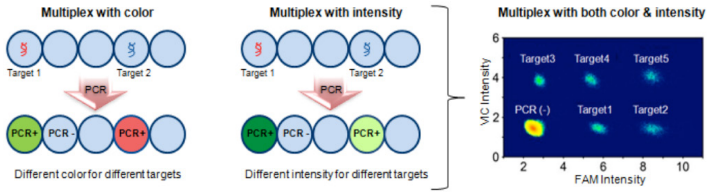
Multiplexing with RainDrop Digital PCR

The RainDrop® Digital PCR System provides unique capabilities and benefits for counting applications such as rare mutation detection, quantifying copy number variation, DNA methylation assessment, and as a pre-test QC and validation tool for DNA sequencing. The power of digital PCR is extended even further with the novel multiplexing capability made possible with millions of droplets. This application note summarizes the simple approach used to develop multiplex assays and provides examples of important multiplexed assays in various research applications.

Simple Multiplexing Principle

By leveraging millions of droplets for single target molecule encapsulation and endpoint PCR assays, the RainDrop dPCR System is able to provide highly sensitive multiplexed sample analysis. dPCR assay multiplexing uses both distinct assay 'colors' and 'intensities' to identify and count the number of each target molecule displayed on a 2-D 'cluster' plot, as shown in Figure 1.

Figure 1:
Multiplexing Assays With Probe Color And Intensity

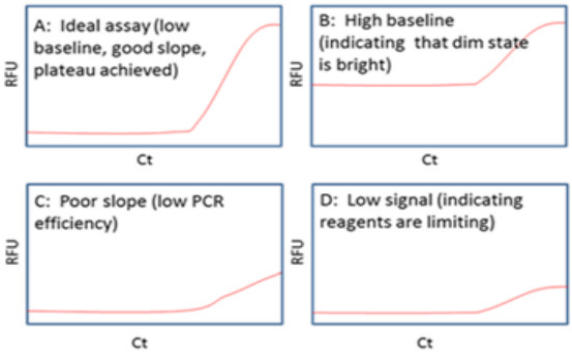


Strategies for Developing Robust Multiplex Assays

A simple three step approach is typically all that is needed to quickly achieve high quality results, with only two steps needed if a qualifying qPCR assay already exists.

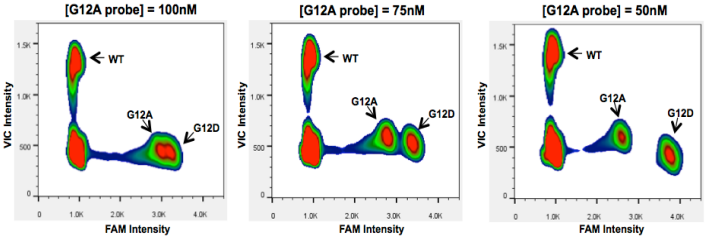
1. Design primers, then probes - Select genetic targets, design PCR primer combinations for each target, and optimize PCR reactions. Primers pairs should be selected that utilize similar annealing temperatures, as they will be run together in multiplex. Avoid primer designs that form secondary structures, are over SNPs, or may be complementary to pseudogenes or homologous regions. Next, design probes and test each assay primer/probe combination by qPCR. For a more comprehensive description of assay design and recommended fluorescent dyes refer to the RainDrop dPCR System Assay Manual. A low noise background and high signal plateau are optimum, as shown in Figure 2.

Figure 2:
qPCR Test Primers and Probes



2. Test by dPCR, optimizing cluster positions and separation by adjusting probe concentrations - Probe concentrations should be limiting in the reaction, and can be easily adjusted to generate clusters that are sufficiently separated from each other for counting. Figure 3 shows an example of how the cluster resulting from an assay for KRAS G12A can be moved relative to the cluster for KRAS G12D and the PCR negative cluster near the origin simply by adjusting the FAM probe concentration (the probe concentrations for WT and G12D are kept constant). Typical primer concentrations used are in the 500-900nM range and probe concentrations from 30-300nM.

Figure 3:
Moving Cluster Position By Varying Probe Concentration



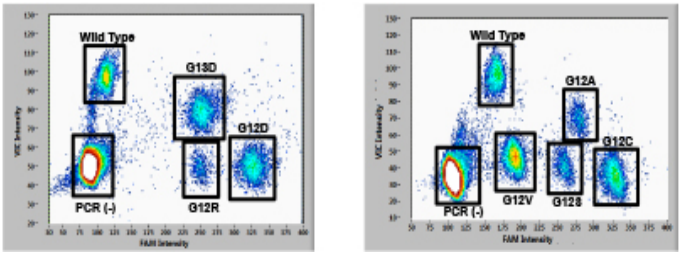
3. Use probe mixtures to assay more targets in off-axis clusters - In addition to positioning clusters along the two dye-intensity axes, clusters may be placed 'off-axis' in the cluster plot by using combinations of labelled probes. In the example shown in Figure 1, the assay for Target 4 uses 50% probe labeled with VIC and 50% probe labeled with FAM (identical Target 4 probe sequences) and the assay for Target 5 uses 25% probe labeled with VIC and 75% probe labeled with FAM (identical Target 5 probe sequences).

Additional details and examples on multiplex assay development are included in the RainDrop dPCR System Assay Guidelines.

Multiplex Capability Demonstrated Across Many Applications

Multiplex assays have been designed and validated with model systems and clinical samples for a variety of applications. These multiplexed assays demonstrate well resolved target clusters that can be identified and counted with accuracy. Two examples are highlighted below in Figure 4.

Figure 4
Fluorescence Intensity Scatter Plots



KRAS Mutation Detection

Two separate multiplexed dPCR panels were created to detect a total of seven different KRAS mutations on codons 12 and 13 (one panel quantifies WT and three KRAS mutations and the second panel quantifies WT and four additional KRAS mutations control DNA shown). A recent publication demonstrated the remarkable capability of these two multiplex panels for evaluating mutations in heterogeneous colorectal cancer FFPE samples and in a 'liquid biopsy' measurement of circulating tumour DNA from patient plasma.[1]

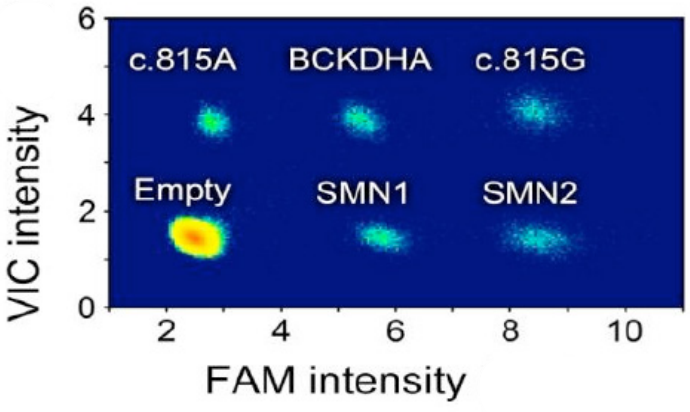
Copy Number and SNP Panel for Spinal Muscular Atrophy

A five-plex dPCR assay was developed for quantifying genes which impact Spinal Muscular Atrophy (SMA). The panel included two genes whose copy number determines disease severity (SMN1 and SMN2), a single nucleotide variant assay for the SMN c.815A>G mutation, and BCKDHA as a standard diploid genome reference. The probes for the SMN1 and SMN2 targets contained only FAM, and for SMN c.815A only VIC. Mixtures of VIC and FAM labelled probes were used for BCKDHA and SMN c.815G. Control spike-in experiments demonstrated the ability to accurately quantify from 0 to 16 genomic copies. The assay was validated with 20 patient samples from Coriell repositories (4 afflicted with SMA, 1 SMA carrier and 15 negative controls). Measured patient genotypes were consistent with their conditions.[2]

Conclusion

Multiplexing assays enables comparative analysis of multiple targets within the same experimental run and maximizes the amount of information that can be gathered from precious samples. The dynamic range provided by the RainDrop dPCR System's ten million droplets enables simple assay multiplexing for use in many counting applications.

Figure 5



References
1. Taly V, et al, Quantitative detection of circulating tumor DNA by droplet-based digital PCR, AACR Poster 2012.
2. Zhong Q, et al, Multiplex PCR: breaking the one target pr color barrier of quantitative PCR, DOI: 10.1039/c1lc20126c.

The RainDrop Digital PCR System is for Research Use Only; not for use in diagnostic procedures.

LCN 50-07047 Rev B