioinformatics* 8, 131.
- Liu S et al. (2010). *CCL3L1* copy number variation and susceptibility to HIV-1 infection: A meta-analysis. *PLoS One* 5, e15778.
- Redon R et al. (2006). Global variation in copy number in the human genome. *Nature* 444, 444–454.
- Speleman F et al. (2008). Copy number alterations and copy number variation in cancer: Close encounters of the bad kind. *Cytogenet Genome Res* 123, 176–182.
- Sudmant PH et al. (2010). Diversity of human copy number variation and multicopy genes. *Science* 330, 641–646.
- Vissers LE and Stankiewicz P (2012). Microdeletion and microduplication syndromes. *Methods Mol Biol* 838, 29–75.
- Weaver S et al. (2010). Taking qPCR to a higher level: Analysis of CNV reveals the power of high throughput qPCR to enhance quantitative resolution. *Methods* 50, 271–276.

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