RainDrop® Assay Guidelines

RainDrop® Assay Guidelines apply to:
RainDrop® & RainDrop *Plus*™ Digital PCR Systems

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Contact

RainDance Technologies, Inc.
749 Middlesex Turnpike
Billerica, MA 01821
Fax: (978) 528-6090 (support)
Web: www.raindancetech.com/support
Email: customercare@raindancetech.com

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CHAPTER 1

Introduction

This chapter includes the following topics:

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Introduction to Digital PCR

Digital PCR (dPCR) is a significant advancement over conventional PCR methods. It can be used to clonally amplify and directly quantify nucleic acids including gDNA, cDNA, or RNA. The key difference between dPCR and traditional methods is in the method for measuring the amounts of nucleic acids; dPCR is far more precise. Conventional PCR carries out one reaction per sample. In dPCR, the sample is separated into a large number of partitions and the reaction is carried out individually within each partition. As a result, each partition contains a negative or positive reaction (“0” or “1”). Nucleic acids are quantified by counting the partitions that contain positive reactions.

Unlike qPCR (quantitative PCR), dPCR is not dependent on the number of amplification cycles to determine the initial amount in the sample, eliminating the reliance on uncertain exponential data to quantify targets, thereby providing absolute quantification. Digital PCR does not require a standard curve because it is counting the amplification of single molecules. The method has been useful for studying variations in gene sequences such as detecting rare mutations, minor alleles, and copy number variants. Absolute quantification is crucial in accurately determining higher CNV levels or quantifying rare variants below the level at which qPCR fails due to competitive amplification of the common DNA. Such capabilities are vital in understanding causative somatic changes, monitoring residual disease, and measuring the impact of therapeutic treatment.

Objective

This document describes the design, preparation, and execution of hydrolysis probe-based digital PCR assays on the RainDrop® or RainDrop *Plus*™ Digital PCR Systems. It is designed to help you achieve optimal assay designs for your application. This document outlines current recommendations for assay components, reagent composition, the preparation of dPCR samples, and troubleshooting tips for dPCR applications related to rare allele detection using the RainDrop System.

This document contains recommendations for:

1. assay design
2. testing and optimization of reagents and thermal cycling
3. approaches for assay validation using control samples
4. troubleshooting

This document does not outline the specific step-by-step instructions for interlacing the assay design with performing runs on the RainDrop or RainDrop *Plus*™ Digital PCR Systems since the exact order of steps may vary depending on the application.

Workflow

The operation of the RainDrop or RainDrop *Plus* Digital PCR System is composed of five main steps, as illustrated in Figure 1-1:

Note: This guide covers steps 1 and 3 of this workflow. It describes the development and optimization of an assay (including some aspects of sample prep) and thermal cycling. The other steps in the process are covered in the *Thunderbolts System/RainDance Source Operator's Manual*, *RainDrop Sense Operator's Manual,* and the *RainDrop Analyst II v1.1 User Manual*.

1. **Prepare the sample.** Sample preparation describes the assembly of reagent components and the DNA template to create an aqueous mix that is suitable for droplet-based PCR.
2. **Create and collect.** The Source Instrument automates the process of converting the sample prepared in step 1 into millions of micro-droplets approximately 5 pL in volume. Perform this step in a pre-PCR room or in a dedicated laminar flow hood using industry standard procedures.
3. **Thermal cycle the droplets.** Thermal cycle the droplets in a standard instrument to complete the PCR reaction. Perform this step in a post-PCR room or in a different location from where the PCR reactions were prepared.
4. **Identify and count.** Load the thermal-cycled droplets onto the Sense Instrument where the instrument reads the intensity of the fluorescence of each droplet. Perform this step in a post-PCR room or in a different location from where the PCR reactions were prepared.
5. **Analyze the data.** Use the RainDrop Analyst II Software to interpret experiment results.

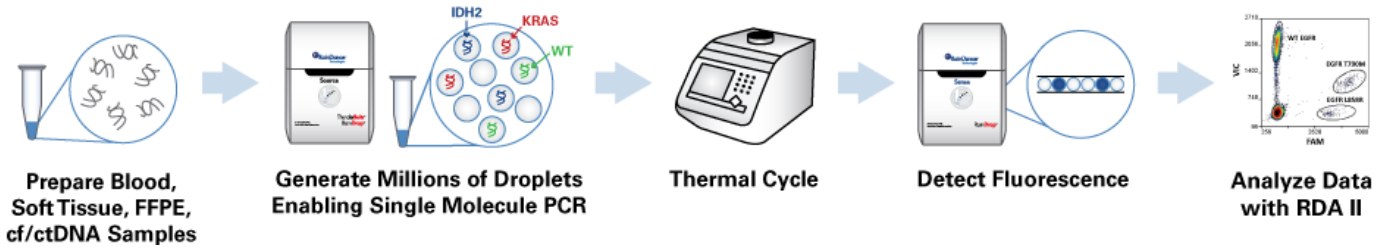

Figure 1-1: Simple Workflow for the RainDrop *Plus* Digital PCR System

CHAPTER 2

Recommended Equipment, Supplies, and Reagents

This chapter includes the following topics:

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Recommended Equipment and Supplies	**page 2-2**
Recommended Reagents	**page 2-3**

Introduction

This chapter lists the equipment, supplies, and reagents required for preparing dPCR samples.

Recommended Equipment and Supplies

The following is a list of the equipment recommended by RainDance Technologies, Inc.

- P2, P10, P20, P200, and P1000 pipettes (routine calibration recommended)
- Sterile aerosol resistant filtered pipette tips
- Vortex mixer
- Mini-microcentrifuge
- 8-Strip PCR Tubes, 0.2 mL (Axygen™ PCR-0208-CP C)
- RainDance Source Chip (30-04295)
- RainDrop® Sense Chip (30-04296)
- RainDrop Elastomer PCR Tube Caps (40-06087)
- Access to Covaris Adaptive Focused Acoustic Instrument or a similar device for shearing DNA
- A thermal cycler with the following specifications, which allows for proper thermal cycling of the emulsion. If you use a thermal cycler that does not meet these specifications, data may be compromised.
  - Thermal cycler block format should accommodate standard 0.2 mL tubes.
  - Adjustable-height heated lids are recommended.
  - The surface of the heated lid should be hard and free of texture.
  - The thermal cycler should allow an adjustable ramp speed.
  - Centrifugal thermal cyclers are not recommended.

Specifically when operating the RainDrop *Plus* System in the fast mode:

- RainDrop Elastomer High Speed Caps (40-08286)

Important: RainDance Technologies recommends using Standard PCR Tube Strip Caps (40-06087) for thermal cycling and subsequently replacing them with the High Speed PCR Tube Strip Caps (40-08286) before transfer to the Sense instrument. Axygen 8-Strip Domed PCR Tube Caps may also be used for thermal-cycling if the cycler does not have an adjustable pressure heated lid. Replace the Domed Caps with Standard PCR Tube Strip Caps or High Speed Tube Strip Caps for Standard Mode or Fast Mode detection on the Sense Instrument respectively.

Recommended Reagents

Table 2-1 lists currently recommended reagents for dPCR-based rare-allele detection application on the RainDrop System.

Table 2-1: Recommended Reagents for the Sample Mix

Reagent	Vendor	Item No.
TaqMan® Genotyping Master Mix	Life Technologies	4371355
TaqMan Probes	Life Technologies, Integrated DNA Technologies, and other vendors	Custom
Oligonucleotide primers	Integrated DNA Technologies	Custom
DNA Controls (genomic or plasmid)	See the note below.	
DNase/RNase-free water	See the note below.	
RainDance 25X Droplet Stabilizer	RainDance	30-07026

Note: RainDance does not recommend a specific vendor for these items. The choice of vendor is at your discretion based upon the assay you are designing or transferring from a qPCR system.

Refer to the following appendices for more information:

- Appendix A “Alternative Recommended Master Mixes”
- Appendix B “Alternative Probe Chemistries”

CHAPTER 3

Assay Design

This chapter includes the following topics:

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In Silico Assay Quality Testing	**page 3-3**
Storing and Handling Primers and Probes	**page 3-3**
Transferring an Existing Assay	**page 3-4**

Introduction

This chapter discusses the TaqMan probe assays for use in detecting rare alleles using the RainDrop® System. It presents recommendations to consider during your assay design. It also discusses transferring an existing assay from another platform for use with the system.

Designing a New dPCR TaqMan Assay

RainDance recommends using hydrolysis probe systems, such as TaqMan MGB (minor groove binders) probes (Life Technologies) or PrimeTime LNA (locked nucleic acids) probes (Integrated DNA Technologies) for detecting rare alleles. This section includes recommendations for design, validation, and storage of probes and primers.

TaqMan Probe-Based Assays for Rare Allele Detection

RainDance currently recommends using TaqMan probe based assays for detecting rare alleles using the RainDrop System. The short design available when using MGB probes or LNA probes results in high specificity, which is necessary for the detection of single nucleotide variants and mutations.

TaqMan MGB Assays are available through Life Technologies, Inc. either as pre-designed SNP assays (http://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-assays/snp-genotyping-taqman-assays.html) that can be purchased pre-mixed or as assays that can be designed in a custom manner for a specific target.

For custom assay designs, use the online ABI Custom TaqMan Assay Design Tool (https://www5.appliedbiosystems.com/tools/cadt/) or design manually using PrimerExpress v3.0. When designing a TaqMan MGB probe dPCR assay manually, consider the following recommendations:

1. Using Life Technologies Primer Express tool, design TaqMan MGB probes with an estimated melting temperature (T_m) of 65-67˚C (MGB moieties inherently increase the primer T_m of the probes).
2. Design TaqMan MGB probes to be as short as possible but no less than 13 nucleotides.
   Tip: Due to the inherent nature of reduced convective heat transfer in droplet PCR, RainDance recommends that you design your assay to favor the higher end of the standard TaqMan T_m range.
3. Avoid stretches of identical nucleotides of 4 or more in the probe sequence.
4. Ensure that the single nucleotide variant target is located as close to the center of the probe as possible.
5. Design primers according to TaqMan standards. Primer T_ms should fall in the 58-62°C range. Calculate T_ms using web-based nearest-neighbor calculation tools such

as Primer3 (http://frodo.wi.mit.edu/) or commercially available oligonucleotide software design tools such as Oligo (http://www.oligo.net/).

6. Ensure that the T_ms of the primers are within 2°C of each other.
7. If multiplexing, be sure the assay design has similar T_ms so that they can perform at equal efficiencies at the same temperatures.
8. Design primers specifically to amplify a target region 75 to 200 bp in length, though ~100 bp is optimal.
9. Perform a BLAST search at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure that you have designed primers so that additional regions are not amplified non-specifically and so that you avoid regions of high homology.
10. Ensure that forward and reverse primers are as close to the probe region as possible, but do not overlap with the probe. If they are too close or if they overlap, hydrolysis does not occur efficiently. The 3′ end of the primer that is on the same strand as the probe should be within 5 bases from the 5′ end of the probe.
11. RainDance recommends that there are more Cs than Gs within the probe region. If Gs exceed Cs within the probe region, design probes for the alternate strand, ensuring that the T_m is maintained as the strand is switched.
12. Due to the natural quenching ability of the G nucleotide, do not design the G nucleotide to be adjacent to the fluorescent moiety.
13. Due to the possibility of mispriming, do not use a 3′ T nucleotide.

For probes containing LNAs, contact IDT technical support (techsupport@idtdna.com) for design assistance.

For information on other probe types and vendors, see Appendix B.

In Silico Assay Quality Testing

If you have designed a new assay or are converting one from qPCR to dPCR, RainDance recommends an upstream evaluation to ensure robust assay performance. Prior to running dPCR reactions, RainDance recommends that you:

- Test primers to ensure amplification of a clean amplicon product.
- Examine the sequence of the amplicon using BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to discern if other regions of high homology could be amplified non-specifically.
- If necessary, perform a standard annealing temperature gradient PCR to discern which annealing temperature is most appropriate to achieve a high quality amplicon product (see “Optimizing Annealing Conditions” on page 5-5).

Storing and Handling Primers and Probes

The following guidelines help you ensure the quality of primers and probes:

- Resuspend lyophilized primers and probes in 1 mM Tris (pH 8.0) to a final stock concentration of 100 µM. You can use 0.1X Tris-EDTA (1 mM Tris, 0.1 mM EDTA, pH 8.0) as an alternative.
- Confirm stock concentrations using a NanoDrop Instrument (Thermo Fisher Scientific) or another spectrophotometric instrument.
- To avoid degradation due to frequent freezing and thawing, RainDance recommends that you aliquot stocks into small volumes for later use and store them at -20˚C.
- To capitalize on the high sensitivity of dPCR assays, use a relatively large amount of amplifiable DNA. To ensure the largest available volume possible for adding DNA to a sample reaction, RainDance recommends that you retain primers and probe aliquots in high concentrations (50 µM to 100 µM).
- For probe aliquots, RainDance recommends using light-restrictive amber tubes to prevent photo bleaching.

Transferring an Existing Assay

You can transfer pre-designed qPCR-based assays to dPCR with little to no optimization. Assemble samples using either the 40X assay mixes as they are provided from Life Technologies or add them as individual components following the recommended reagent concentrations (see “Preparation of the dPCR Sample” on page 4-7). To optimize your assay for use in the RainDrop System, follow the sections below; in particular, RainDance recommends using an upstream in silico and empirical qPCR validation to verify that the reagents are performing as expected.

CHAPTER 4

Sample Preparation

This chapter includes the following topics:

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Shearing Genomic DNA	**page 4-3**
Cleaning up Sheared Genomic DNA	**page 4-4**
QC and Quantify the Fragmented Genomic DNA	**page 4-4**
DNA Control Template	**page 4-5**
Input DNA Calculation	**page 4-5**
Preparation of the dPCR Sample	**page 4-7**
Workspace Considerations	**page 4-8**

Introduction

This chapter explains recommended procedures and precautions for preparing the DNA template and for preparing the sample mix for use in the RainDrop® System. It includes a list of recommended equipment, reagents, and consumables.

The sample preparation protocols described in this document assume that you are familiar with the contents of this chapter, use the recommended equipment and consumables, and implement all recommendations.

The process of DNA preparation consists of four key components:

- **Sample Type**. The type and quality of specimens that are recommended for evaluation on the RainDrop System.
- **Shearing.** A Covaris Adaptive Focused Acoustic instrument and the Blue miniTube protocol for fragmenting DNA.
- **Cleanup.** The Qiagen MinElute® procedure for cleaning up sheared genomic DNA.
- **QC and Quantification.** A spectrophotometer for quantifying and validating the sheared genomic DNA samples.

DNA Sample Preparation

Sample Type

These sample types have been shown to work with the RainDrop System:

- Cell-free DNA or cDNA from blood samples
- Genomic DNA or cDNA from solid tumors and tissue specimens
- Genomic DNA or cDNA from cell lines
- Formalin-fixed, paraffin-embedded (FFPE) tissue specimens

Note: If your sample type is not listed, contact RainDance for additional recommendations.

You can evaluate most DNA sample sources for rare allele detection. Use a Thermo Fisher Scientific NanoDrop Instrument or a comparable spectrophotometer to analyze the $A_{260/280}$ ratios. Ensure that the 260/280 ratio falls within the range of 1.7 to 1.9. RainDance recommends using high quality upstream extraction and purification methods. You can perform a standard or quantitative PCR reaction prior to dPCR to ensure that the DNA is free of inhibitors and is amplifiable.

It is important that the DNA template is approximately 3 kb in length or shorter before proceeding to the emulsion generation on the Source Instrument. DNA originating

from cell-free blood-based DNA or FFPE specimens, or cDNA from any source, should not require additional fragmentation. DNA that is too short will have a low level of amplifiable fragments. RainDance recommends that you run a gel to verify fragment size. You can also use an Agilent Bioanalyzer or equivalent instrument to determine the fragment size.

Processing unsheared genomic DNA is generally acceptable if the loading is less than 0.75% of the anticipated droplets, which for human DNA is 80 ng or less for 25 µL reactions, or 160 ng or less for 50 µL reactions. You should consider if multiple targets are possibly located on the same fragment of template DNA. If co-localized targets are present, then data analysis will be challenging because a disproportionate fraction of droplets will exhibit signal arising from multiple targets.

Purification and Concentration of FFPE DNA

Human cancer specimens are often archived as FFPE specimens. This FFPE archival process typically results in fragmenting the DNA. As such, it is not necessary to physically shear or fragment DNA from FFPE specimens. However, residual inhibitors may affect PCR performance, so it is important to purify and concentrate FFPE DNA samples prior to use.

RainDance recommends the Zymo Research DNA Clean & Concentrator Kit (Cat# D4013) for purifying FFPE DNA samples. Alternatively, you can use the Epigentek Concentrator kit (Cat# P-1006-1) as well. There are several other purification methods available that are compatible with the RainDrop System. Contact your Field Applications Scientist or email support@raindancetech.com if you require further assistance.

Shearing Genomic DNA

As discussed above, the target range for shearing standard genomic DNA for dPCR is 2 to 4 kb in length. The optimal fragment length is 3 to 4 kb. Shorter fragments are acceptable; however, you must validate PCR products to ensure unbiased amplification of the target controls. To obtain the most consistent results, RainDance recommends using the Covaris Adaptive Focused Acoustic instruments for shearing genomic DNA. For more information, consult the Covaris recommended protocol for the Blue miniTUBE (http://covarisinc.com/wp-content/uploads/pn_010299.pdf).

If you are unable to shear using the Covaris system, you can use a variety of other devices to produce DNA of the appropriate size (3 kb). As an alternative, you can shear DNA using a Nebulizer; the methodology is described at: https://tools.thermofisher.com/content/sfs/manuals/nebulizer_man.pdf.

If you want to use alternative shearing methods, contact RainDance for additional recommendations and tips.

Cleaning up Sheared Genomic DNA

If you shear DNA using the Covaris Adaptive Focus Acoustic instruments, no clean up is required. For other shearing methods, RainDance recommends using the Qiagen MinElute® columns for cleaning up sheared genomic DNA. Below is a sample protocol that has been used successfully in conjunction with RainDance's products. For more information and current procedures, consult the Qiagen MinElute Handbook at: https://www.qiagen.com/us/resources/resourcedetail?id=fa2ed17d-a5e8-4843-80c1-3d0ea6c2287d&lang=en.
The MinElute purification kit has a maximum binding capacity of 5 µg and is appropriate for the purification of double-stranded DNA 70 bp to 4 kb in length.

The following lists recommended reagents and supplies for purifying sheared genomic DNA using a Qiagen MinElute kit:

- Qiagen MinElute Kit
- Sterile aerosol-resistant filtered pipette tips
- 5 M NaCl
- 3 M Na-Acetate, pH 5.2
- 10 mM Trizma-HCl, pH 8.0
- Laminar flow cabinet/PCR workstation
- Mini-microcentrifuge
- Thermo Fisher Scientific NanoDrop Instrument or a comparable spectrophotometer

MinElute Clean Up

1. Add the following reagents to your genomic DNA template:
   - Add a 1:10 ratio of 5 M NaCl to the sheared genomic DNA.
   - Add Qiagen PB (binding) buffer (including pH indicator) at ratio of 5X the volume of sample PB buffer to sample volume (the maximum capacity of the column reservoir is 800 µL).
   - Add 10 µL 3 M Na-Acetate, pH 5.2.
2. Mix well and load onto the Qiagen MinElute column.
3. Continue with the standard Qiagen MinElute protocol.
4. Elute each sample off the column with 11 µL of the Qiagen Elution Buffer (EB) (10 mM Trizma-HCl, pH 8.5).

Note: There are several alternative purification methods available that are compatible with the RainDrop System. Contact RainDance Support for further assistance or for recommendations for different cleanup kits.

QC and Quantify the Fragmented Genomic DNA

The expected yield should be sufficient to achieve the desired lower limit of detection (LLOD), as described in "Input DNA Calculation" on page 4-5. To achieve maximal sensitivity and DNA loading, the sample should be concentrated to ~1 µg/µL.

1. Dilute 1.5 µL of the re-suspended fragmented genomic DNA sample with 1.5 µL 10 mM Trizma-HCl, pH 8.0.
2. Quantify 1.5 µL of the fragmented genomic DNA by optical density measurement with a NanoDrop spectrophotometer or a similar instrument.
3. Run approximately 200 ng of the diluted fragmented genomic DNA on a 0.8% agarose gel to ensure that you obtain the correct size range of fragmented genomic DNA. An alternative is to run ~10 to 50 ng on a high sensitivity chip on the Agilent Bioanalyzer.

DNA Control Template

RainDance recommends using representative target control DNA samples such as wild-type human genomic DNA from Promega (item #G3041). RainDance also recommends using cell-line genomic DNA or previously characterized samples as controls for rare variants and mutations. If genomic DNA is unavailable for a particular target of interest, RainDance recommends using linearized plasmid DNA or synthetic oligonucleotides.

For circularized plasmid DNA, RainDance recommends that you incorporate a restriction enzyme cut site into circularized plasmid control samples. You can digest and subsequently purify the plasmid with a Qiagen MinElute column following the manufacturer's recommendations. QC and quantify the digested and purified plasmid template following the recommendations above; "QC and Quantify the Fragmented Genomic DNA."

Alternatively, use synthetic oligonucleotides as template controls. You can purchase synthetic oligonucleotides as single-stranded ultramers (http://www.idtdna.com/pages/products/dna-rna/ultramer-oligos) or double-stranded gBlocks (https://www.idtdna.com/pages/products/genes/gblocks-gene-fragments) from Integrated DNA Technologies. These synthetic oligonucleotides are synthesized in the 3′ to 5′ direction. With this synthesis strategy, a certain fraction of the synthesized oligonucleotides will not be full-length.

RainDance recommends that the oligonucleotides are designed such that the target length is notably increased on the 5′ end to account for the incomplete synthesis (see http://www.idtdna.com/pages/products/dna-rna/ultramer-oligos for further guidance). If a large portion of your template DNA is not the full length of your amplicon, PCR will either not occur or in some cases, the efficiency will be compromised and the overall drop fluorescence will exhibit high variability (clusters will not be well defined and may be difficult to gate).

Input DNA Calculation

RainDance recommends that control samples are evaluated by incorporating the target DNA at concentrations that span the dynamic range of samples to be evaluated. The LLOD of a given reaction is influenced by the number of copies of target DNA that are evaluated and the statistical confidence of mutation calling (see *RainDrop Analyst II v1.1 User Manual*). It is important to determine the number of copies of DNA that should be evaluated in order to reach the desired assay sensitivity.

Presently, RainDance recommends DNA loading up to ~10% target occupancy. For example, 5 million droplets (5 pL in volume) can be created from a 25 µL reaction, and can contain up to 500,000 target molecules of DNA (roughly 1.5 µg of human genomic DNA), while 10 million droplets will be generated from a 50 µL reaction and can contain up to one million target molecules of DNA, which is roughly 3 µg of human genomic DNA.

Calculate the Number of Target Copies (Genome Equivalents)

Use one of the following approaches to calculate the number of target copies contained in a given sample.

Note: The number of genomic equivalents = the amount of total DNA / the weight of the genome.

Note: The molecular weight of one diploid copy of the human genome is ~6.6 pg, while the human genome is approximately 3 billion base pairs in length.

1. Use a simple calculator to estimate the number of template copies here: https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html#/legacy=www.thermoscientificbio.com
2. Perform the full calculation as follows:
   On average, one base pair (bp) weighs 650 Daltons. As such, you can calculate the molecular weight (MW) of a double stranded DNA template of known length by multiplying the genome length (in bp) by the weight of 1 bp (650 Da):
   - MW of genome (or plasmid/virus) = (Length in bp x 650)
     e.g., human genome = $3 \times 10^9 \times 650 = 1.95 \times 10^{12}$
   - 1 mole of DNA (i.e., MW in grams) is equivalent to Avogadro's constant (6.022×10^{23} molecules). Therefore, the weight of 1 genome (molecule):
   - 1 molecule (g) = (Length in bp x 650)/(6.022×10^{23})
   - e.g., human genome = ($3 \times 10^9 \times 650$)/(6.022×10^{23}) = $3.23\ 10^{-12}$g or 3.23 pg
   - Calculate the target copy number of Genome Equivalents (GE) as follows:
   - GE = (Weight of DNA) / (Weight of 1 genome)
   - e.g., GE in 1.65 µg Human DNA = 1.65 / (3.23×10^{-6}) = 510,836
   - 1 ng of human genomic DNA contains ~300 genome equivalents. Human Genomic DNA at a concentration of 200 ng/µl has ~60,600 genome equivalents/µL.

Calculate the Target Occupancy

You can calculate target occupancy by dividing the number of expected droplets by the estimated number of target copies (genome equivalents). For example,1.5 µg of human

genomic DNA has roughly 500,000 target copies of DNA, which in 5 million droplets result in 10% DNA occupancy.

Target occupancy = (expected number of droplets) / (number of target molecules)

Preparation of the dPCR Sample

Following Table 4-1 below, assemble the PCR master mix for the appropriate number of samples to be evaluated. For uniformity, calculate the required reagents needed for the number of samples to be prepared. Prepare a single master mix whose volume is sufficient to analyze all samples.

Table 4-1: Recommended Reagent Components

Reagent Mix	Per Reaction
TaqMan Genotyping Master Mix (Life Technologies, Item #4371355)	1X
25X Droplet Stabilizer (RainDance)	1X
Custom primers	0.5 to 0.9 µM*
TaqMan Probes (Life Technologies, IDT, custom or pre-designed assay)	0.2 µM
DNA template	Variable
DNase/RNase-free sterile water	Bring to volume

Caution: To avoid creating air bubbles, dispense the sample slowly.

*** Note:** You can use a primer concentration of 0.5 µM, however, if you use a pre-designed 40X assay mix from Life Technologies, a 0.9 µM concentration is acceptable. Due to the confined space in picodroplet-based PCR, high amplicon concentrations produced throughout PCR can compete with probe hybridization and reduce the final cluster signal. Reducing the primer concentration lessens the amplicon-probe competition in the later stages.

RainDance recommends that prior to use, you filter and autoclave all water, Tris, TE, or other reagents used in the resuspension or preparation of primers, probes, DNA, and sample stocks. A 20 µM filter has proven sufficient to remove debris that could potentially destabilize the emulsion. Any debris or particulate material may result in destabilization of the emulsion.

Workspace Considerations

Follow standard pre-PCR laboratory practices to avoid contamination that could lead to false positive amplification. The sensitivity and LLODs of dPCR assays may be affected by the occurrence of contamination and false positive amplification.

To reduce the risk of false positive amplification due to contamination, RainDance recommends the following practices:

- Maintain a clean working space. Clean lab benches and equipment periodically with freshly diluted 10% bleach solution or decontamination reagents such as DNA-ExitusPlus (http://www.applichem.com/en/shop/product-detail/as/dna-exitusplustrade/).
- Maintain separate work areas dedicated to pre-PCR sample preparation, emulsion generation on the RainDance Source Instrument, PCR amplification and thermal cycling, post-PCR reading of emulsions on the RainDrop Sense Instrument, and data analysis.
- Be careful to avoid contamination of samples, reagents, equipment, and the pre-PCR space.
- Use new and clean personal protection equipment when preparing samples for PCR amplification.
- Use a laminar flow cabinet/PCR workstation that contains UV decontamination. (RainDance recommends that you routinely decontaminate bench tops and equipment in your pre-PCR environment.)
- To reduce the risk of contamination, never bring highly-concentrated plasmid controls (>5000 copies/µL) or amplified PCR products into the pre-PCR room.
- Use aerosol-resistant pipette tips and decontaminate pipettors frequently.
- Keep caps closed whenever possible to reduce the risk of introducing debris into samples.
- Dispense the sample slowly to avoid forming air bubbles throughout the sample preparation step.
- For safety and biohazard guidelines, refer to the Safety section in the Life Technologies TaqMan Genotyping Master Mix Protocol (http://tools.invitrogen.com/content/sfs/manuals/cms_039282.pdf).

CHAPTER 5

Thermal Cycling

This chapter includes the following topics:

Introduction	**page 5-2**
Thermal Cycler Requirements	**page 5-2**
Thermal Cycling Parameters	**page 5-2**
Observing Thermal Cycled PCR Tubes	**page 5-3**
Ramping Speed	**page 5-4**
qPCR Primer and Probe Test	**page 5-5**
Optimizing Annealing Conditions	**page 5-5**
Annealing Test Using qPCR	**page 5-5**
Annealing Test Using dPCR	**page 5-7**

Introduction

This chapter describes important considerations for thermal cycling for dPCR reactions.

Thermal Cycler Requirements

- Thermal cycler block format should accommodate standard 0.2 mL tubes.
- RainDance recommends adjustable-height heated lids (if these are unavailable, you can use hard silicone inserts (VWR 1011-006), but it is imperative that evaporation and condensation are limited. RainDance cannot guarantee maximal performance if you use this approach.)
- The surface of the heated lid should be hard and free of texture. Soft, foam or gel-based heated lids do not provide sufficient pressure to prevent evaporation and condensation. A textured or domed surface may damage the tube strip cap.
- Adjustable ramp speed. 0.5°C/second.
- Centrifugal thermal cyclers are not recommended.

Thermal Cycling Parameters

You can thermal cycle dPCR reactions on standard thermal cyclers with Peltier units. RainDance does not recommend using centrifugal thermal cyclers for droplet PCR. It is important to use a thermal cycler with a heated lid to control sample evaporation and condensation. RainDance does not recommend using oil overlays.

Note: If your thermal cycler allows you to define the sample volume in the program, use 75 µL. The 75 µL volume is an approximate final volume of emulsion and oil that is generated on the Source Instrument.

RainDance recommends using the master mix manufacturer-recommended thermal cycling conditions specific to the master mix that is used. When using the Life Technologies Genotyping Master mix, apply standard thermal cycling conditions for TaqMan probes as defined in Table 5-1 with the addition of a final polymerase deactivation step and a slower ramping speed.

Table 5-1: Thermal Cycling Conditions for 2XGenotyping Master Mix

Step	Temp	Time	Cycles
Polymerase activation	95°C	10 min	1
Denaturation	95°C	15 sec	45
Annealing & Extension	60°C	1 min	45
Final hold	98°C	10 min	1

Table 5-1: Thermal Cycling Conditions for 2XGenotyping Master Mix

Step	Temp	Time	Cycles
Cool down and storage	4°C		

Observing Thermal Cycled PCR Tubes

The following images may help you identify conditions that may cause problems when thermal cycling your PCR tubes.

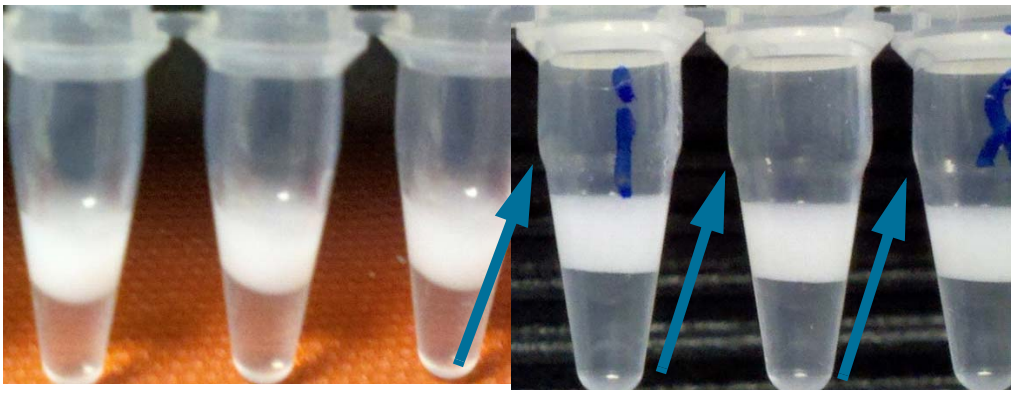


Figure 5-1: Comparison of Good Tubes and Misshapen Tubes

Confirm that your thermal cycler does not cause the tubes to become misshapen. The tubes on the left have been properly thermal cycled; the tubes on the right were damaged during thermal cycling. Tubes can become misshapen when the thermal cycler contains an atypically shaped heating block or when too much pressure is applied from the lid. Misshapen tubes can interfere with the way the PCR tubes work with the RainDrop® Sense Chips.

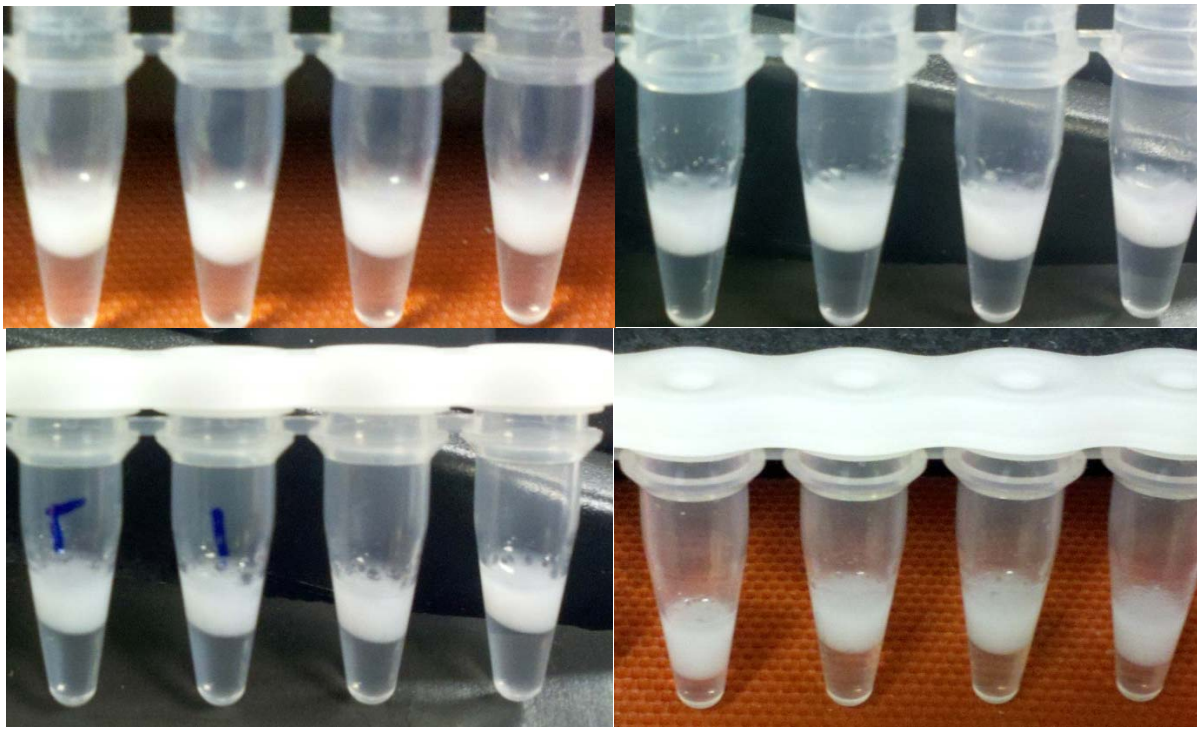


Figure 5-2: Examples of PCR Tubes

When sufficient pressure is not applied by the heated lid, condensation and evaporation can occur during thermal cycling. Evaporation can also occur if the lids are not placed on the tubes correctly. The tubes in the upper left have been properly thermal cycled. The tubes in the upper right show a large amount of condensation on the sides of the tubes. The tubes on the lower left show air bubbles at the surface of the emulsion likely caused by evaporation and condensation, whereas those on the right exhibit evaporation in some of the tubes (as indicated by the variability in overall volume). Evaporation and condensation may result in generating spurious noise and clusters within the dPCR landscape.

Ramping Speed

Heat transfer occurs more slowly in emulsified samples than it does in bulk sample PCR. As such slower ramp speeds prove beneficial to equilibrate temperature exposure across the droplet population.

Note: RainDance recommends using a slow ramp speed of approximately 0.5°C/second. If you cannot achieve a slow ramp speed, you can increase the hold times by 1.5x to improve the PCR performance.

qPCR Primer and Probe Test

RainDance recommends that you run an assay test with qPCR prior to dPCR. RainDance also recommends using standard positive, negative, and no-template control samples to ensure that assays result in robust amplification, successful probe hydrolysis, and assay specificity. This test may also be beneficial to pre-screening newly prepared template samples in order to quantify the proportion of amplifiable DNA.

To ensure clean and robust amplification and detection of the target, run a gradient PCR to discern which annealing temperature is most appropriate to achieve specificity between the controls (see “Optimizing Annealing Conditions” on page 5-5). Of note, the LLOD of the assay can be influenced by the degree of probe specificity and the occurrence of false positive amplification as well as inefficient amplification and probe hydrolysis.

Optimizing Annealing Conditions

As with standard PCR and qPCR, it is important to empirically determine the reaction annealing temperature such that assay specificity is achieved. Because dPCR allows for the individual identification of single molecules, it is important to design and effectively optimize the specificity of the oligonucleotide system. As outlined below, RainDance recommends using qPCR to optimize annealing temperatures prior to optimizing dPCR. RainDance recommends designing the assay so that you can use an annealing temperature of 60°C with most PCR master mixes for hydrolysis probes.

Annealing Test Using qPCR

RainDance recommends that you first test assays in a qPCR setting to ensure high probe specificity and robust amplification. Evaluate a simple gradient analysis of the annealing temperature (typically appropriate annealing temperatures occur between 50°C and 65°C). When using the Life Technologies TaqMan Genotyping Master Mix, perform the extension step at the standard 60°C.

The ideal annealing temperature promotes robust amplification without compromising specificity. Specificity is typically more critical than the end point fluorescence intensity to improve the lower limit of detection of an assay.

Referring to Figure 5-3, panels A and D show the matched VIC target and VIC assay and FAM target and FAM assay respectively. Panel A shows the effect of temperature on the amplification of the VIC target by the VIC assay, while panel D shows the effect of temperature on the amplification of the FAM target by the FAM assay. For both assay/target pairs, 55°C gives the highest end point signal, while 68°C and 72°C give the lowest.

Panels B and C show the cross reactivity of the FAM assay to the VIC target, or the VIC assay to the FAM target, respectively. The FAM assay shows little amplification of the VIC

target (panel B). However the VIC assay shows significant amplification of the FAM target at annealing temperatures of 48-55°C (panel C). The cross reactivity drops dramatically at an annealing temperature of 62°C and higher.

Based on this data the ideal annealing temperature is 62°C, which gives minimal cross talk and acceptable end point signal.

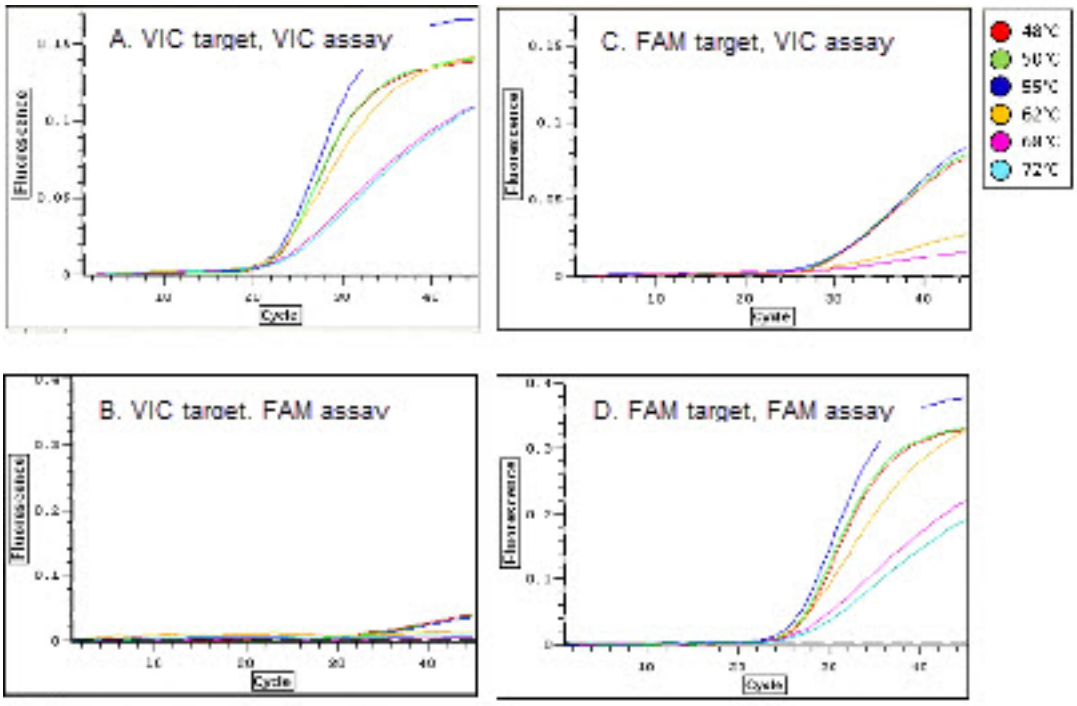


Figure 5-3: Annealing Gradient Test. Panels A and C contain the VIC assay. Panels B and D contain the FAM Assay. Panels A and B contain the VIC target. Panels C and D contain the FAM target.

After identifying an appropriate annealing temperature using qPCR, RainDance recommends that you confirm the annealing temperature using dPCR evaluation and the appropriate positive controls to limit the occurrence of false positive and false negative amplification.

Annealing Test Using dPCR

After you qPCR test the annealing temperatures, RainDance recommends that you confirm annealing temperatures in a dPCR setting to ensure robust amplification and high probe specificity. Rather than using a two-step PCR cycling protocol, use a traditional three-step PCR to improve the reaction performance (as presented in the following table), which fixes the annealing temperature at 60°C. A three-step PCR allows for the annealing temperature of the assay to be incorporated, while allowing for the extension to be performed at the temperature that is optimal for the polymerase.

Table 5-2: Recommended Three-step PCR Cycling Protocol to Accommodate for an Altered Annealing Temperature using 2x Genotyping MM

Step	Temp	Time	Cycles
Polymerase activation*	95°C	10 min	1
Denaturation	95°C	15 sec	45
Annealing	T_a°C gradient	15 sec	45
Annealing & Extension	60°C	45 sec	45
Final hold	98°C	10 min	1
Cool down and storage	4°C		1

*Note: If you use a master mix other than 2x Genotyping from Life Technologies, adjust the activation step according to the master mix recommendations.

As in standard PCR, the three steps are composed of a denaturation step, annealing step, and an extension step. Evaluate a simple gradient analysis of the annealing temperature (typically appropriate annealing temperatures occur between 50°C and 65°C).

You can identify optimal annealing temperatures by quantifying the following events:

1. Cluster separation
   - **a)** Maximize the distance between two positive target clusters.
   - **b)** Maximize the distance from a positive target cluster to a negative cluster.

2. False positive and false negative error
   - **a)** When an assay is poorly optimized, non-specific amplification can pull one target towards the other target. As the incorrect probe is hydrolysed, it can result in pulling the droplet into the gating area of the alternative target and result in false positive scoring.

As presented in Figure 5-4, you can optimize the annealing temperature to reduce non-specific probe hydrolysis and to increase amplification efficiency. Panel A demonstrates how you can improve probe specificity. For this particular assay, increasing the annealing temperature to 59°C reduces the non-specific probe hydrolysis observed at 50°C, increases the distance between the positive clusters, and drives the positive clusters closer to the FAM and VIC axes.

You can improve amplification efficiency by optimizing the annealing temperature as well. Figure 5-4 shows that reducing the annealing temperature to a temperature such as 50°C improves the amplification. In addition, increasing the annealing temperature to a temperature such as 68°C inhibits the reaction. The goal is to find a temperature that optimizes probe specificity while still achieving robust amplification.

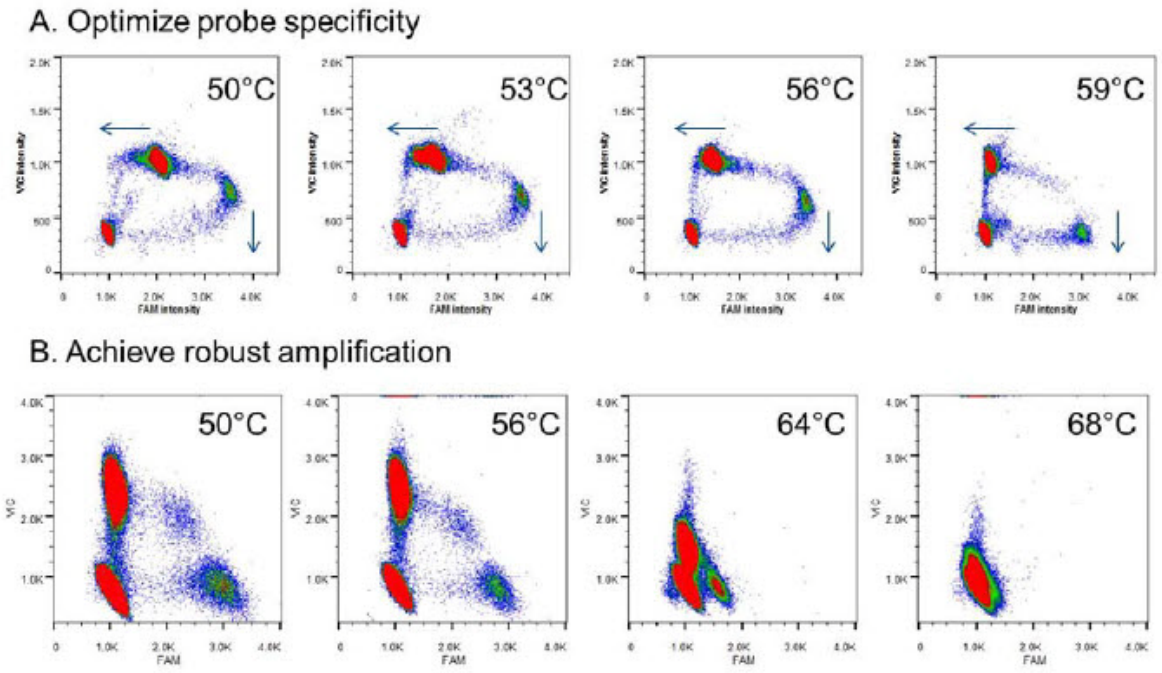


Figure 5-4: Probe Specificity and Reaction Efficiency. Optimizing the annealing temperature proves beneficial for controlling assay non-specificity (panel A) as well as improving the robustness of the assay efficiency (panel B).

CHAPTER 6

Multiplexing with RainDrop® Digital PCR

This chapter includes the following topics:

Introduction	**page 6-2**
Strategies for Developing Robust Multiplex Assays	**page 6-2**

Introduction

By leveraging millions of droplets, the RainDrop® System is able to provide superior multiplexing capability by combining both color and probe intensity techniques to create high resolution two-dimensional data plots (Figure 6-1).

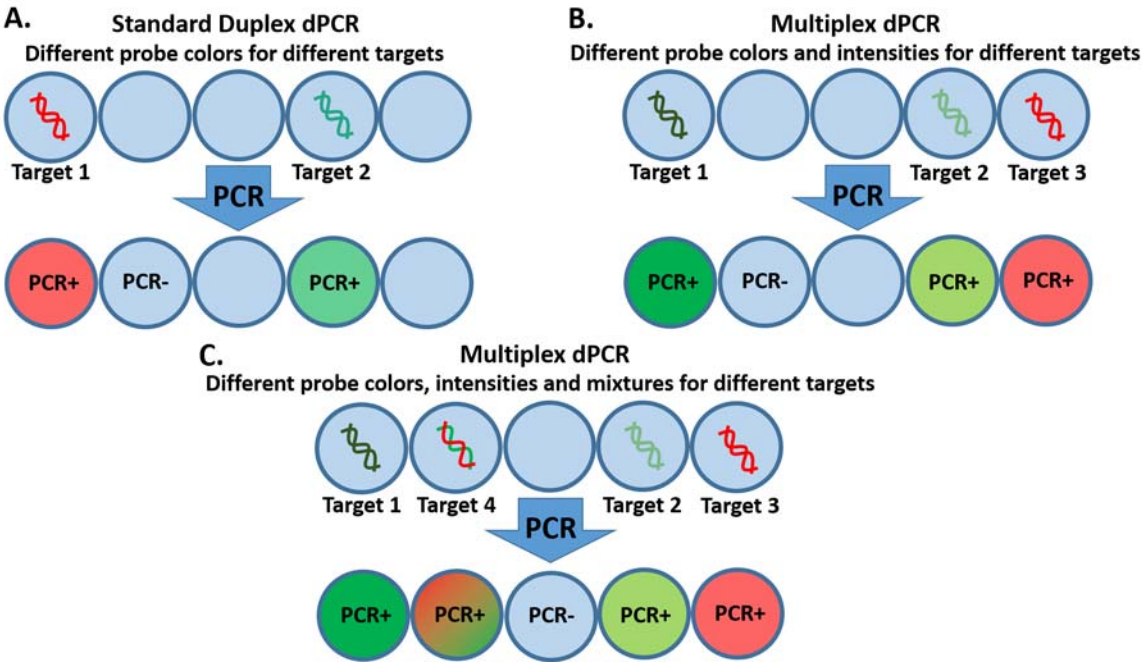


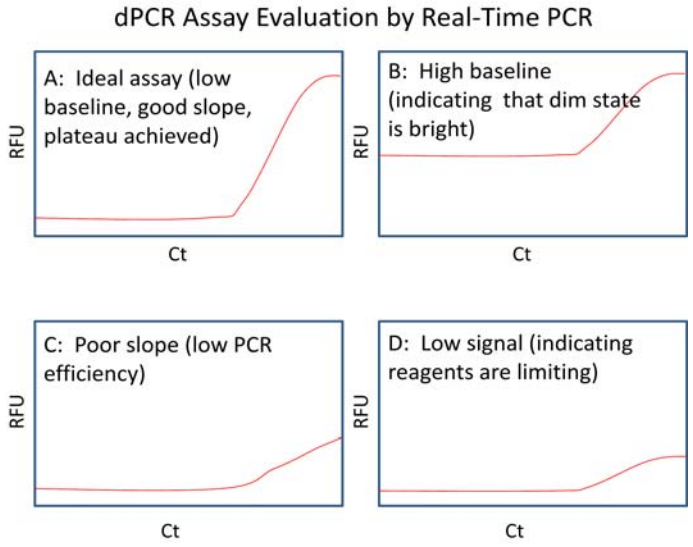
Figure 6-1: Methods for Multiplexing Digital PCR. A standard duplex assay (A) can be built upon by adjusting probe intensity (B) as well as mixing probe fluorophores (C) to assess for >2 targets of interest within the same sample.

Note: The sensitivity of a dPCR assay designed to detect variants may be different when run as a single assay than when it is combined with other assays in a multiplex format. Multiplexing assays often results in a decline of sensitivity for some of the assays. The sensitivity of a dPCR assay when run as a multiplex must be determined experimentally.

Strategies for Developing Robust Multiplex Assays

1. Select target assays and design multiple PCR primer combinations per assay. Optimize PCR efficiency by end-point and/or qPCR (SYBR Green). Design primers that avoid excessive dimerization and formation of secondary structures. This is particularly critical as the complexity of primer composition increases. Avoid primer designs that may be complementary to pseudogenes.

2. With primers identified, design corresponding fluorescent probe(s). Test primer/probe combinations as single or duplex reactions (WT_VIC/MUT_FAM) for each assay by qPCR. Assess baseline noise floor (without baseline subtraction), growth curve slope and maximum RFU signal.

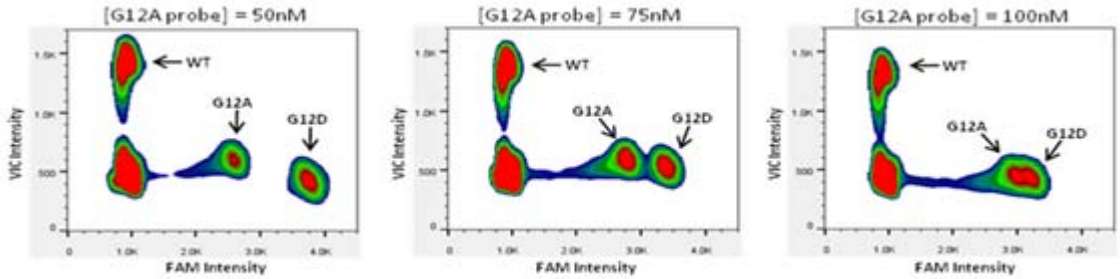


(Optional: Optimize primer/probe concentrations using qPCR before testing using dPCR. This is a relatively quick way to test for allele specificity of each probe in a duplex.)

3. Test each duplex assay using dPCR using 500 nM primer concentration and a titration of 50-100-150-200 nM of MUT and WT probes.
4. Assess cluster signal-to-noise (S/N) for each probe concentration per assay and construct a preliminary map of expected cluster positions based on S/N data from titration experiments. Note which assays generate ‘droplet drag’ from the negative (NEG) cluster to the expected cluster. Assays with clusters that drag should be assigned positions closer to the NEG cluster (by limiting probe concentration). Assays without drag should be placed further from the NEG cluster by using higher probe concentrations.
5. To begin construction of the desired multiplex assay mix, start with a single duplex assay and continue to add duplex assays one by one in successive fashion. Note that the cumulative background fluorescence increases proportionally with each duplex assay addition. As this results in a decrease of cluster S/N, more judicious positioning of clusters is required as the number of assays increases.
6. When starting with assays whose clusters have similar S/N, modulation of corresponding concentrations, lower concentrations results in movement towards the NEG cluster and higher concentrations away from the NEG cluster.

   Probe concentrations should be limiting in the reaction and can be easily adjusted to generate clusters that are sufficiently separated from each other for gating. The following image 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-

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 500-900 nM range and probe concentrations from 30-300 nM. If any assays generate ‘droplet drag’, they should be assigned positions closer to the NEG cluster by reducing probe concentration..



7. When starting with assays whose clusters exhibit varying S/N, try probes for each assay at the same concentration.
8. In addition to locating clusters along either the X or Y axes, clusters may be positioned in the remaining 2-D space by using a combination of VIC-labeled FAM-labeled probes for the corresponding assay.
9. Using a higher proportion of the VIC probe will place the cluster in the 2-D space closest to that axis. The converse is true with higher proportion of FAM probe.
10. Gene expression multiplex assays may be more specific than SNP multiplex assays due to a single probe interrogating a single gene. However, the challenge is to ensure minimal primer-primer interaction.
11. Multiplex SNP assays interrogating multiple mutations under single gene locus may benefit from using one primer pair with multiple probes. This allows for uniformity in terms of PCR efficiency across all probes in the multiplex. The challenge with this situation is the need for very high probe specificity among all the probes interrogating the same locus.

CHAPTER 7

Diagnosing and Minimizing Coalescence in Digital PCR Data

This chapter includes the following topics:

Introduction	**page 7-2**
What does coalescence look like?	**page 7-2**
Where does coalescence come from, and how can it be reduced?	**page 7-4**
Can coalesced droplets be removed from the data for data clarity purposes?	**page 7-5**

Introduction

Often, multiple targets are interrogated in a single sample, such as wild-type (WT) and mutant alleles of a gene for rare allele detection, or target and reference genes for copy number or gene expression analyses. Under optimal conditions, the droplet partitions remain intact and separate from each other after droplet generation on the RainDance Source Instrument, through thermal cycling, and ultimately droplet reading on the RainDrop® Sense Instrument. However, certain conditions may cause multiple droplets to merge together, or “coalesce,” mixing their contents and ultimately producing clusters with mixed fluorescence signals. This section discusses how to identify coalescence in digital PCR data, possible factors contributing to it, and potential ways to reduce it.

What does coalescence look like?

When droplets containing different targets merge during thermal cycling, the combined large droplet now contains templates and hydrolyzed probes for each target. During reading on the RainDrop Sense Instrument, these larger droplets are split back into smaller droplets, each individual small droplet subsequently containing some positive fluorescence for both targets. As a result, coalescence most often shows up on 2-D digital PCR plots as extra “clusters” with intermediate fluorescence for each target in between the two positive clusters. If the occupancy of each target in the sample is high and relatively equal, these clusters may be closer to the middle of the plot (A). If the occupancy of one target is much higher than the other, the coalescence cluster may appear closer to the higher-occupancy target’s axis (B).

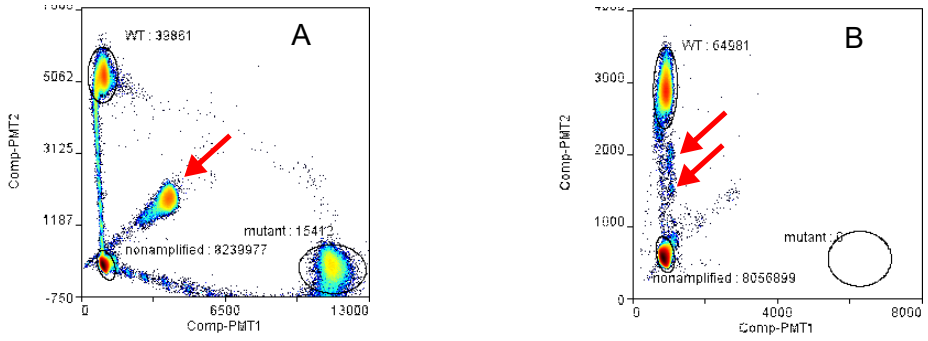


Figure 7-1: A- An example of a 2-target digital PCR data plot with an extra cluster due to coalescence, indicated by a red arrow. B- An example of a 1-target digital PCR; arrows illustrate coalescence.

The best way to distinguish a coalescence cluster from a true extra positive cluster, or a double-occupancy cluster is to view the data in a “1-D” plot. To do this in RDAII, first perform spectral compensation so your positive clusters are at a 90-degree angle. Next, change the X-axis to “Events” and adjust the axis scale to include all of the droplets for that sample. The Y-axis may be either Comp-PMT1 or Comp-PMT2; note that coalescence may be more obvious in one channel than the other, so you may want to try setting the Y-axis to each option.

Samples without coalescence will typically show two concentrated horizontal lines of droplets that extend uninterrupted across this “Events plot”: one with low fluorescence, representing the PCR-negative droplets, and one with high fluorescence, representing the PCR-positive droplets in that channel (Figure 2).

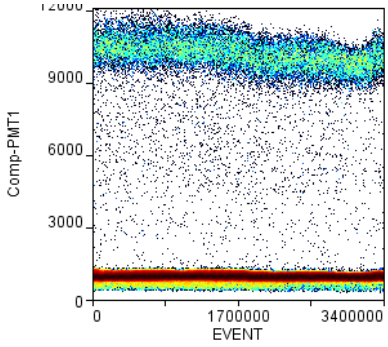


Figure 7-2: One-dimensional plot of Events versus Comp-PMT1 with no coalescence. Two horizontal lines are observed, representing the PCR-negative (low fluorescence) and PCR-positive droplets (high fluorescenc**e**).

For samples with coalescence, the events plot will reveal extra clumps of droplets, typically closely spaced along the Events axis, and with fluorescence in between the PCR-negative and PCR-positive droplets (Figure 3). Droplets from coalescence typically appear as vertical lines when data is viewed in the Event plot view. This pattern is observed due to the large coalesced droplets being divided into smaller droplets as they are pushed through the Sense Chip microfluidics.

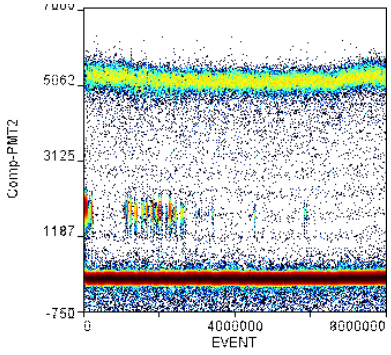


Figure 7-3: One-dimensional plot of Events versus Comp-PMT1 with visible evidence of droplet coalescence, indicated by red box.

Where does coalescence come from, and how can it be reduced?

The greatest contributor to coalescence is unfavorable conditions within the PCR tubes during thermal cycling. For a list of circumstances that may contribute to coalescence and possible corrective actions, see Table 1 below. For clarification or individualized discussions pertaining to your lab environment or equipment, email support@raindancetech.com.

Table 7-1: Potential contributors to coalescence and possible corrective actions

Potential Contributor	Suggested Corrections
Improperly sealed PCR tube caps. If the tube/cap seal is incomplete, air circulation may cause evaporation and coalescence within samples.	• When applying the elastomer caps to the strip tubes, massage the cap onto each sample's tube for several seconds. • Alternatively, try thermal cycling with hard plastic domed caps. Replace with elastomer caps before loading tubes onto the Sense Instrument.
Insufficiently heated thermal cycler lid. A heated lid is required to prevent evaporation and condensation of the oil and sample on the sides of the PCR tubes.	• Confirm that the cycler is programmed to turn on the heated lid to 105°C. Higher temperature may be required for certain thermal cycler models to prevent excessive condensation on tubes between the lid and the sample. • Carefully confirm that heated lid is functioning.
Improperly tightened thermal cycler lid. If the lid is too loose, sufficient pressure will not be applied to seal the elastomer caps onto tubes. If the lid is too tight, tubes may become distorted, compromising the lid-cap seal.	• If your thermal cycler has a torqueing lid, tighten the tension dial until the tension limit is reached, usually indicated by audible clicks. • If your thermal cycler lid is only manually adjustable, turn the tension dial until some tension is felt, and add no more than ¼ revolution of the dial. • If your thermal cycler lid tension is automatically set by the cycler, refer to the manufacturer to ensure the tension is set appropriately.
Insufficient droplet stabilizer concentration. The Droplet Stabilizer provided in the RainDrop Consumables kits is required at 1x in each sample to ensure emulsion stability.	• Verify that you have added Droplet Stabilizer to your samples to 1x final concentration. • Verify that the expiration date of your Droplet Stabilizer stock solution has not expired.

Table 7-1: Potential contributors to coalescence and possible corrective actions

Incompatible PCR master mix. Commercially available or in-house PCR master mix recipes may contain or lack additives that can impact droplet stability. RDT has not exhaustively tested the spectrum of master mixes available.	• If possible, test your reactions in a different master mix. The TaqMan Genotyping Master Mix (Thermo Fisher Catalog #4371355) has been tested and can serve as a recommended comparator. • Check with RainDance Technologies Support regarding the choice of master mix.
Static build-up on PCR tubes, equipment, or personnel handling tubes Static can build up on plastic ware, clothing/lab coats, or equipment. Static charges can significantly compromise the digital PCR droplets. Be particularly aware of this source of coalescence when the air is very dry e.g aggressive air conditioning or low external temperatures.	• Cotton (non-polyester) lab coats and nitrile gloves are recommended. Avoid wearing synthetic clothing, which can generate static charges with movement. • Store PCR tubes in gray anti-static plastic bags designed for computer components. • Install well-grounded anti-static mats below the thermal cycler and in the reaction set up area. • Treat PCR tubes, pipet tips, and plastic racks used in reaction assembly with an anti-static gun. • Consider using anti-static wands mounted over the reaction prep area.

Note that depending on the degree of coalescence in the samples, droplets may read on the Sense Instrument without any indication of coalescence. However, coalescence due to poorly heated cycler lids or improperly sealed tubes may result in a large proportion of droplets being of an unexpected size, and the Sense Instrument may flag these samples as “Wet.” In addition, if not obvious in the 2-D scatter plots, coalescence may be indicated by a lower than average “Intact” population reported by the filters applied to the data in the RainDrop Analyst or RainDrop Analyst II software. Refer to the potential contributors and suggested corrections in the table above.

Can coalesced droplets be removed from the data for data clarity purposes?

The RainDrop Analyst II software includes gating functionality that allows you to selectively remove data from a data plot. In the event that the data shows clear evidence of coalescence in an Event plot, use gating to remove the unacceptable data, i.e., a form of NOT gating. We strongly recommend that you consider the suggested actions above first to minimize coalescence. If coalescence-based clusters that confound clear data presentation persist, this feature may be utilized.

CHAPTER 8

Troubleshooting

This chapter includes the following topics:

Introduction	**page 8-2**
Amplicon Quality	**page 8-2**
Confirm Primer and Amplicon Quality in Silico	**page 8-2**
Confirm Empirical Amplicon Quality	**page 8-2**
TaqMan Probe Quality Evaluation	**page 8-2**
Preparing the qPCR Samples	**page 8-2**
Analysis	**page 8-3**
Recommendations	**page 8-4**
Additives	**page 8-4**
Magnesium Chloride	**page 8-5**
Tetramethylammonium Chloride	**page 8-6**
Droplet Stability	**page 8-6**
False Positives and False Negatives	**page 8-7**
DNA Loading	**page 8-8**
Sample-to-Sample Cluster Movement	**page 8-8**
Additional Troubleshooting Information	**page 8-10**

Introduction

This troubleshooting section provides examples of potential issues that may arise while optimizing dPCR reactions and recommendations for how to address them. If you encounter other issues, contact RainDance for support.

Amplicon Quality

Confirm Primer and Amplicon Quality in Silico

RainDance recommends designing the assay to use an annealing temperature of 60°C. RainDance recommends that you examine the sequence of the amplicon using BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to discern if other regions of high homology are amplified non-specifically. It is important to ensure that non-target regions such as pseudogenes are not amplified unintentionally.

Confirm Empirical Amplicon Quality

You can test primers independently (without probes) to ensure amplification of a clean amplicon product. You may need to perform a standard gradient PCR to discern which annealing temperature is most appropriate to achieve the highest quality amplicon. You can subsequently analyze the products using the Agilent BioAnalyzer or run them on an agarose gel to ensure that you have generated a single amplicon.

TaqMan Probe Quality Evaluation

The objective of this protocol is to evaluate the quality of your TaqMan probes through the use of qPCR. Probe quality and fluorescence influences the location of gates that are used in defining the positive cluster gates. As such, reduced quality or lot-to-lot variability in probe stocks may require that cluster gating is adjusted during the analysis process. To reduce this effect, RainDance recommends that you assess the quality of your TaqMan probe.

The following protocol describes an approach for comparing probe stocks. You can perform this comparison between probes of the same fluorescent moiety (for example, in a situation in which one probe yields unfavorable cluster intensity when compared to another) or use it to compare probes from differing lots.

Preparing the qPCR Samples

1. Prepare samples containing the test lot.
   a) Determine the number of samples to be evaluated.

b) Assemble the reagent components into a master mix to achieve normal reagent concentrations as defined in Table 4-1. Mix gently. RainDance recommends a reaction volume of 25 µL.

2. Follow the same protocol in step 1 above to prepare samples using the control lot of TaqMan Probe.
3. For each sample mix, dispense 25 µL into each PCR tube.
4. Load the capped tubes into the qPCR instrument, and follow the instrument manual for setting up thermal cycling and signal collection protocols. RainDance recommends that assay-specific pre-optimized thermal cycling profiles are used.

Analysis

Most qPCR instrumentation software contains a function to display the amplification curve without the background subtraction called the raw fluorescent units or RFU. You can use this option to calculate the fluorescence gain (ΔF, the fluorescence intensity at the endpoint minus the fluorescence intensity at the first cycle). Use the fluorescence gain to determine the degree of lot-to-lot variability in probe quality.

For a detailed evaluation, calculate the mean of ΔF of replicate samples for each lot and compare it to a reference lot. RainDance has observed that comparable ΔF values between test lots and reference lots (Figure 8-1) correlates to assay performance in dPCR. If you observe large differences between probe lots, it is likely that you will observe the cluster movement in the dPCR landscape.

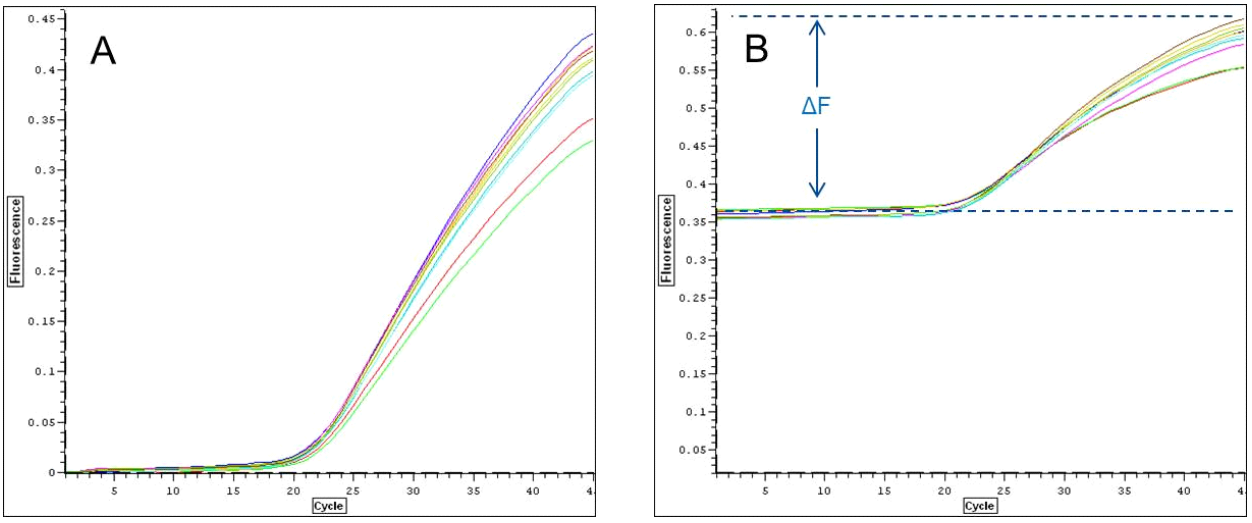


Figure 8-1: Amplification Curves Showing Change in Baseline Fluorescence

Panels A and B present amplification curves as viewed in standard software of a real-time thermal cycler. Panel A presents the data in a standard fashion that uses the baseline subtraction. Panel B presents the data without the baseline subtraction.

Recommendations

- A 10% or more decrease in the ΔF in a test lot correlates to reduced performance of the dPCR assay.
- A 30% decrease in the ΔF correlates to dPCR reaction failure (Figure 8-2).
- This protocol can be applied to alternative hydrolysis probe chemistries as well.

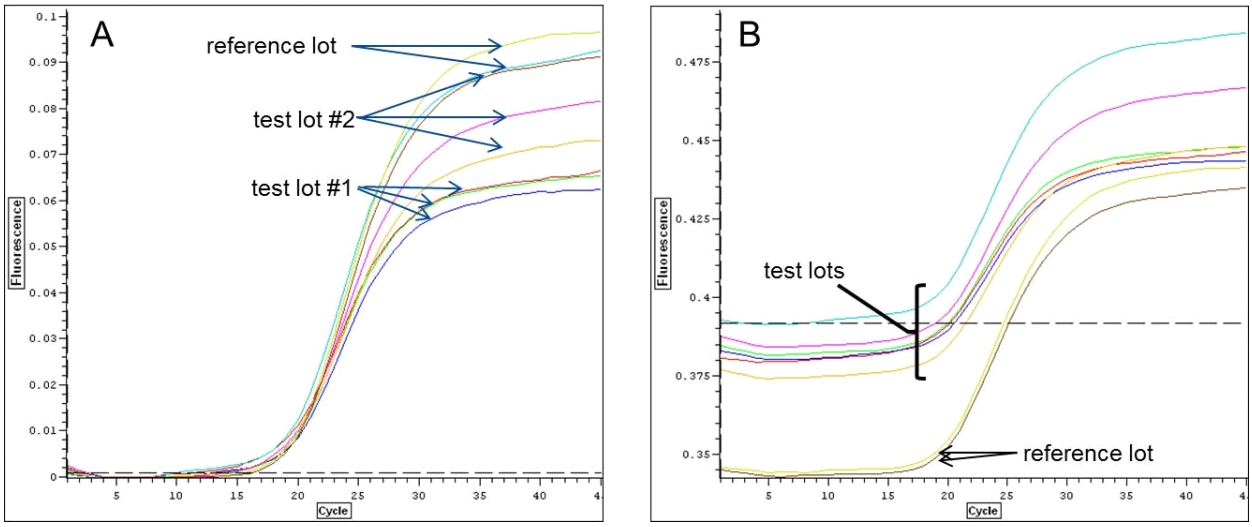


Figure 8-2: Amplification Curves Showing Test Lots and Reference Lots

Panels A and B present amplification curves as viewed in standard software of a real-time thermal cycler. Panel A presents the data in a standard fashion that uses the baseline subtraction. Panel B presents the data without the baseline subtraction. In each panel, two test lots have been evaluated in comparison to the reference lot. Both test lot #1 and test lot #2 exhibited approximately a 15% decrease in ΔF and exhibited poor performance in dPCR relative to the reference lot.

Additives

As in standard PCR, reagent additives can improve reaction efficiency and specificity. When you use the TaqMan Genotyping Master Mix, it is not typically necessary to supplement the reagent mix with additional additives. However, in rare cases, additives such as magnesium chloride and tetramethylammonium chloride have proven beneficial. The following sections present scenarios in which you can evaluate an additive while troubleshooting. The benefit is often assay-specific, so RainDance recommends using non-critical control DNA for this type of testing.

Magnesium Chloride

Magnesium chloride ($MgCl_2$) is a common reagent in standard PCR that serves as a cofactor to DNA polymerase. In standard PCR, the concentration of $MgCl_2$ is typically optimized for each assay, but should always exceed the total dNTP concentration. In dPCR, it is similarly an important component to consider while optimizing your reaction. Most polymerase master mixes for TaqMan or hydrolysis probe reactions contain $MgCl_2$ at a final concentration of 3 mM to 5 mM. As with standard PCR, it is important to optimize the final $MgCl_2$ concentration to ensure robust and specific polymerase activity (Figure 8-3). If you are investigating an alternative polymerase system, RainDance recommends that you evaluate a titration of $MgCl_2$ to determine the appropriate concentration required to achieve optimal performance.

1. Prepare a series of samples.
2. Incorporate $MgCl_2$ at a variety of final concentrations. Typically, increasing the $MgCl_2$ concentration in 0.5 to 1.0 mM increments is helpful to determine the optimal range.
3. Increasing the $MgCl_2$ often improves amplification efficiency but it is important to ensure that you are not increasing non-specific amplification detrimentally.
4. The best condition is a final concentration that maximizes PCR efficiency without compromising probe specificity.

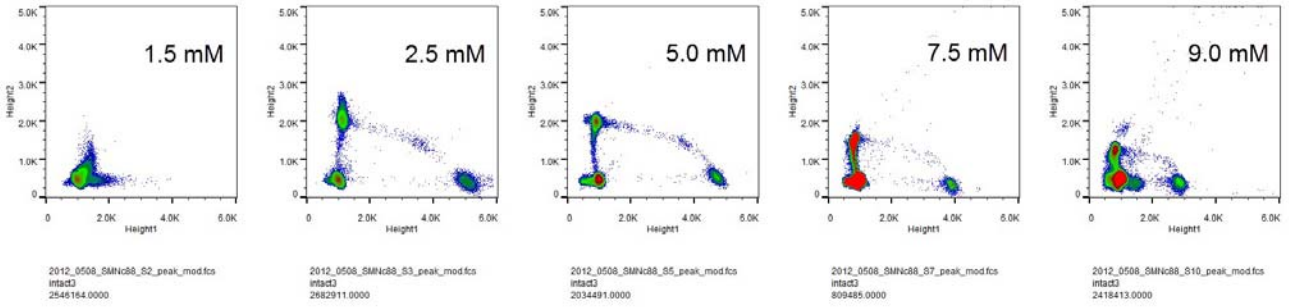


Figure 8-3: Final concentrations of magnesium chloride can be optimized to improve PCR performance. As observed, the addition of 2.5 mM $MgCl_2$ results in robust amplification and increased cluster separation. If the concentration of the $MgCl_2$ is too low, such as the 1.5 mM concentration observed here, the polymerase will be inefficient. If the $MgCl_2$ concentration is too high, such as the 7.5 mM and 9.5 mM concentrations observed here, the polymerase activity will be increased and will likely result in non-specific amplification that competes with the amplification of the true target.

Tetramethylammonium Chloride

Tetramethylammonium chloride (TMAC) is often used to enhance hybridization but studies have also demonstrated that TMAC can improve primer and probe specificity by increasing the melting temperature of the reagent mix. If a reaction exhibits non-specificity, you can add TMAC to the PCR mix. RainDance recommends an assay-specific titration study to determine the appropriate concentration. RainDance has observed that 10-15 mM TMAC is often effective in improving probe specificity (Figure 8-4).

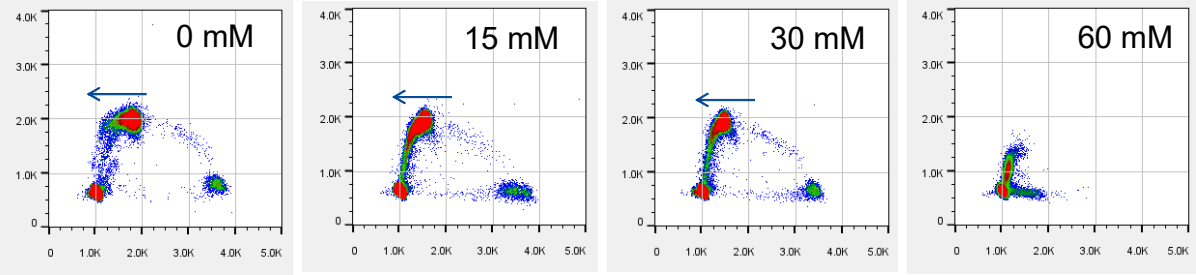


Figure 8-4: Adding TMAC can improve specificity. For example, adding 15 mM of TMAC reduces the amount of the FAM probe that is incorrectly incorporated in the VIC-positive drops. In addition, it improves the VIC cluster tightness and proximity to the axis. Add it with care; it may reduce the distance between the positive and negative clusters.

Droplet Stability

When the emulsion quality or droplet stability is compromised, the droplet landscape may reflect the creation of a variety of artifacts. These artifacts can fall within the target gates and detrimentally affect the droplet counts within those gates.

There are a variety of events that can result in poor emulsion quality. The presence of debris or particulate matter in the reagents may destabilize the drops. Coalescence of drops can occur due to droplet instability or as the result of excessive evaporation and condensation that may occur during thermal cycling. Make sure the heated lid is seated properly; you can place spare tube strips and tube strip caps in the empty wells surrounding the samples to balance the lid of the thermal cycler. See Chapter 7 for more information on how to prevent coalescence. RainDance does not recommend using oil overlays. Contact RainDance for further guidance.

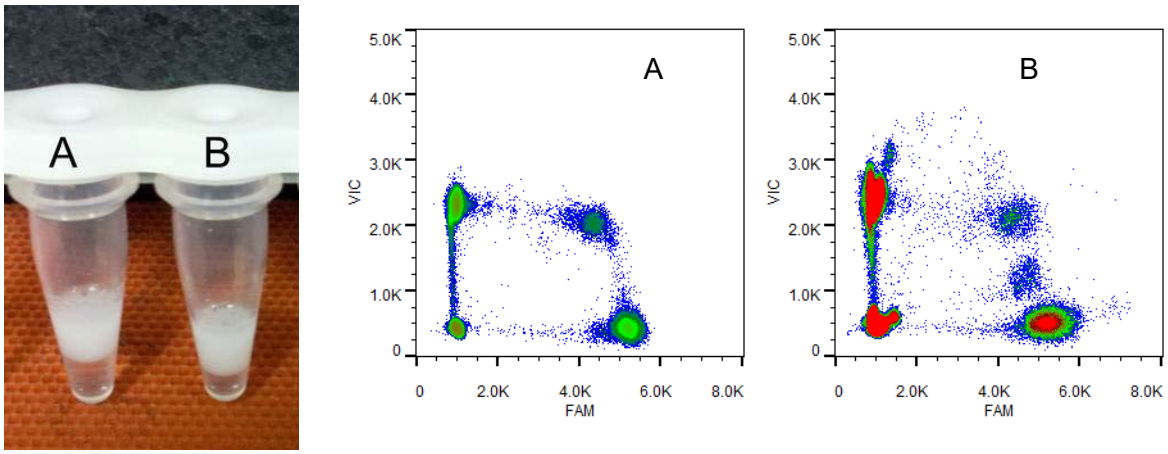


Figure 8-5: Viewing Droplet Instability. Droplet instability can create unfavorable artifacts that can interfere with droplet clustering and gating. Artifacts may form due to droplet instability and make it difficult to gate clusters. The photo exhibits evaporation in Tube B. The evaporation produces artifacts that are visible in the dPCR plot in panel B.

False Positives and False Negatives

It is imperative that you use clean reagents that are free of contaminants in dPCR reactions; RainDance recommends that you keep pre-PCR and post-PCR spaces isolated to reduce the risk of airborne amplicons from contaminating reagents. This applies to general lab use outside of dPCR-related work as well. The high sensitivity of the RainDrop® System reveals the presence of reagent or workspace contamination that may not be detected with other less sensitive methods (including qPCR).

Poorly designed assays may result in the creation of false positive drops, which you can identify when these counts are higher than expected. Detection of pseudogenes also might lead to unexpected clusters. Typically, the pseudogene count scales with the DNA loading. Ensure that you are using amplicons designed to unique regions of the genome such that pseudogenes are not amplified and do not falsely interfere with molecular counting.

If you observe lower than expected positive counts, validate the DNA quality. RainDance recommends that you evaluate the control DNA of a known concentration to ensure that amplification performance is accurate and to gauge the percentage of DNA that is amplifiable. During sample preparation and the shearing of the DNA, it is expected that a portion of the DNA will be rendered unamplifiable. Similarly, upstream storage and treatment of the sample influence the proportion of the DNA that is amplifiable. For example, FFPE DNA is often damaged during the fixation process; as such, it is likely that a portion of this DNA is not amplifiable.

DNA Loading

RainDance recommends that control samples are evaluated using target DNA at concentrations that span the dynamic range of samples to be evaluated. It has been observed that relative endpoint fluorescence is lower at higher loading concentrations of DNA. RainDance recommends DNA loading occupancies up to ~10%.

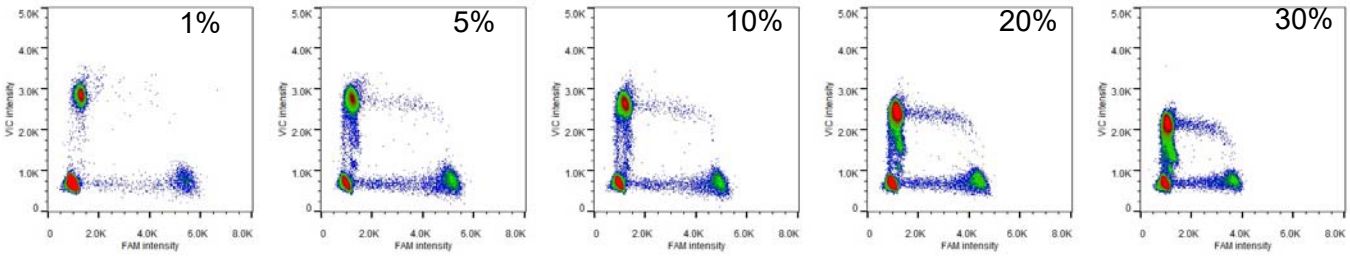


Figure 8-6: DNA Loading Influences the dPCR Landscape

As DNA occupancy (the estimated number of target molecules per the number of drops evaluated) increases above a target of 10%, PCR performance often declines. A reduction in PCR performance can decrease the distance between the positive and negative clusters. Additionally, increasing the DNA occupancy above a 10% target occupancy can result in the formation of artifacts and background noise that can interfere with target gates. If samples are known to have high occupancies of target we recommend that you use the High Occupancy run mode on the Sense Instrument.

Sample-to-Sample Cluster Movement

RainDance has observed a sample-to-sample decline in cluster intensity throughout a run, for example sample A to sample H from a single chip, for some assays and some polymerases. In this situation, the intensity of the fluorescence in the negative cluster is actually increasing putatively due to post-PCR enzymatic activity. The intensities of the positive clusters appear to decline because the dPCR cluster landscape is normalized to the negative clusters. RainDance has observed that some assays are more susceptible to this than others.

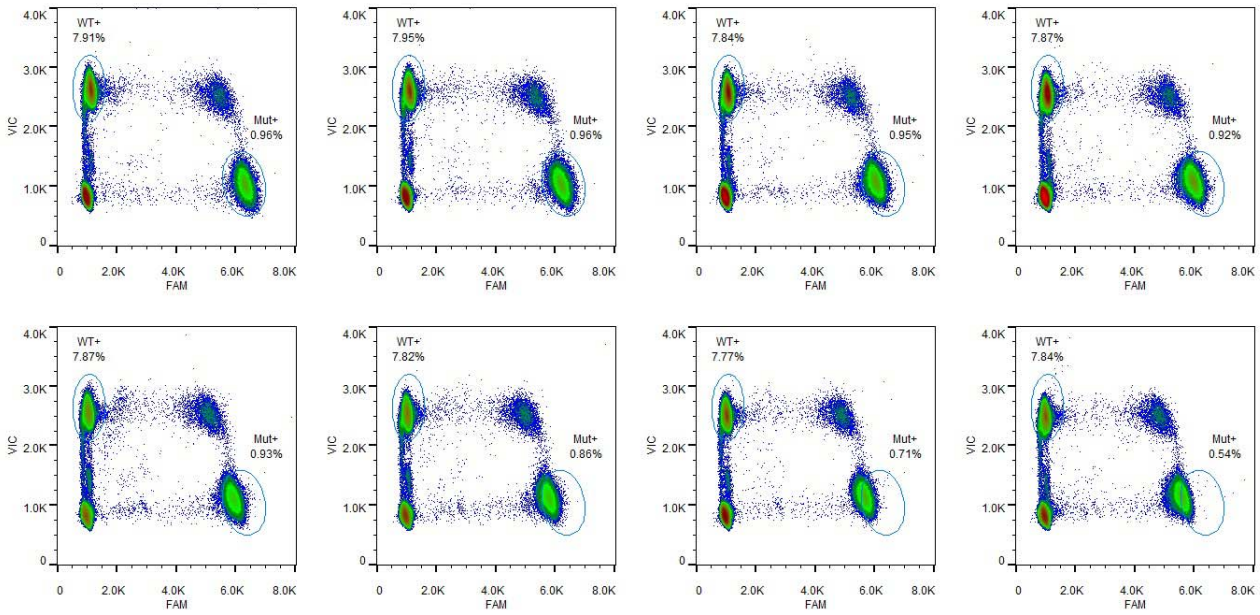


Figure 8-7: You may observe a sample-to-sample decline in cluster intensity for some assays and some polymerases throughout a run (for example, sample A to H from a single chip).

Running assays in the FAST mode on the RainDrop Sense Instrument minimizes or prevents this cluster movement in most cases. Refer to the *RainDrop Sense Operator's Manual* for more information.The 10 minute soak at 98°C at the end of the thermal cycling protocol also helps to reduce polymerase activity. If a pronounced decline in cluster intensity from sample A to sample H persists, increase this soak time. For potentially problematic assays, another alternative is to use a polymerase master mix that is aptamer-based, such as the Hot Start Taq 2X Master Mix (https://www.neb.com/products/M0496-Hot-Start-Taq-2X-Master-Mix, Catalog# M0496L) from New England BioLabs. The aptamer binds reversibly to the enzyme, which inhibits polymerase activity at temperatures below 45°C.

Additionally, an analysis-based approach for resolving the sample A to sample H decline is to use AutoGates, which can adjust gates from sample to sample to accommodate small changes in cluster position. Another analysis based approach or resolving the sample A to sample H decline is to create slightly enlarged gates that accommodate the cluster movement. Refer to the *RainDrop Analyst II v1.1 User Manual*, for further information on analysis and gating strategies.

Additional Troubleshooting Information

The following table details additional information for use while optimizing your assay.

Table 8-1: Troubleshooting dPCR

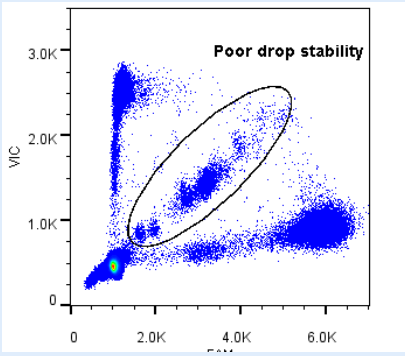
Observation	Possible Problem	Solution
 Poor drop stability.	Static discharge can build up, much like dragging your feet across a carpet in extremely dry conditions.	A humidifier can help increase the humidity in the lab space. Antistatic mats, wands, or guns can help discharge static.
	Debris in samples from reagents or DNA samples may destabilize the emulsion.	Filter reagents such as water or Tris through a Millipore Millex-FG 0.2 micron filter prior to use (http://www.millipore.com/catalogue/item/slfgx13nl). Purify and concentrate DNA templates using the Qiagen Minelute kits if the template is less than 4 kb in length. If the DNA is longer, use Millipore Microcon filters (http://www.millipore.com/catalogue/module/c113861).
	Improperly sheared DNA (or DNA that is too long) may interfere with drop generation.	Evaluate DNA template size using an Agilent Bioanalyzer or agarose gels. If you are concerned that the DNA is not fully fragmented, re-shear or renebulize the DNA to the appropriate length.
	Thermal cycler heated lid is inadequate.	Ensure that the temperature of the heated lid is ~105°C, such that evaporation and condensation do not occur.

Table 8-1: Troubleshooting dPCR

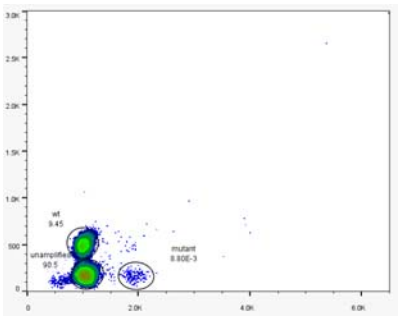
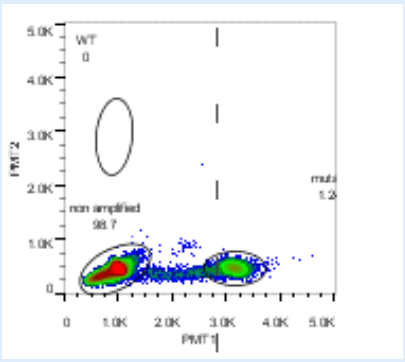
 Low bright to dim ratio.	Stock probe solution is very bright in its quenched state, probe possibly degraded. Poor efficiency of amplification or probe hydrolysis. Too much probe added.	Run a qPCR reaction and analyze without the baseline subtraction. Compare baselines across other assays that exhibit strong signal intensity. Do not freeze thaw probes. Keep aliquots in regular use at 4 degrees. Decrease the amount of probe.
 One cluster is missing.	No target signal.	Ensure that the probe was added. Ensure that the probe is functioning well by analyzing data through qPCR. Confirm that the probe design is adequate and that it does not contain a 5’G that had the potential to quench the fluorescent signal.

Table 8-1: Troubleshooting dPCR

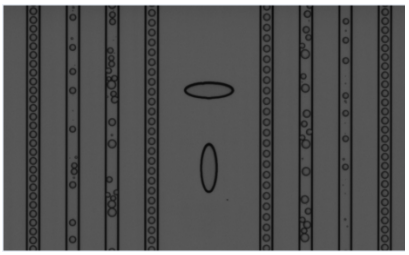
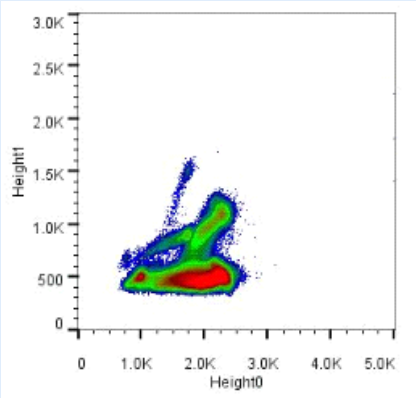
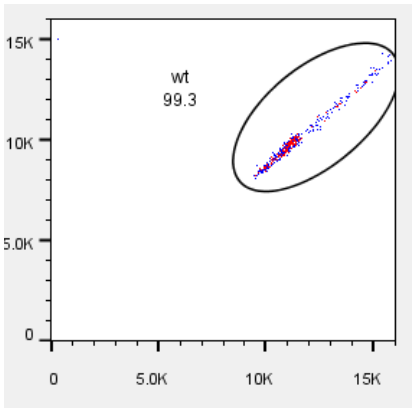
 Poor droplet generation.	DNA is improperly sheared or is not sheared.	Evaluate DNA template size using an Agilent Bioanalyzer or agarose gels. If you are concerned that the DNA is not fully fragmented, the DNA can be re-sheared or re-nebulized to the appropriate length.
	DNA may contain debris.	Regents such as water or Tris can be filtered through a Millipore Millex-FG 0.2 micron filter prior to use (http://www.millipore.com/catalogue/item/slfgx13nl). DNA templates can be purified and concentrated using the Qiagen Minelute kits if the template is less than 4 kb in length. If the DNA is longer, Millipore Microcon filters can be used (http://www.millipore.com/catalogue/module/c113861).
	Too much unsheared genomic DNA is added.	Reduce the amount of unsheared DNA in the sample, or shear the DNA to 3 kb or smaller.

Table 8-1: Troubleshooting dPCR

 Poor cluster resolution.	Thermal cycler heated lid is inadequate, or thermal cycler wells are not deep enough	Ensure that the temperature of the heated lid is ~105°C, such that evaporation and condensation does not occur. Use a marker to mark the top of the emulsion on the tubes and place in thermal cycler to ensure emulsion is below top of thermal cycler wells.
	Not enough droplet stabilizer added.	Droplet stabilizer is at a 25x concentration – 1.0 µL should be added to 25 µL reactions, and 2.0 µL should be added to 50 µL reactions.
 Positive droplets are too bright.	Too much probe added to reaction.	If probes were not part of a premix check the resuspension and dilutions volumes, there may be an error in the calculations.
	Probe too bright in dim state.	Review length of probe and quencher, possibly order with a different darker quencher, redesign so probe is shorter, or both.

Appendix A

Alternative Recommended Master Mixes

This chapter includes the following topics:

Alternative Recommended Master Mixes	page A-2

Alternative Recommended Master Mixes

RainDance currently recommends using Life Technologies TaqMan Genotyping Master Mix (4371355); however, the RainDrop® System is flexible and allows you to use a variety of probe-based polymerase and buffer systems. RainDance recommends using a master mix that contains a hot start polymerase to reduce the potential for nonspecific priming and the formation of primer-dimers. RainDance recommends high fidelity polymerases for rare allele detection and does not recommend highly processive or fast polymerases. As general lab practice, when examining rare alleles through PCR-based applications, RainDance recommends that you consider using dUTPs and uracil DNA glycosylase to reduce the risk of contaminating reagents and DNA samples.

A number of master mixes have been evaluated for use with the RainDrop System; those that have presented the most favorable data are presented in Table A-1. As with standard PCR, it is important to recognize that the inherent amplification and hydrolysis efficiency among assays varies. Due to the proprietary nature of most commercially available master mixes, it is likely that thermal cycling conditions or reagent concentrations will require some optimization (see “Annealing Test Using dPCR” on page 5-7). Common optimization approaches include altering the annealing temperature, slowing ramping speeds, or changing the magnesium chloride concentration.

Table A-1: Recommended Top Performing Master Mixes for Use on the RainDrop System

Vendor	Master Mix	Catalog #
New England BioLabs	Hot Start Taq 2X Master Mix	M0496L
Qiagen	Type-it Fast SNP Probe PCR Mix	206042
Quanta Biosciences	AccuStart™ Genotyping ToughMix®	95115
Clontech/Takara Bio	Premix Ex Taq™ (Probe qPCR)	RR390A
Sigma	JumpStart™ Taq ReadyMix™	P2893
Kapa BioSystems	KAPA™ PROBE FAST qPCR Kit, Master Mix (2X) Universal	KK4701
Life Technologies	TaqMan Universal PCR Master Mix I	4304437
Life Technologies	TaqMan Universal PCR Master Mix II, no UNG	4440043
Life Technologies	TaqMan Universal PCR Master Mix II, with UNG	4440042

Appendix B

Alternative Probe Chemistries

This chapter includes the following topics:

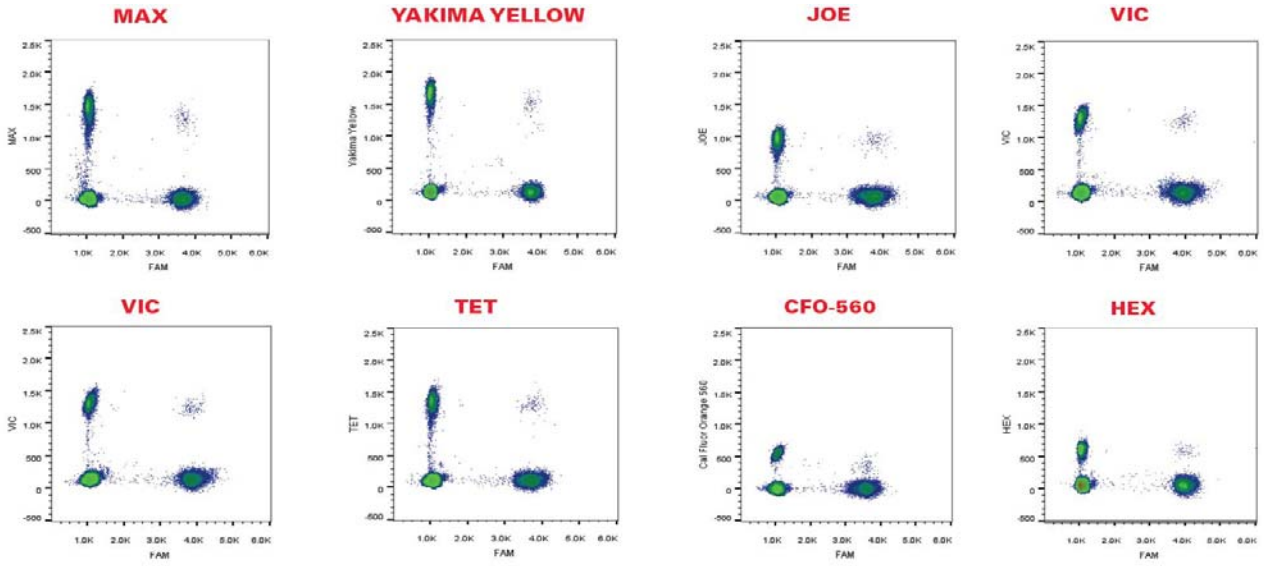
Introduction	**page B-2**
Selecting Fluorescent Dyes	**page B-2**
Tamra-quenched TaqMan Probes (Life Technologies)	**page B-2**
ZEN Double-Quenched Probes Prime Time® Probes (Integrated DNA Technologies)	**page B-3**
LNA Prime Time Probes with ZEN Dual-Quenching (Integrated DNA Technologies)	**page B-3**
BHQplus™ Probes (Biosearch Technologies)	**page B-3**

Introduction

This appendix describes a collection of alternative probe chemistries that you can use with the RainDrop® System.

Selecting Fluorescent Dyes

While TaqMan MGB probes are recommended for high specificity, you can run other probe systems on the RainDrop System. It is important to select fluorescent moieties that are compatible with the optical spectrum of the instrument. Fluor molecules should possess an excitation wavelength within 485 – 491 nm. Acceptable emission spectrum of the selected fluorescent dyes should be within 498 – 524 nm for Channel 1 and 532-554 for Channel 2. 6FAM is universally available for most probe chemistries; as such, RainDance has not examined alternative dyes for channel 1. However, as VIC alternatives, RainDance has demonstrated detection of MAX, Yakima Yellow, JOE, and TET. CFO-560 and HEX are detected very poorly.



Tamra-quenched TaqMan Probes (Life Technologies)

RainDance recommends TaqMan MGB probes over traditional Tamra-quenched TaqMan probes because they have demonstrated superior mismatch detection essential for high specificity in rare allele detection. Due to their long length, Tamra-quenched TaqMan probes tend to be more tolerant of variability within the sequence composition of the probing region; as such, they may not perform with the specificity necessary for single nucleotide mismatch detection. These probes demonstrate efficient specificity when there is more than one nucleotide differentiating the target, or when the rare allele is characterized by a short insertion or deletion. Thus, when properly designed, RainDance has demonstrated that

Tamra-quenched TaqMan probes can be used for rare allele detection within the RainDrop System. Follow standard recommendations when designing Tamra-based probes for insertion- and deletion-based assays.

In addition to Primer Express, you can use the commercially available software program Beacon Designer (http://www.premierbiosoft.com/tech_notes/TaqMan.html) to design Tamra-based probes.

ZEN Double-Quenched Probes Prime Time® Probes (Integrated DNA Technologies)

Similar to Tamra-quenched TaqMan probes, standard ZEN probes are typically long probes and may not be appropriate for mismatch rare allele detection. However, the attribute of this probe design is the inclusion of dual-quenching. The placement of the internal ZEN quencher decreases the distance between the dyes and quenchers. The dually-quenched state of the PCR negative drops provides increased signal-to-noise ratio allowing for better cluster separation in the end-point droplet landscape. You can design Zen-based Prime Time assays using IDT's online tool PrimerQuest (http://www.idtdna.com/PrimerQuest/Home/Index).

LNA Prime Time Probes with ZEN Dual-Quenching (Integrated DNA Technologies)

You can add a ZEN dual-quenching moiety as a custom modification to the LNA Prime Time probe to allow for dual-quenching discussed above. This approach increases the signal-to-noise ratio from the ZEN quencher in addition to achieving the high specificity of the LNA Prime Time probes. To design LNA Prime Time Probes with ZEN dual-quenching, contact IDT technical support. These probes require a custom quote.

BHQplus™ Probes (Biosearch Technologies)

The BHQplus™ probes by Biosearch Technologies offer features similar to that of a TaqMan MGB probe. The probes contain duplex stabilizers that allow for short probe designs appropriate for mismatch rare allele detection. You can design BHQ plus probes using the Biosearch RealTimeDesign™ program (http://www.biosearchtech.com/realtimedesign). Standard designs are appropriate for use within the RainDrop System.

Appendix C

Glossary

Amplicon

Amplified sequence of DNA in the PCR process. This may also be called the PCR product.

Applications

Specific uses of the RainDrop® System, such as rare allele detection, copy number variation, and gene expression. Applications are intended to provide a specific capability or performance level.

Chip

One-time use microfluidic device on which assays are run.

Cleanup

A method for purifying the DNA template, often completed after a treatment of the nucleic acid template (such as shearing) and is typically performed to remove inhibitors or undesirable residual reagents. A cleanup procedure can also be used to control the concentration of the nucleic acid template.

Coalescence

Undesired merging of two or more droplets.

Droplet

Microscopic aqueous phase partition in an oil background.

Emulsion

A collection of droplets of aqueous material surrounded by oil. In the context of this protocol the oil contains a surfactant that prevents coalescence of droplets.

FAM

6-carboxy fluorescein. FAM is the most commonly used reporter dye at the 5′ end of a TaqMan probe. In allelic discrimination assays, the two probes are usually labeled by VIC (more abundant wild-type allele-specific probe) and FAM (the variant allele-specific probe) for best spectral non-overlapping combination.

Hydrolysis probe

One of the main fluorescence-monitoring systems for DNA amplification. These probes are hydrolyzed by the 5′ to 3′ exonuclease activity of Taq Polymerase during PCR. TaqMan probes are examples of hydrolysis probes.

LLOD

LLOD, lower limit of detection, is the lowest concentration for which there is a 95% confidence that a sample will be accurately identified as positive (i.e., the limit at which a true sample will not be measured to be negative).

Minor groove binders (MGBs)

Hydrolysis probes with conjugated minor groove binder (MGB) polyamides form stable duplexes with target DNA, allowing shorter probes to be designed. TaqMan probes containing MGBs will have higher melting temperatures and increased specificity over standard TaqMan probes.

Multiplexing

Simultaneous analysis of more than one target in the same reaction.

No template controls (NTCs)

NTCs include all of the PCR reagents except the template. No product should be synthesized in the NTC reaction. If a product is amplified, this indicates contamination (fluorescent or PCR products)

Polymerase Chain Reaction (PCR)

An established methodology for the exponential amplification of specific regions of nucleic acid template.

Peltier unit

A solid state element that provides both heating and cooling in a PCR machine.

Reagents

Consumable materials or components used in a biochemical reaction performed to measure, examine, or amplify.

Reproducibility

A measure of consistency across multiple instruments. Assess by running the same sample multiple times across two or more instruments.

Run

A *run* is any set of samples, processed as a batch. If one sample is processed at a time, then a run and a sample are the same. A run on the RainDrop System consists of droplet creation/

collection on the Source Instrument, thermal cycling, and identification/counting of droplets on the Sense Instrument.

Shearing

DNA shearing is a process used to prepare DNA for analysis. Shearing is typically achieved through the use of sonification or nebulization. DNA is generally sheared to a desired fragment length.

TaqMan probe

A dual-labeled specific hydrolysis probe designed to bind to a target sequence with a fluorescent reporter dye at one end (5′) and a quencher at the other (3′). Assays using TaqMan probes are also called 5′ nuclease assays.

Template

The nucleic acid sample used to amplify the target sequence. Assessment of template integrity/quality is one of the MIQE requirements.

Template (Input) Sample

Aqueous solution containing DNA template and reaction components. Typically referred to as the Sample.

Waste

Materials generated in the course of a run or maintenance activity on the RainDrop System. Waste materials are intended for disposal by the user, and are not retained for subsequent analysis or archival purposes.

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Raindance Technologies, Inc.
749 Middlesex Turnpike
Billerica, MA 01821
Fax: (978) 528-6090 (support)
Web: www.raindancetech.com/support
Email: customercare@raindancetech.com