RainDance Technologies

TECHNICAL NOTE

Recommendations for Developing a Multiplex Digital PCR Assay

Jennifer B. Jackson, RainDance Technologies Inc., 749 Middlesex Turnpike, Billerica, MA 01821, USA.

Abstract

Digital PCR (dPCR) is one of the most sensitive and precise methodologies for quantification of nucleic acids and is the preferred approach for the detection of low frequency mutations in a high wild-type background. For detecting minor allele frequency (MAF) using standard dPCR, a duplex assay is utilized to differentiate between wild-type and a mutant single nucleotide polymorphism (SNP) enabling absolute quantification. Recently, however, many investigators have pushed beyond the two probe experimental design and have developed multiplex assays to assess multiple mutations within a single sample.[1,2,3] Multiplex dPCR for sequences located in genes such as EGFR and KRAS that have multiple cancer-relevant SNPs, are the most requested assay designs as the presence of these mutations determine a clinical course of action. The following technical note describes in detail the recommended workflow for optimizing a multiplex dPCR assay for detecting multiple SNPs from a single sample.

Introduction

The RainDrop® Digital PCR System provides the unique capability of absolute quantification by leveraging millions of droplets for single target encapsulation and amplification, providing highly sensitive detection of low frequency events such as rare cancer mutations. Using TaqMan-based chemistries and endpoint PCR, wild-type alleles and mutant variants can be detected and differentiated by the use of two distinctly labeled fluorescent probes, for the RainDrop platform either a red or a green fluorophore (Figure 1A). By ensuring that each droplet only contains either 0 or 1 target molecule, droplets will fluoresce based on the presence of either the normal or mutated sequence of interest. This ensures that even very low frequency target molecules can be detected in a vast background of wild-type template making dPCR the most sensitive detection methodology available.

One of the major advantages of the RainDrop Digital PCR System is the ability to manipulate and fine-tune positive droplet cluster locations thereby enabling assay multiplexing to assess >2 targets (SNPs) within the same well. Using the standard two color probe set-up (Figure 1A), additional targets can be assessed by designing a probe with either the red or green fluorophore, and adjusting probe concentration to alter fluorescence intensity (Figure 1B) and produce a distinct cluster for that target. Furthermore, for higher order multiplexing, designing probes containing both colors for a single target (Figure 1C) enables the shifting of that population between the red and green clusters. Overall, this capability maximizes the amount of information that can be extracted from a single sample which is of particular importance for low yield samples such as circulating tumor DNA (ctDNA).

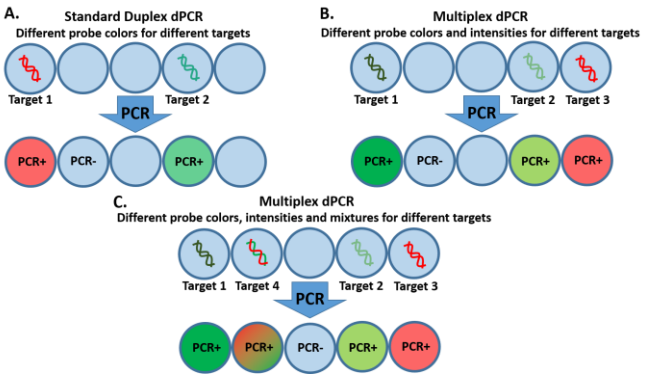

Figure 1: Methods for Multiplexing Digital PCR. A standard duplex assay (A) can be built upon by adjusting probe intensity (B) as well as mixing probe fluorophores (C) to assess for >2 targets of interest within the same sample.

Methods and Results

Assessment of Duplex Assays in dPCR

1. For multiplexing in dPCR, we recommend a maximum of 3 mutant SNPs plus one wild-type (total of 4 positive clusters) to ensure ample cluster separation of the different SNP populations.
   a. Wild-type probes should be designed with a red fluorophore (VIC, HEX, or TET) which will orient the wild-type population to the y-axis.
   b. Mutant probes should be designed with the FAM fluorophore. Mutant probes can also be designed with a red fluorophore to shift mutant clusters away from the x-axis (FAM axis).

2. Primer pairs and probes for each loci and SNP should utilize similar annealing temperatures to minimize amplification bias or allelic dropout.

3. To assess specificity and to optimize multiplexing protocol, you will need positive control DNA. These should include:
   a. Wild-type DNA to test mutant probe cross-reactivity.
   b. Plasmid DNA containing the individual mutations or SNPs of interest (preferred).
   c. Heterozygous cell line DNA with wild-type and mutant alleles if plasmid DNA is not available.

4. We recommend building upon successfully validated qPCR duplex assays. For assessment in dPCR, test duplex SNP assays (2 primers and 2 probes for wild-type and mutant sequence) using appropriate controls to determine specificity.
   a. Use 500nM of both primers and 200nM of each probe to test on wild-type DNA alone and plasmid DNA containing the mutant of interest alone or heterozygous cell line DNA to assess specificity. No template controls (NTCs) should also be run to assess non-specific probe hydrolysis.
   b. The assay with the least cross-reactivity of the mutant probe with the wild-type DNA alone is the best candidate assay to begin building the multiplex (Figure 2).

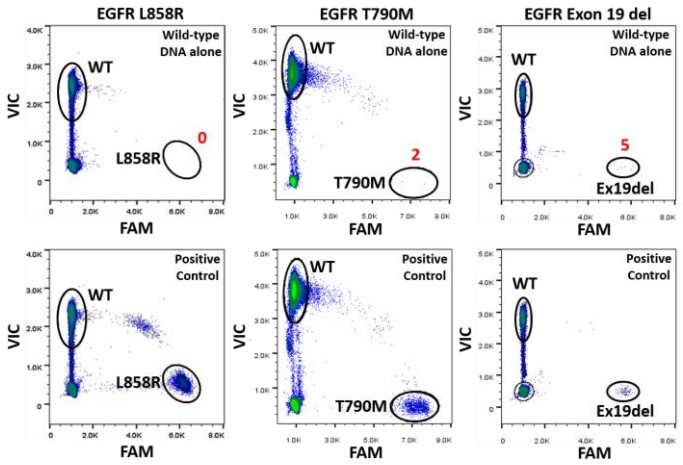

Figure 2: Assessment of individual duplex assays. Assays for EGFR L858R, T790M, and exon 19 deletion are individually assessed for false positive background (counts in red) in dPCR prior to multiplexing. The assay which produces the lowest background will serve as the foundation for building the multiplex by adding in primers and/or probes for additional targets/SNPS.

5. Using the wild-type probe from the duplex assay with the least amount of false positive noise in the wild-type control (L858R assay in this case), individually assess the other mutant probes (T790M and Exon 19 deletion) in combination with the wild-type probe using appropriate controls to determine specificity of each duplex assay.

6. If the mutant probe cross-reacts with wild-type DNA, switch the wild-type probe to the wild-type sequence for the mutant that is displaying the cross talk.

Building a Multiplexed Assay in dPCR

1. After determining which wild-type probe is optimal for pairing with the individual mutant probes, add an additional mutant probe along with the appropriate primers (if necessary) to the duplex assay. Using a mix of VIC- and FAM-labeled probe for EGFR T790M, the mutant cluster is shifted away from the EGFR L858R mutant cluster on the x-axis (Figure 3).

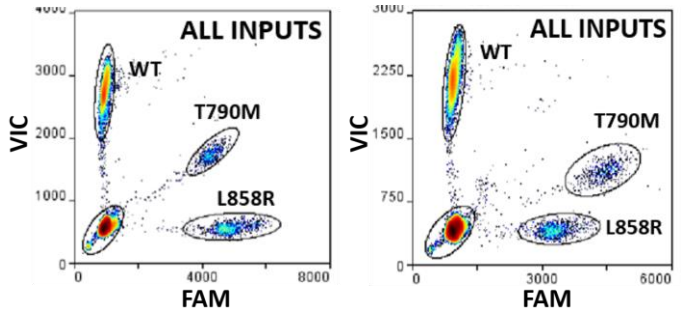

Figure 3: Building and optimizing a 3-plex assay. Wild-type EGFR (WT) and T790M and L858R targets are simultaneously detected in dPCR. By adjusting the T790M FAM-labeled probe, the T790M cluster is shifted up the x-axis from left to right by mixing 200nM T790M-VIC probe with either 100nM (left plot) or 200nM (right plot) T790M-FAM probe.

2. Test a range of concentrations of VIC- and FAM-labeled probes (50-200nM) for the shifted mutant SNP to determine optimal positioning of the cluster. The left and right plots in Figure 3 show the position of T790M mutant cluster when the T790M probes are at 200nM VIC/100nM FAM and 200nM VIC/200nM FAM, respectively. Note the movement of the T790M cluster to the right when increasing the FAM probe concentration.
   a. Run the multiplex assay with wild-type DNA only, wild-type+mutant 1, wild-type+mutant 2, all inputs, and NTC to look for any potential cross talk.
   b. (Optional) If using mutant plasmids, run the assay with mutant 1 and mutant 2 alone and in combination to assess whether the wild-type probe is specific or cross-reacts the mutant sequence.

3. Repeat step 2 for additional SNPs. In Figure 4, an exon 19 deletion (del746-750) is incorporated into the EGFR multiplex assay. Using 200nM of VIC-labeled EGFR T790M probe, the T790M cluster is shifted to the y-axis above the wild-type cluster (wild-type-VIC probe at 50nM). The EGFR L858R cluster is shifted along the y-axis above the exon 19 deletion mutant by spiking in VIC-labeled L858R probe.

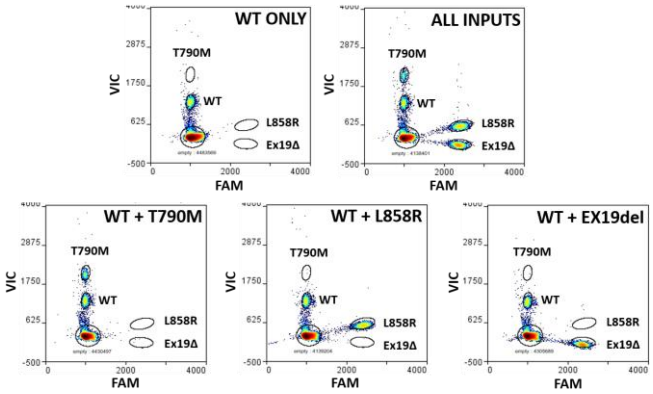

Figure 4: Adding additional SNPs to a multiplex assay. Wild-type EGFR (WT), an exon 19 deletion (Ex19Δ), T790M, and L858R targets are simultaneously detected in dPCR. Using the T790M-VIC probe, the mutant population is shifted above the wild-type cluster on the y-axis. The L858R mutant cluster is shifted up the y-axis above the Exon 19 deletion cluster by spiking in L858R-VIC probe. Testing wild-type DNA alone and in combination with each mutant DNA will assess for false positive signal for each probe.

4. Run the full multiplex assay with wild-type DNA only, wild-type+mutant 1, wild-type+mutant 2, wild-type+mutant 3, all inputs, and NTC.

5. (Optional) If using mutant plasmids, run the assay with mutants 1, 2, and 3 alone and in all combinations.

6. Confirm that the percentage of mutant signal stays constant in all test combinations to further validate the absence of probe cross talk.

7. If possible, run assay with known samples or reference standards to validate assay performance and accuracy.

Conclusions

Multiplexing in Action

With the use of low yield DNA samples for detection of rare variants such as those found in ctDNA, multiplexing dPCR assays is quickly becoming an essential methodology. It enables comparative analysis of multiple targets within the same experimental run and maximizes the amount of information that can be gathered from precious samples. Implementation of multiplexing on the RainDrop dPCR platform has recently been demonstrated by researchers at the National Cancer Center Research Institute in Tokyo, Japan. They successfully designed a KRAS multiplex dPCR assay to assess four of the most common codon 12 and 13 mutations (G12D, G12V, G12R, and G13D) in pancreatic ductal adenocarcinoma patients (PDAC) accounting for more than 90% of all KRAS mutations found in this cancer type.[2] Using reference DNA for wild-type and mutant KRAS for cluster gating (Figure 5A), circulating cell-free DNA (ccfDNA) from the blood of either a healthy patient (Figure 5B) or PDAC patients (Figure 5C and D) was assessed for presence of KRAS mutation. ccfDNA from the healthy control had no detectable mutations while the ctDNA from PDAC patients exhibited mutations at two different loci. Overall, the study found that the majority of patients with inoperable PDAC had detectable ctDNA and concluded that the detection of KRAS mutations in the blood may provide a prognostic and diagnostic tool to direct therapeutic strategies in PDAC cases.

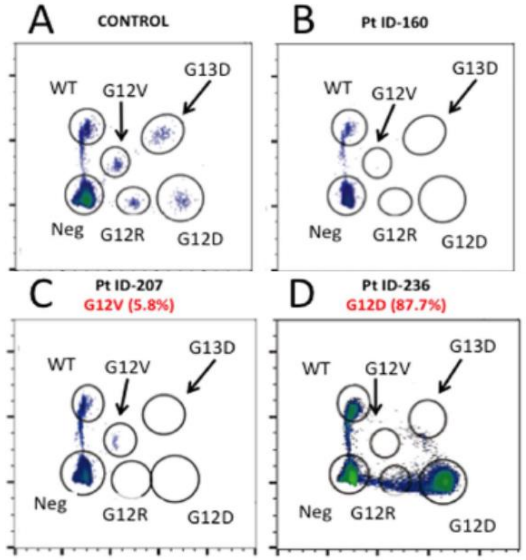

Figure 5: KRAS 5-plex dPCR assay for detection of KRAS mutations in plasma ccfDNA. As a positive control, wild-type and mutant KRAS genomic DNA reference standards were used for cluster gating (A). Representative views of dPCR plots using plasma ccfDNA show no mutant KRAS alleles in a healthy control sample (B), G12V-mutant KRAS alleles (C) and G12D-mutant KRAS alleles (D) in the blood of patients with PDAC.

References

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www.RainDanceTech.com

Support@RainDanceTech.com

LCN 50-08231 Rev A (June 2016)


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