

Digital PCR Multiplexing Assay for Gene Copy Number

This application note presents the results of a novel 5-plex droplet-based digital PCR assay to identify gene copy variations and SNPs using a Spinal Muscular Atrophy (SMA) model. Assay performance demonstrates accurate quantification and superior reproducibility across a set of samples and a wide range of copy number levels.

Copy Number Variation

The DNA copy number of a genomic locus is the number of copies of the DNA in that region relative to either a single control sample or a pooled reference population. Copy number variations (CNVs) include loci gains or losses, and have been associated with familial cancer² and other complex diseases³. New whole-genome genotyping platforms provide the ability to interrogate multiple CNVs and SNPs, and have begun to yield associations between such variants and disease phenotypes⁴. However, these methods are sensitive to DNA quality⁵ and are limited in their accuracy when genotyping multi-allelic loci. Locus specific methods are being employed to complement whole genome approaches, particularly for complex diseases where effect sizes are likely to be small. Such targeted methods must provide sufficiently accurate data to limit the potential for false positive or negative results as a result of differential bias. Current approaches, including qPCR, may exhibit differential amplification efficiencies between test and reference loci which can generate inaccuracies, particularly for high copy number measurements where the relative difference in ratio between test and reference products can be quite small⁶. Digital PCR, with its superior accuracy and precision, is a promising new technique for quantifying CNV.

CNV and SNPs in Spinal Muscular Atrophy

A Spinal Muscular Atrophy (SMA) panel was selected due to its clinical significance and complex genetics. SMA is the second-most prevalent fatal neurodegenerative disease and affects 1:10,000 live births⁷. SMA is most often caused by homozygous absence of exon 7 within the "survival of motor neuron 1" (SMN1) gene⁸, however the severity of the condition is modulated by the number of gene copies of SMN2, with prognosis ranging from lethal to asymptomatic over 1–5 copy numbers⁹. Accurate quantitation of SMN2 copy number is important for clinical prognosis and genetic counseling. Aside from large deletions of SMN1, a number of single point mutations or short deletions/duplications within the same

Figure 1
Fluorescence Intensity Scatter Plot

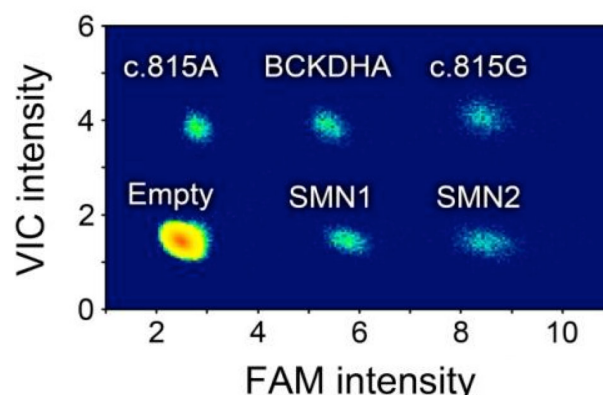
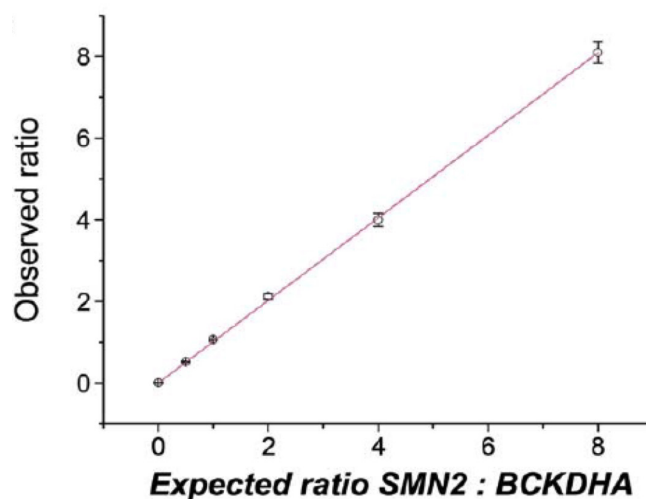


Figure 2



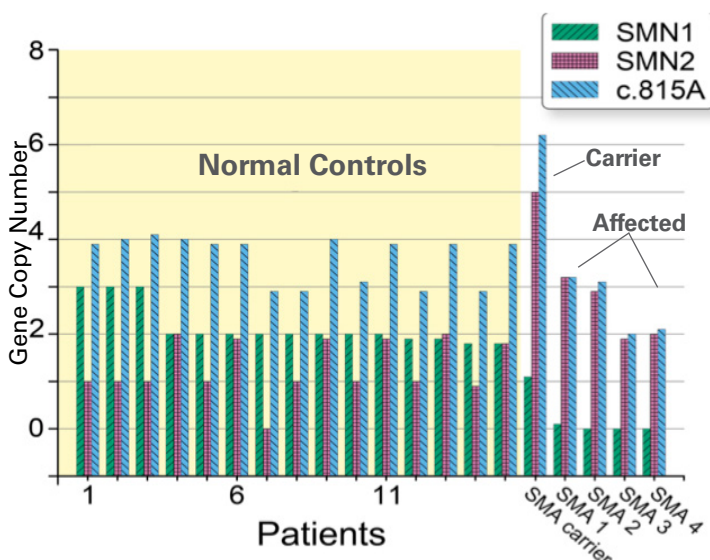
gene also account for 4% of cases of SMA.¹⁰ The multiplexed dPCR assay contains both copy number assays for SMN1 and 2 and an assay for one of the prevalent SNPs (c.815A>G).

Panel Design

A 5-plex dPCR assay was developed for common genetic variants impacting SMA, including two copy number assays for SMN1 and SMN2 genes with BCKDHA as a reference, and a SNP assay for the c.815A>G mutation. Two different color fluorophores, FAM and VIC, were used to uniquely identify each assay. The probes for SMN1 and SMN2 contained only FAM, and for c.815A only VIC. Mixtures of VIC and FAM-labelled probes were used for BCKDHA and c.815G. (Figure 1). Six clearly separated populations were clearly evident, five for the assay panel and one for the “empty” PCR negative droplets. Additional information about multiplexing dPCR assays can be found in the Multiplexing dPCR Application Note.

First a model system with varying concentrations of plasmid DNA was used to quantify changes in the target gene copy number relative to the reference gene, BCKDHA. In Figure 2 the ratio of gene copies for each sample in the serial dilution are plotted against expected values, yielding an excellent linear fit ($R^2=0.9997$, slope =1.01) across the full range of 0-16 copies of SMN2 per cell. (Normal reference is two copies per cell).

Figure 3



Clinical Performance

Next, twenty different patient samples from the Coriell cell repositories were analyzed: four afflicted with SMA, one SMA carrier and 15 negative controls. The measured patient genotypes were consistent with their phenotypes.¹¹ The patients afflicted with SMA each had zero copies of SMN1 (Patients SMA 1–4), the SMA carrier had just one SMN1 copy, and the negative controls all had either two or three copies (patients 1–15). SMN2 ranged from zero to two copies across the normal controls, five copies for the carrier, and two to three copies for the affected samples. Three unrelated individuals had three copies of SMN1, occurring at a rate of 20% which is similar to a previous report for healthy individuals.¹² The SNP assay revealed that all patients carried the normal c.815A genotype and no instances of c.815G were observed.

Conclusion

The RainDrop® dPCR System enables accurate and precise quantification of copy number and SNP markers for important clinical applications using multiplexed assay panels. The platform enables use of multiplexing, allows uses of different chemistries, and can be used for additional applications including expression analysis and detection of rare variants. RainDrop’s ten million droplets per sample produce superior performance over qPCR methods and other dPCR techniques which have fewer reactions.

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