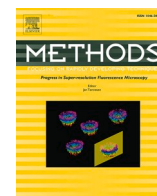




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In pursuit of sensitivity: Lessons learned from viral nucleic acid detection and quantification on the Raindance ddPCR platform

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ABSTRACT

Sensitive PCR detection of viral nucleic acids plays a critical role in infectious disease research, diagnosis and monitoring. In the context of SARS-CoV-2 detection, recent reports indicate that digital PCR-based tests are significantly more sensitive than traditional qPCR tests. Numerous factors can influence digital PCR reaction sensitivity. In this review, using a model for human HIV infection and the Raindance ddPCR platform as an example, we describe technical aspects that contribute to sensitive viral signal detection in DNA and RNA from tissue samples, which often harbor viral reservoirs and serve as better predictors of disease outcome and indicators of treatment efficacy.

1. Introduction

Highly accurate and sensitive detection of the nucleic acids of causative agents has proven instrumental in the diagnosis and monitoring of infectious diseases, as evidenced during the ongoing COVID-19 pandemic and the HIV global epidemic. Although the mainstay assays in disease detection and follow-up have been RT-qPCR assays, another PCR derivative, digital PCR (dPCR), has shown significant advantages particularly in patients exhibiting low viral load, due to its higher sensitivity and robust performance in challenging clinical samples. In this review, we first summarize the technical principle of dPCR technology and its advantages. We then describe the major technical aspects that contribute to sensitive viral nucleic acid detection and quantitation in dPCR, including inhibitors, factors specific to viral DNA quantification, and factors specific to viral RNA quantification. Most of the discussion uses a human HIV infection model and the Raindance ddPCR platform as an example, but the technical considerations presented herein are applicable and can be adapted to a broad range of

applications where highly sensitive nucleic acid detection is required.

1.1. The critical role of PCR assay sensitivity as highlighted during the COVID-19 pandemic

The ongoing COVID-19 pandemic has highlighted the crucial importance of the sensitive detection of viral nucleic acids, since any inaccurate diagnosis caused by insufficient assay sensitivity could undermine efforts at containment of the pandemic (e.g. asymptomatic yet infected persons receiving a false diagnostic result might not be isolated, thereby posing a risk to others). Two reports in early 2020 from Wuhan, China also caused concern about false negative RT-qPCR test results in patients with apparent COVID-19 symptoms. In one of the studies [1], a total of 205 throat swabs, 490 nasal swabs and 142 sputum samples were collected and analyzed (median = 3 per patient). The authors found that during the first week after illness onset, a significant fraction of samples (40% of throat, 27% of nasal, 11% of sputum) were falsely classified as negative with a regulatorily approved RT-qPCR assay. In a second report

Abbreviations: cART, combination antiretroviral therapy; COVID-19, Coronavirus disease 2019; Ct, threshold cycle; CT, Computed Tomography; dMIQE, Minimum Information for Publication of Quantitative digital PCR experiments; dPCR, digital PCR; ddPCR, droplet digital polymerase chain reaction; FFPE, formalin-fixed paraffin-embedded; HIV, human immunodeficiency virus; HO, high occupancy; HPV, human papillomavirus; HTLV, human T-cell leukaemia virus; LoD, limit of detection; MGB, minor groove binder; miRNA, microRNA; NGS, next generation sequencing; NHP, non-human primate; PIV, parainfluenza virus; qPCR, quantitative (real time) polymerase chain reaction; RSV, respiratory syncytial virus; RT, reverse transcriptase; RT-dPCR, reverse transcription digital polymerase chain reaction; RT-qPCR, quantitative reverse transcription (real time) polymerase chain reaction; SARS, severe acute respiratory syndrome; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SIV, simian immunodeficiency virus; SNP, single nucleotide polymorphism; SNV, single nucleotide variant; STLV-1, simian T cell lymphotropic viruses type 1; STLV-2, simian T cell lymphotropic viruses type 2; Tm, melting temperature.

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[2], a total of 173 patients who were hospitalized due to acute respiratory symptoms and a chest CT typical of COVID-19, or SARS-CoV-2 being detected in at least one respiratory specimen, were studied. In only 67% of this group of patients, SARS-CoV-2 RT-qPCR tested positive in at least one respiratory sample taken on days one through seven of hospitalization. A more recent systematic review [3] of 34 other studies involving 12,057 confirmed COVID-19 cases found that up to 54% of COVID-19 patients may have had an initial false negative RT-qPCR result. Collectively, these results point toward an estimated average sensitivity of available RT-qPCR-based tests that allows about 70% of infected individuals to be correctly diagnosed, a percentage that is substantially lower than desired.

Two additional studies evaluated the use of digital droplet PCR (ddPCR) in COVID-19 testing and compared the ddPCR performance with that of RT-qPCR. In the first study [4], the authors assessed the ability of a ddPCR-based test to detect COVID-19 in individuals with suspected infection, and compared the ddPCR results to those obtained with three commercial RT-qPCR kits. In total, the study covered 103 symptomatic individuals suspected to have SARS-CoV-2, 75 close contacts and 16 recovering patients. The overall diagnostic accuracy of the reverse transcription ddPCR (RT-ddPCR) assay was 93% with an overall sensitivity of 90% and specificity of 100%. More specifically, in individuals that already had COVID-19-like symptoms, the sensitivity of SARS-CoV-2 detection was improved from 28% (by RT-qPCR) to 87% (by RT-ddPCR). The RT-ddPCR test also reduced the occurrence of false positive results among close contacts from 21% to 1%. In addition, the RT-ddPCR test showed that elderly people recovering from COVID-19 tend to have higher viral loads for longer periods of time, indicating that these patients would need to remain quarantined longer to prevent further spread of infection among the general population. In the second study [5], researchers examined whether RT-ddPCR could detect SARS-CoV-2 among throat swabs (a common sample type, yet notorious for containing low viral loads that are prone for high rates of false negative results) of 57 patients who tested negative using RT-qPCR but later developed COVID-19 disease. The authors found that RT-ddPCR was 94% accurate in detecting SARS-CoV-2 and was 500 times more sensitive than RT-qPCR. This same study also included 14 convalescent patients that had received two negative RT-qPCR results. Re-testing their samples with RT-ddPCR found that 9 of the 14 patients still hosted detectable levels of the virus and should have remained in quarantine longer. A more recent study [6] also found that a SARS-CoV-2 RT-ddPCR assay was significantly more sensitive than an RT-qPCR assay on saliva samples, expanding the utility of the RT-ddPCR assay to saliva testing for repetitive sampling and to testing individuals for whom nasopharyngeal swabbing is not possible. These RT-ddPCR and RT-qPCR comparison study results strongly suggest that more sensitive detection of SARS-CoV-2 can potentially lead to improved quarantine practice and earlier treatment.

1.2. PCR applications in viral detection

One of the most prevalent applications of PCR has been for the detection of viruses. The ability to quantify virus load has had a huge impact on the study of infectious disease progression [7] and has helped to clarify association between specific viral sequences and clinical symptoms [8]. Historically quantitative PCR (qPCR) has been instrumental in sensitive detection and accurate measurement of viral groups that are significant for human health. These include: (1) Positive-strand RNA viruses. Several RT-qPCR assays have provided critical prognostic information for clinical management of diseases involving enteroviruses [9], noroviruses [10], and rhinoviruses [11]. These diagnostic assays are significantly more sensitive than traditional methods such as viral culture [12] and proved to be of critical importance in geographical areas of viral outbreaks such as during the SARS pandemic [13], and have been instrumental to identifying, isolating, treating patients and defining the epidemiology of the coronavirus during the COVID-19

pandemic [14–16]. (2) Negative-strand RNA viruses. This group includes the measles and mumps viruses, various viruses that target the respiratory tract such as influenza virus types A and B, respiratory syncytial virus (RSV) and parainfluenza virus (PIV) types 1–4 [17–21]. Codetection of SARS-CoV2 and influenza A virus was also reported recently [22]. (3) Double stranded RNA viruses including human rotaviruses. qRT-PCR assays have been used to delineate the epidemiology and pathogenesis of this group of etiological agents that can lead to severe and potentially life-threatening symptoms in infants and young children [23]. (4) Retroviruses. Viruses in this group, which includes HIV and HTLV (human T-cell leukaemia virus) and related viruses in animal models such as simian immunodeficiency virus (SIV), simian T cell lymphotropic viruses type 1 and 2 (STLV-1 and 2), and others, contain RNA-dependent DNA polymerases that copy the viral RNA genomes into DNA, which is then integrated into the host genome [24,25]. In the case of HIV, development of qRT-PCR assays that can detect and quantify low level (i.e. single digit copy level) HIV-1 RNA led to a significant increase in the accuracy and clinical relevance of HIV testing [26], however, such assays' utility has been limited by the extreme sequence heterogeneity of HIV-1. (5) DNA viruses. These include six different DNA virus families that cause a large variety of diseases in humans. Viruses in this category include human papillomavirus (HPV), hepatitis B, adenoviruses, herpesviruses, and poxviruses, among others [27].

1.3. Limitations associated with quantitative real time PCR (qPCR)

qPCR assays have several limitations. (1) Being reliant on reference material-generated standard curves, qPCR data quality is influenced by how accurate the quantification of such reference material is. In addition, in many scenarios, precisely quantified reference material may not exist. (2) Sequence heterogeneity in viruses such as HIV-1 interferes with primer and/or probe binding to target sequences and can lead to under-quantification or non-recognition of targets with significant sequence divergence. As new subtypes and clades are identified, regions which were thought to be highly conserved and housed primer and probe sequences in earlier assay designs can show sequence divergence, which necessitates design of new assays or modification of older assays. (3) In some infectious diseases, tissue sites have been found to be better predictors of disease outcome and indicators of treatment efficacy [28], and consequently there is a need to sensitively detect and accurately quantitate viral nucleic acids in tissues (as opposed to e.g. plasma) from infected individuals or animal models. However, inhibitors that co-purify with DNA and/or RNA (especially from tissues) have been shown to present challenges during qPCR and RT-qPCR quantification and therefore can limit the utility of qPCR assays in these applications.

1.4. Digital PCR concept, advantages and limitations

Digital PCR (dPCR) takes a different approach to determine the quantity of target nucleic acid molecules within a sample. Instead of depending on PCR threshold cycle (Ct) values and standard curves as in real-time PCR (qPCR), digital PCR achieves signal detection and quantitation through partitioning each reaction into up to millions of picoliter reactions. Depending on the platform employed, samples are partitioned into individual reaction wells on chips/arrays, or into oil emulsion droplets (this latter category instruments are known as droplet digital PCR (ddPCR) platforms). These mini-reactions are thermocycled to reach PCR endpoint, the numbers of negative and positive reactions are counted, and the copy number of target molecules in the original sample is calculated based on Poisson distribution. In comparison to qPCR, digital PCR benefits from direct absolute quantitation of analyte without relying on external standards or calibration curves, and is therefore not influenced by inaccuracy introduced during reference and/or standard quantitation. As digital PCR quantitation is based on end point PCR product detection, this method is less susceptible to

amplification efficiency variations, which often occur in Ct-dependent qPCR when there are primer and/or probe mismatches due to sequence heterogeneity, or inhibitors in samples. Additional advantages associated with digital PCR include higher quantification precision especially in lower target template copy number range, as well as greater multiplexing ability because of the platform's unique amplitude or ratio-based higher order multiplexing [29].

The term “higher order multiplexing” refers to multiplexing that enables counting more targets than the fluorescent detection channel number. (The majority of digital PCR systems currently perform detection in two optical channels, with the exception of the Fluidigm BioMark™ and EP1™ systems and the Stilla Naica™ System, which allows multiplexing in four and three optical channels, respectively.) The principle behind higher order multiplexing is that the final fluorescence signal intensity (i.e. amplitude) in each partition is determined by a combination of factors such as probe-dye type(s), probe concentration(s) [30] and potential mixing ratio, primer concentrations and the pre-amplification target type and amplicon size [31]. As each partition is represented as an individual event in a 2-dimensional scatter plotting space, multiple targets can be quantified at the same time by properly deconvoluting the plotting pattern(s) and unambiguously assigning a cluster to a signal type. Different scenarios regarding primer, probe and dye combination for various applications and detailed technical considerations regarding factors contributing to accurate quantification (such as linked targets, specificity, effects of partition-specific competition, template type, conformation etc.) were covered in several excellent previous publications [29,32–36]. For example, in [29], four scenarios for duplex reactions and four additional scenarios for higher order multiplexing were described.

Due to the fact that digital PCR is more precise and sensitive than quantitative PCR (qPCR) in a number of test systems and applications, it has achieved wide applications in areas other than virus/pathogen detection such as mutation detection/quantification, gene expression and miRNA analysis, copy number variation determination, as well as reference standard and NGS library quantification [37–56].

Compared to qPCR, Digital PCR traditionally was perceived to present two related constraints: limited dynamic range and somewhat narrow range of per reaction nucleic acid input. By definition, dynamic range in digital PCR is determined by partition number that is made available for each sample. On several chip- and array-based platforms such as Fluidigm BioMark HD, QuantStudio 3D Digital and JN MedSys Clarity and Clarity Plus, the partition number per sample is between 10,000 to 45,000, and on a couple of ddPCR (i.e. oil emulsion-based) platforms (such as Stilla Naica System and BioRad QX100/200 instruments), partition number per sample is in the range of 20,000 to 30,000. On one hand, these relatively low partition numbers usually require dilution of many input samples to attain accurate measurements [57]. On the other hand, platforms with lower dynamic range capacity in general are limited in their total sample input (which consequently translates into lower detection sensitivity), as each partition should ideally contain at most one target molecule. In some ddPCR platforms [58] overloading each reaction with nucleic acids above a certain threshold amount was reported to cause significant deformation of droplets, a decline in the number of droplet generated, and a significant inhibition at the quantitation step (i.e. fewer target-containing droplets reaching the fluorescent intensity detection threshold at the end of thermocycling).

1.5. Raindance ddPCR platform: high quantification dynamic range and applications

In the accompanying protocol article [59], we describe utilizing the Raindance digital PCR method to achieve ultrasensitive detection of viral DNA and RNA that are extracted from animal tissues and cells. The Raindance ddPCR platform partitions each sample into 10 million droplets, and this significantly expanded dynamic range (6 log)

compares favorably to the quantification dynamic range achieved in qPCR systems [46,47,59,60]. Independent studies in HIV DNA analysis and cancer mutation (KRAS) genotyping confirmed the Raindance ddPCR platform's capability of performing accurate and precise quantification over six orders of magnitude [60].

Raindance digital PCR system has been used to detect viruses [46,47,60–65], cancer mutations or rare disease somatic mosaicism [30,60,66–74], gene/DNA copy number [34,75], single nucleotide polymorphism (SNP) [34,76], fusion gene transcript [77], and genetically engineered traits [57].

In sections 2, 3 and 4, technical considerations for achieving maximal detection sensitivity are detailed in part based on the Raindance ddPCR platform. Researchers are also recommended to refer to a few excellent articles by the dMIQE working group for more general guidelines that apply to all digital PCR applications [35,36].

1.6. Simian immunodeficiency virus (SIV)-infected Rhesus macaque as a model for ultrasensitive viral detection

Rhesus macaques infected with simian immunodeficiency virus (SIV) represent an excellent model of human HIV infection. Many features of infected monkeys closely parallel those of infected humans, such as viral infection, pathogenesis and cure strategy responses [24,25,78–80]. SIV-infected rhesus macaques serve as an ideal system to study ultrasensitive detection of viruses for two main reasons: (1) Especially in antiretroviral agent-treated Rhesus macaques, SIV nucleic acid levels can be extremely suppressed. Being able to detect any remaining viral nucleic acid in these animals is critical for evaluating and comparing the efficacy of different treatment regimens. Consequently, nucleic acids extracted from these animals provide a suitable system to study methods and assays that are designed and intended to detect ultra-low level viral target (s). (2) Necropsied Rhesus macaque tissues serve as an indispensable source for studying viral quantities in various tissue reservoirs. For example, about 30 different macaque tissues are routinely analyzed in non-human primate (NHP) research and quantified for viral nucleic acids to provide insights into viral spread and its timeline to various parts of the body in model animals (e.g. [81]). Being able to apply ultrasensitive detection techniques to a tissue/site of interest can enable in-depth understanding of the viral pathogenesis mechanism and dynamics in animal models and humans.

2. Inhibitors in viral nucleic acid quantification

One major factor that often confounds viral nucleic acid quantification is inhibitors that are introduced during sample procurement or co-purify with specimen-derived nucleic acids during extraction. Much of the published literature on DNA inhibition has focused on inhibiting molecules or substances that may interfere with cell lysis, the capture of components necessary for DNA extraction, or cause DNA degradation and/or inhibit DNA polymerase amplification of target DNA [82]. These include excess salts, collagen, humic acid, hematin, melanin, indigo dye, ionic detergents, alcohols and phenol–chloroform used in DNA extractions [83–90], among others. Common sample types of animal or human origin that are known to contain inhibitors include blood, tissues [46] and body fluids (reviewed in [91]). Several main mechanisms have been proposed regarding how inhibitors may interfere with PCR reactions. These include binding of inhibitors to DNA polymerases such as Taq to block enzyme activity; interaction of the inhibitor with the DNA template which leads to blocking of primer/probe binding sites and prevention of amplification; and interaction of the inhibitor with cofactors such as Mg^{2+} or other components of PCR leading to decreased processivity.

A number of methods have been developed to improve PCR amplification in the presence of inhibition [85,92–99]. For example, inhibitor-containing substrate can be avoided during sample processing through special processing method, or be removed during nucleic acid

extraction. Samples can be diluted to reduce the input template amount (and consequently inhibitors, especially inherent inhibitors). In addition, DNA polymerase amount in PCR reaction can be increased, and additives, PCR enhancers and inhibitor-tolerant polymerases can be used to provide resistance to inhibitors. It is noteworthy that methods that are used to improve PCR performance in some applications may not benefit other applications. For example, reducing template input and performing additional sample cleanup can greatly improve genotyping type of application's outcome, however, these treatments likely will not improve results for applications where sensitive target detection (such as for viruses) in a high amount of background material is required, due to limiting the total sample input or loss of input template (and simultaneous loss of pathogen target) during the purification process. An additional complication is that it is often difficult to differentiate between the inhibitory effect of genuine inhibitors from the quantity of nucleic acid input, as a large amount of nucleic acids can be inhibitory even in the absence of additional inhibitors. This will be further discussed in Section 3.1.

For RNA samples, endogenous inhibitors and trace amounts of reagents used during RNA isolation can lead to inhibition during reverse transcription and/or PCR step. Sources such as animal tissues (and to a lesser extent, cells) frequently present issues. For example, heme compounds, urea, fats, humic acids, bile salt and heavy metals that are inherent in the samples, and certain reagents introduced during sample procurement or extraction can both present serious issues to reliable and reproducible quantification [46]. For example, many reagents used to lyse cells contain detergents. TRIzol reagent, commonly used for RNA extraction from cells and tissues, contains phenol. Salts such as guanidinium chloride, guanidinium isothiocyanate, ammonium acetate, and lithium chloride, and alcohols such as isopropanol and ethanol are used in multiple steps during RNA isolation and precipitation. RNA derived from FFPE samples may still contain formalin and paraffin. Some of the inhibitors, such as the anticoagulant heparin, can be avoided at the sample procurement step. Other inhibitors can be difficult to remove. Through negatively affecting cDNA production and/or PCR efficiency, inhibitors can lead to lowered sensitivity of viral detection (even in cases where an optimally sensitive PCR assay is used) and complicate the interpretation of viral load results. In addition, they make it difficult to compare the RT-PCR results from different subjects or different samples from the same subject. It was previously demonstrated that the inhibitory effect on RNA quantification of these common inhibitors can be effectively reversed by using a high processivity reverse transcriptase, SuperScript IV, during cDNA synthesis. This combined with Raindance ddPCR system at the PCR step, could overcome inhibition in RNA samples that were severely inhibited (more than 99.99%) in qRT-PCR [46]. This finding can potentially expand the repertoire of tissue RNA samples that can be directly analyzed without the need for removing inhibitors, especially in scenarios where the identity of the inhibitor is unknown (also see Section 4.4). In comparison, although subjecting the same severely inhibited RNA samples to sequential precipitations with isopropanol and lithium chloride could also completely remove the inhibition [46], RNA recovery after lithium precipitation was often compromised, even more so when the sequential precipitation approach was used, and this effectively reduced the sample input (due to RNA loss during precipitation) and consequently the test sensitivity. The precipitation method is therefore especially harmful in situations where the target viral RNA load is extremely low, as under such conditions, quantitative recovery is essential for optimal signal detection. In fact, any inhibitor removal method that is unable to achieve consistent quantitative recovery will likely lead to a detection sensitivity issue due to reduced effectual sample input in downstream analysis.

3. Technical considerations specific to viral DNA quantification

3.1. Total DNA input upper limit

As more input DNA is included in each PCR reaction, the copurifying inhibitor(s) amount also increases, which can partly explain the inhibitory effect on PCR of a large amount of input DNA. On the other hand, the amount of template input can also directly contribute to PCR inhibition. Proposed mechanisms for this phenomenon include DNA competing with Taq polymerase for Mg²⁺, obstructed diffusion of large polymerase molecules by a large amount of DNA packed in the confined space of the reaction vessel, and double-stranded DNA itself being a direct inhibitor of DNA polymerase [100]. Nucleic acid extracted from tissues can present special challenges during PCR quantification due to co-purified inhibitors (see 2), as tissues often contain complex matrix. Additionally, in some diseases such as HIV, tissue sites have been found to serve as better predictors of disease outcome and indicators of treatment efficacy [28], highlighting the value of sensitive detection of viruses in tissues from infected individuals or animal models. One technical difficulty often encountered during analysis of antiviral drug-suppressed samples, however, is that the target viral signals are present at extremely low levels, which often necessitates testing a large amount of total nucleic acid to detect and quantify the authentic target signal, and the large amounts of background nucleic acid from tissues (i.e. compared to nucleic acid extracted from plasma) can contribute to quantitation inhibition. Consequently, sensitivity limitations of many tissue PCR assays derive mainly from the quantity of input nucleic acid that is allowed in each reaction before inhibition occurs due to the combination of actual inhibitors and the background nucleic acid amount in the reaction. An important ramification of reaction inhibition is that low levels of viral target signal (e.g. as rare as 1 copy of viral nucleic acid in the background of 10 million cells equivalent of genome DNA) may not be detectable due to inhibition, even when an optimized (i.e. single copy assay with a limit of detection (LoD) of 3 copies per reaction) PCR assay is used.

For droplet digital platforms involving water-in-oil droplets, the effect of proper upper limit of nucleic acid input per reaction manifests itself at two distinct stages. (1) Droplet formation step. It was shown that for certain ddPCR platforms, the maximal DNA input in each reaction was 3 µg, above which a significant droplet number decrease and droplet deformation were observed (for example, see [58]). (2) Chemical reaction step. On these same ddPCR platforms, significant reaction inhibition was observed when the total DNA input was above 1.5 µg in each reaction, leading to under-quantification of viral target. Consequently, the BioRad ddPCR platform protocol specifically calls for an upper limit of 1 µg DNA (fragmented) and less than 60 ng intact DNA for each 20 µL reaction [101]. In comparison, on the Raindance ddPCR platform, up to 8 million cell genome equivalent of DNA was included in each 50 µL reaction without compromising the physical integrity of the droplets [46]. In addition, up to 4 million cell genome equivalent of DNA in each reaction did not cause appreciably reaction inhibition [46]. It is noteworthy that the same sample, when used in the quantity of 4 million cell equivalent of DNA in each qPCR reaction, led to 99.99% inhibition in viral signal quantitation. The Raindance ddPCR platform, therefore, drastically improves the nucleic acid input upper limit in each reaction. As the total DNA input is further increased, the distance between clusters becomes less distinct (Fig. 1 A-D). When total DNA input is 5 million cell equivalent and above in each reaction, there are no distinct signal clusters (Fig. 1 E-H). Thus for signal quantification, it is recommended that the total DNA input is no more than 4 million cell DNA equivalent in each 50 µL ddPCR reaction on this platform.

Sensitivity of analysis for a particular sample can be considered to be composed of innate assay sensitivity and sample input. Innate assay sensitivity measures to what extent an assay can detect the target signals that are present. For example, a single copy assay with a LoD of 3 copies of target template per reaction yields a 95% positive rate in a collection

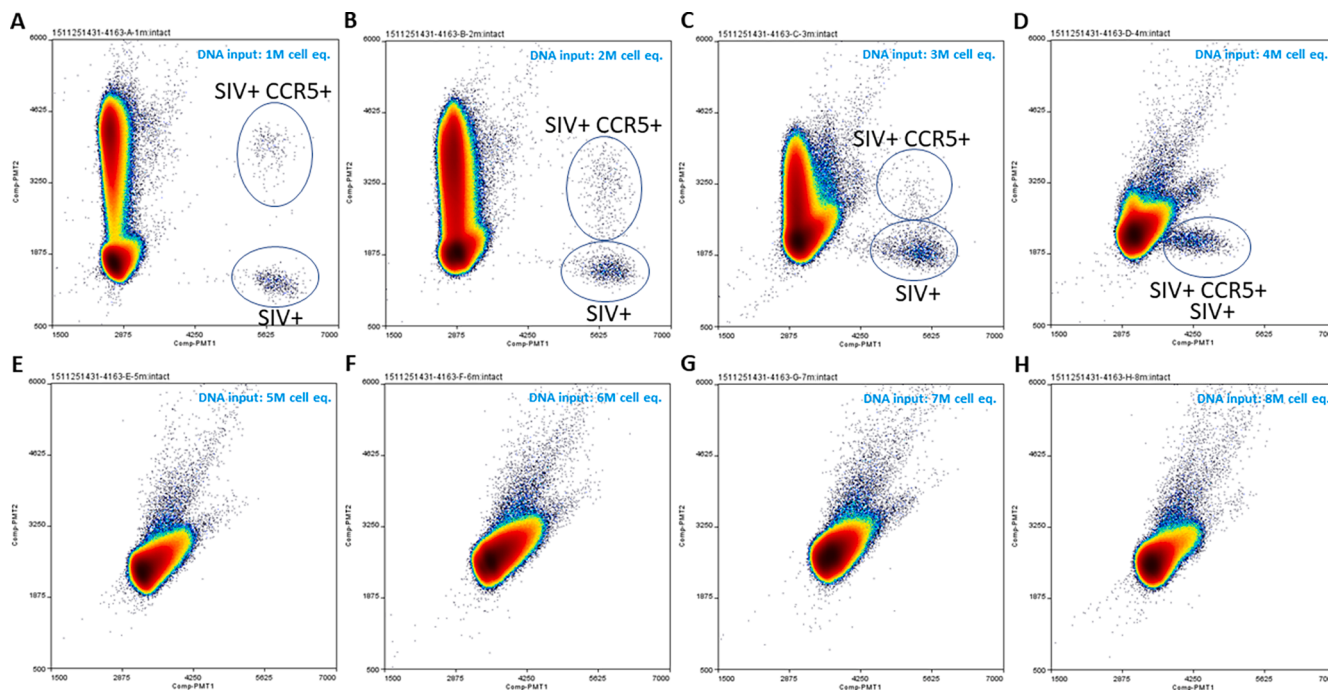


Fig. 1. DNA input upper limit test on the Raindance ddPCR platform. Each 50 μ L, SIV and CCR5 duplex ddPCR reaction was loaded with 1 million to 8 million cell equivalent genomic DNA extracted from the duodenum of an SIVmac239-infected rhesus macaque (animal 313–08) that was treated with combination anti-retrovirals. Note that as the input DNA amount was progressively increased, the separation between clusters was less distinct, and when the total DNA input in each reaction was 5 million cell equivalent or more, there were no distinct signal clusters. SIV+, target cluster containing SIV signal. SIV+ CCR5+, dual occupancy cluster.

of reactions into which an average of 3 copies of target templates are distributed. The 95% positive detection rate is determined by the fact that under this condition, 5% of the reactions will contain no template, which means a “perfect”, single copy assay will yield a positive detection signal every time at least one copy of the template is present in a reaction. In comparison, an assay with a LoD of 5 copies of template per reaction will only yield a positive detection rate of about 80% in a collection of reactions into which an average of 3 copies of target templates are distributed, and reaches 95% positive detection rate when on average the reactions contain 5 copies of target templates, a significant drop in detection sensitivity compared to the single copy assay. What this translates to is that even when a suboptimal assay (for example, an assay with a LoD of 5 copies per reaction) is used, detection sensitivity can be partly compensated by supplying the reaction with a large quantity of total input nucleic acid (and increased target template proportionally), therefore enabling detection of rare events. In HIV cure research for example, if the HIV DNA viral load in a patient is reduced to 1 viral DNA per million cells under combination antiretroviral therapy (cART), assuming an assay with a LoD = 5 copies is used and if each reaction allows 5 million cells input, then in 95% of the cases, a positive signal can be detected. Similarly, for a reaction that allows a 3 million cell input to achieve 95% detection in the same sample, the assay needs to have a LoD of 3 copies per reaction. Therefore, adding more DNA to a reaction alleviates the requirement for the development of super-sensitive assays, since not all genes and genome regions are amenable to the development of single copy (LoD = 3 copies per reaction) assays.

In this context, the finding that the RainDance ddPCR platform can tolerate significantly more input DNA than the Bio-Rad ddPCR platform, even when the reaction volume factor was adjusted, was interesting. The average RainDance droplet size is about 5 picoliter, which is \sim 200 fold smaller than the Bio-Rad droplet size. This difference in droplet size can be converted to many more droplets in a RainDance reaction compared to a Bio-Rad reaction with the same starting reaction volume, and this in principle can allow better partitioning of inhibitory molecules and DNA template molecules into distinct droplets (i.e. separate reaction

chambers) to minimize inhibition. In addition, as excessive DNA input itself can inhibit PCR by competing for polymerase binding [100], the ddPCR dropletization step can theoretically create target sequence-containing droplets in which the effective ratio of the target sequence versus background DNA is many orders of magnitude higher than such a ratio in a qPCR reaction, and this should reduce competition for polymerase and increase PCR success rate in target sequence-containing droplets.

As an important contributor to the overall assay sensitivity is the total amount of nucleic acid that can be analyzed in each PCR reaction without introducing significant inhibition, many qPCR methods have attempted to sidestep the sample input limit issue through diluting or splitting samples until there is no observable inhibition. This can conceivably complicate the workflow and significantly lengthen the sample analysis time, as effort is required to identify the optimal (defined as maximal input without inhibition) per-reaction input for each individual sample using this approach. On the digital PCR side, the BioRad ddPCR platform manufacturer’s recommended 1 μ g input upper limit only corresponds to 0.15 million mammalian cells equivalent input in each reaction. For analyzing samples, e.g. from animals subjected to HIV curative interventions, that contain low levels of viruses, large quantities of DNA are required to be tested to detect single digit number of viruses out of 10 million cells (corresponding to 66 μ g of DNA input). In such scenarios, the BioRad ddPCR platform’s throughput will prove severely limited. Consequently, ddPCR assays performed on the RainDance system can circumvent certain limitations associated with qPCR and the BioRad ddPCR platforms due to its ability to tolerate significantly more DNA.

3.2. Nucleic acid fragmentation

With ddPCR, in addition to input DNA amount/concentration, fragment size can also affect droplet uniformity during dropletization, as viscosity plays a critical role in emulsification as well as the dynamics of droplet stability [102]. For example, the viscosity of highly concentrated

intact genomic DNA can change the average droplet volume, interfere with droplet number, and affect DNA quantification accuracy. Consequently, DNA sample fragmentation and fragment size quality control are important steps prior to ddPCR reaction. The most commonly used DNA fragmentation methods currently include:

(1) Heating. This is usually performed by incubating the samples at 95 °C for 10 min and is the least expensive method. However, heating was found to cause greater than expected copy numbers in ddPCR quantification, likely due to denaturation and insufficient re-annealing of complementary strands resulting in partitioning of single-stranded DNA templates into separate droplets. Consistent with this, one study demonstrated that heat-fragmented genomic DNA in ddPCR measured 49–72% more than expected (double-stranded) input copies. In comparison, Covaris acoustically sheared DNA (below) showed quantitation that is consistent with input (89–94%) [103]. Heating may also increase background mutation rate as heating at high temperature causes deamination of cytosine to uracil [104]. Due to these considerations, the heating fragmentation method is not recommended for ddPCR quantification.

(2) Enzyme digestion. This approach can be performed with either a site-specific restriction endonuclease [105], or with an enzyme (such as Fragmentase) that randomly generates double-strand breaks [106].

(3) Acoustic shearing based on focused ultrasonic energy, where vibrations produce gaseous cavitations in the liquid that shear or break high molecular weight DNA molecules through resonance vibration [106]. Side-by-side comparison between enzyme digestion and acoustic shearing showed the two methods had equivalent performance when considering factors such as target DNA recovery. Restriction enzyme digestion therefore can be a simple and cost-effective alternative to acoustic shearing for ddPCR sample pre-analytic preparation (also see cost discussion below). However, a couple caveats need to be highlighted regarding the restriction digestion method. a) The fragmentation efficiency of several restriction enzymes on the particular genome DNA type needs to be tested to identify the top performer; in addition, it needs to be confirmed that neither the targets nor any reference amplicons have the enzyme recognition site(s) and that the enzyme(s) is insensitive to methylation. b) Efficiency for target DNA detection can be modestly lower in restriction enzyme-treated samples compared to sonicated samples, when digested DNA sample is directly used in PCR reaction, potentially due to the inhibitory effect on PCR by the component in restriction enzyme storage or reaction buffers. On the other hand, purifying the DNA fragments after the restriction digestion step can also potentially lead to reduced DNA recovery.

(4) Fragmentation using a biopolymer shredding system such as QIAshredder, which shears the high molecular weight genomic DNA (and other high molecular weight cellular components) through homogenization. The QIAshredder microcentrifuge spin-columns have been used for DNA preparation in ddPCR for the detection of integrated HIV DNA [107] and mitochondrial DNA [108] in clinical samples. In HIV DNA detection, the QIAshredder/spin-column method outperformed the digestion method, and this improved efficiency was attributed to reduced sample viscosity and a decreased likelihood of salt inhibition on PCR reactions as compared to enzymatic digestion [107]. In a study that performed a side-by-side comparison between QIAshredder and restriction digestion as pre-analytic methods, QIAshredder was shown to allow better target detection sensitivity and a significant decrease in sample preprocessing time [109].

(5) Nebulization, an additional DNA fragmentation process in which a DNA solution is forced through a small hole in the nebulizer unit to create fine mist (composed of random, mechanically sheared, heterogeneous mix of double-stranded DNA fragments); the size of the fragments obtained by nebulization is determined mainly by the speed at which the DNA solution passes through the passageway, the pressure of the blowing gas (compressed nitrogen or air) through the nebulizer, the solution's initial viscosity and the temperature. The main advantage of this method is that the size of the resulting DNA fragment can be well

controlled and shows a distribution over a narrow range. However, documented application of DNA nebulization as pre-analytic preparation in ddPCR has been lacking in published literature thus far.

Two additional factors to consider regarding the fragmentation options (in particular, among (2), (3) and (4)) are equipment requirement and consumable/reagent cost. While the enzyme digestion method only requires an incubator, the sonication-based method and QIAshredder require an ultrasonicator (such as a Covaris M220 Focused Ultrasonicator) and a centrifuge, respectively. Whereas each enzyme digestion-based reaction can cost as low as \$0.01, consumable cost for the other two methods can vary from \$1.40 per sample for QIAshredder to \$7.00 per sample for Covaris sonication-based method.

Sheared genomic DNA cleanup is another important pre-analytic step consideration. While acoustic-based shearing does not require cleanup, other shearing methods may do. It is worth noting that filter-based columns, which are frequently used for nucleic acid cleanup, have a maximum binding capacity and are usually optimized for the purification of nucleic acid molecules in a certain length range (e.g. shorter nucleic acid fragments may not be recovered during the purification process) (also see 4.1). These facts need to be taken into account as a sufficient DNA yield is needed to achieve the desired lower limit of detection (LLOD) in downstream ddPCR quantitation. In addition, to achieve maximal sensitivity and DNA loading, some samples need to be concentrated after cleanup. (Alternatively, high concentrations (such as 100 µM) of primers and probes can be used to ensure that the largest available volume possible of DNA can be added to a sample reaction.)

Regarding the optimal fragmentation length, Raindance's assay guideline recommends a target range for shearing genomic DNA to 2 to 4 kb in length, with an optimal fragment size of 3 to 4 kb. Vitomirov et al [109] compared the correlation between target detection performance and the DNA fragment size (fragmentation obtained by sonication) and reached a similar conclusion: 2 kb DNA fragment size provided the best detection sensitivity when compared to other fragment sizes tested: 200 bp, 500 bp, 800 bp and 5 kb. One potential contributing factor to lower detection sensitivity when DNA is sheared to shorter fragments is that the chance of introducing breaks in the middle of the PCR amplicon has an inverse relationship to the fragment size. To ensure that the correct size range of fragmented genomic DNA has been obtained for downstream ddPCR application, an aliquot of fragmented DNA can be visualized after fractionation on an agarose gel, or alternatively, be analyzed using a microfluidic chip on the Agilent Bioanalyzer.

3.3. Assay design

Digital PCR assay probe and reaction/master mix condition are the two most important factors that determine the data quality, as assessed by assay background signal, cluster separation (including the distance between two positive target clusters, and the distance from a positive target cluster to a negative cluster), cluster diffuseness, and the agreement between target input amount and digital count result. While most applications put the highest priority on accurate signal count (i.e. sub-optimal conditions can cause significant under-quantification of target, as shown in [47]), research areas such as HIV cure studies especially prioritize on clean assay background, as there is the need to differentiate between true negatives from severely suppressed low signals under cART treatment conditions, as well as on the assay's ability to quantify ultra-low levels of viral signals, a pre-requisite allowing differentiating among different regimens and monitoring patient responses to treatments. In a recent study [47], a variety of master mixes combined with different probes were tested to compare data quality in low level viral signal detection and quantitation on the Raindance ddPCR platform. In this particular test system, the minor groove binder (MGB) probe and TaqMan genotyping master mix combination was found to be the best assay condition, while other assay/condition combinations suffered from various aspects such as equivocal background signal, low signal count, extra signal clusters, etc. As the inherent amplification and

hydrolysis efficiency among different assays/designs vary, when feasible it is recommended that multiple assay designs and conditions be explored, possibly combined with variations in PCR cycling conditions to allow selection of the optimal assay conditions for individual application needs. As mentioned in section 1.5, higher order multiplexing in digital PCR can be achieved by combining both color and probe intensity to generate high resolution 2-dimensional data plots. However, due to the fact that the performance (e.g. sensitivity) of a digital PCR assay can be very different when run as a single-plex assay as opposed to combining with other assays in a duplex or multiplex format, assay performance characteristics should be assessed empirically under different conditions. For example, multiplexing can lead to sensitivity drop for some digital PCR assays, and it is normal to observe the cumulative background fluorescence to increase as the complexity of primer composition increases (i.e. as more assays are added). Careful design of oligo sequences to avoid potential intra- and inter-assay primer dimers, adjusting primer/probe concentrations, switching the fluorophore(s), are among the common approaches to improve assay performance during multiplex digital PCR assay optimization.

It is understood that for certain applications, the requirement for low background signals may not be as stringent. In these cases, a wide selection of probe chemistries can potentially be used, including TAMRA-quenched TaqMan probes, double-quenched probes, LNA probes with dual quenching and BHQplus probes. These probes each have distinct advantages. For example, TAMRA-quenched TaqMan probes are typically longer (30–40 bases), and this feature can facilitate targeting genomic regions of low complexity such as in AT-rich transcripts through providing greater assay design flexibility. In general, a master mix that has been optimized for end-point PCR will likely prove superior to master mixes for which the primary utility was not for end-point PCR (e.g. qPCR master mixes). This is important when attempting to adapt a qPCR assay (i.e. validated in a qPCR master mix) to a digital PCR platform (see 3.4). Two practical considerations include reagent cost and product availability, factors that are especially relevant in core laboratory and diagnostic application settings, which process large numbers of samples and may require minimal batch-to-batch reagent variation.

3.4. Considerations involved in transferring an existing qPCR assay onto a ddPCR platform

Direct transfer of validated qPCR assays to digital PCR platforms has been documented [110–114]. However, this usually will require optimization and possible assay redesign. For ddPCR platforms with a closed master mix system, the qPCR assay primers and probe(s) sequence lengths typically need to be adjusted, taking advantage of manufacture-provided online digital PCR assay design tools, to yield oligos with melting temperatures that are compatible with their respective dPCR master mix condition. On occasion oligo sequences may need to be totally redesigned. For an open, flexible platform such as Raindance, which does not specify a defined master mix condition, optimization (and possibly redesign) is still required to achieve optimal assay performance, although the reaction condition choices are not limited, and numerous custom as well as commercial master mixes can be tested to identify the best-performing assay-condition combination. For example, on the Raindance platform, a variety of probe-based polymerase and buffer systems can be used, as long as the master mix contains a hot start polymerase to reduce or eliminate the potential for nonspecific priming and primer-dimer formation. A conservative, yet systematic approach would be to (1) first select several master mixes or conditions that are to be tested; (2) modify the primer and probe design of an existing, validated qPCR assay, based on the unique composition of each master mix (e.g., melting temperature requirement); (3) test the assay design(s) in each master mix, based on each master mix's recommended condition (primer, probe concentration and PCR cycling conditions); (4) select the best performing condition/assay design combination. If necessary, move the design region(s) of primers and probe (i.e. redesign assays) and

repeat steps (3) and (4). It is noteworthy that the published PCR cycling conditions for most commercially available master mixes are usually based on qPCR reactions, and therefore when used in ddPCR reactions, it is possible to perform further optimization such as by altering the annealing temperature, slowing ramping speeds, or increasing the magnesium concentration. Magnesium chloride often serves in PCR reactions as a cofactor to DNA polymerase. Although increasing its concentration can improve amplification efficiency, it can also possibly promote detrimental non-specific amplification. Therefore, the optimal condition will be a final magnesium chloride concentration that ensures robust polymerase activity (i.e. PCR efficiency) but does not compromise probe specificity. One additive that can potentially improve reaction specificity is tetramethylammonium chloride (TMAC). TMAC has been shown [115,116] to improve primer and probe specificity by increasing the melting temperature of the reagent mix. As its benefit can be assay specific, the optimal concentration of this additive often needs to be determined through a custom titration test based on individual assays.

3.5. Reagent sufficiency consideration, especially that of primers and probe(s)

Reagent concentrations, especially primer and probe concentrations, in qPCR reactions are rarely of concern, as the reagents supplied (i.e. on the scale of 100 to 900 nM concentration) usually can more than sustain the PCR cycling. (As a matter of fact, low oligonucleotide concentrations are often preferred in qPCR and qRT-PCR applications since these can minimize both the cross reaction/assay background and assay cost.) This appears not to be the case in digital PCR reactions. For example, a “cluster-squeeze” phenomenon (i.e. two clusters move closer to each other) was observed [47], pointing toward potential reagent (e.g. primers and probes) exhaustion, although the assay primer and probe concentrations were in the 100–900 nM concentration range. ddPCR on the Raindance platform segregates the reagents and templates of a 50 μ L reaction into 10 million 5 picoliter-sized droplets. The advantage of this partition is that target templates can be enriched in some droplets to reduce the target template-containing droplets' genetic background, consequently leading to lowered competition and inhibition. The potential disadvantage, however, is that the reagents involved in the PCR reaction are also evenly distributed into the droplets, and a ddPCR reaction within each droplet is limited to the quantity of the reagents contained in that droplet. The relative sensitivity of the cluster separation (the distance of which reflects target-containing droplets' signal intensity) to primer and probe concentrations (as observed in “cluster squeeze” cases) is consistent with the possibility that the amount for primers and probe(s) (and potentially other components in the reactions as well) are not in great excess as is the case in real time PCR reactions where the reaction components in the whole reaction volume are theoretically accessible to the positive reactions. For example, a 50 μ L PCR reaction containing 600 nM of each PCR primer has about 2 million copies of each primer in each Raindance droplet after dropletization. Assuming 100% PCR efficiency, the primers in a target-containing droplet will be exhausted after 21 thermal cycles during PCR. Comparatively, primers in target-containing droplets will be exhausted after ~20 thermal cycles if the PCR reaction is started with a 200 nM primer concentration. In this latter scenario, each positive droplet's signal intensity on average will be about half of that of the previous reaction's positive droplets. (A similar conclusion can be drawn for the probe, which is another “consumed” reagent during the PCR reaction.) “Cluster squeeze” caused by reagent insufficiency can usually be resolved by increasing the primer and/or probe concentrations. However, care should be taken not to overload reagents into the system, as higher background was observed with the higher concentrations of reagents [47]. In addition, DNA concentration measurement should be used as an independent parameter of template input, as “cluster-squeeze” can lead to inaccurate DNA quantification based on control gene cluster signal(s).

One other condition that was observed to cause “cluster squeeze” was when DNA input was large (e.g. 3 million cell genome equivalent in each reaction; Fig. 2, D and H), as is the case when DNA from a cART-suppressed subject is tested (i.e. a low level of viral signal needs to be detected from a large amount of total DNA). In this case, we attempted to alleviate the “cluster-squeeze” issue (at least for the single and double-occupancy target-containing clusters) through using the high occupancy (HO) setting during the “sense” step. However, it was observed that the HO setting did not lead to appreciably improved cluster separation of the single occupancy target cluster and double occupancy cluster (whose distance parallels that of the control assay cluster and the negative cluster on the y-axis). However, to the advantage of HIV cure research which involves detecting low level virus in infected-individual’s tissue or cells samples, even when a relatively large amount of total DNA (e.g. 3 million cell equivalent of genomic DNA) was added in each reaction, the two SIV target-containing clusters could still be reasonably well separated and quantified (under both HO and non-HO settings).

3.6. Dead/lost volume consideration

As microfluidics are invariably used at the dropletization step of ddPCR platforms, it is not unusual that dead/lost volume, the portion of the internal volume that is out of the flow path, is frequently observed. For example, although Raindance platform is supposed to generate 10 million droplets at the dropletization step for each 50 μ L reaction, we frequently obtain between 8 million and 9 million droplets after the “Sense” step, the step where the droplets are imaged and “positive/negative” signals are assigned. This indicates that about 10% of the droplets (based on theoretical calculation) did not reach the signal reading step and were lost either during dropletization or during transferring to the “Sense” chip. For most applications, the lost reaction volume/droplets can be compensated for when performing the final calculation. For ultrasensitive applications such as those aiming to detect a single viral signal among millions of cells, one needs to keep in mind that when no viral signal is detected, there is an \sim 10% chance that

the negative results were due to the dead/lost volume that is trapped on the device hardware and consumable(s). Appropriate reporting should therefore take into account the dead/lost volume factor, especially in cases where no replicate samples are available for duplicate testing.

4. Technical considerations specific to viral RNA quantification

4.1. RNA quantitative recovery

Quantitative or near quantitative recovery of RNA from tissue samples is important for viral quantitation [81,117], as it is critical to ensure all or most viral RNA molecules are preserved in the purified nucleic acid sample to maximize detection sensitivity and ensure representation of various molecular signatures. In general, precipitation-based methods allows better recovery of RNA in samples compared to column-based methods, as RNA yields in the latter are usually limited by the column/silica membrane’s binding capacity. In addition, precipitation-based methods also tend to preserve the small RNA molecules that are often lost when using column-based methods. It is also important to note that different products/kits used to prepare purified RNA from tissue and cell samples can vary significantly in the amount and the integrity of the RNA samples recovered. Similar quantitative recovery considerations apply to DNA quantitative recovery as well (also see 3.2).

One cost-effective and efficient method for quantitatively recovering RNA and DNA from tissues and cells utilizes TriReagent, a monophasic solution containing phenol and guanidine thiocyanate. During the procedure, after tissue homogenization in this solution and centrifugation, RNA of all sizes can be precipitated from the upper, aqueous phase, while DNA can be isolated from the interphase and phenol phase through back extraction and precipitation [59]. In comparison, to quantitatively recover viral nucleic acids from plasma samples, the following procedure proved to be robust, rapid and convenient: in this procedure viruses are first concentrated from plasma through centrifugation, contaminating proteins are digested away with proteinase K, guanidine thiocyanate is used to dissociate protein-nucleic acid complexes, and nucleic acids are recovered via alcohol precipitation with

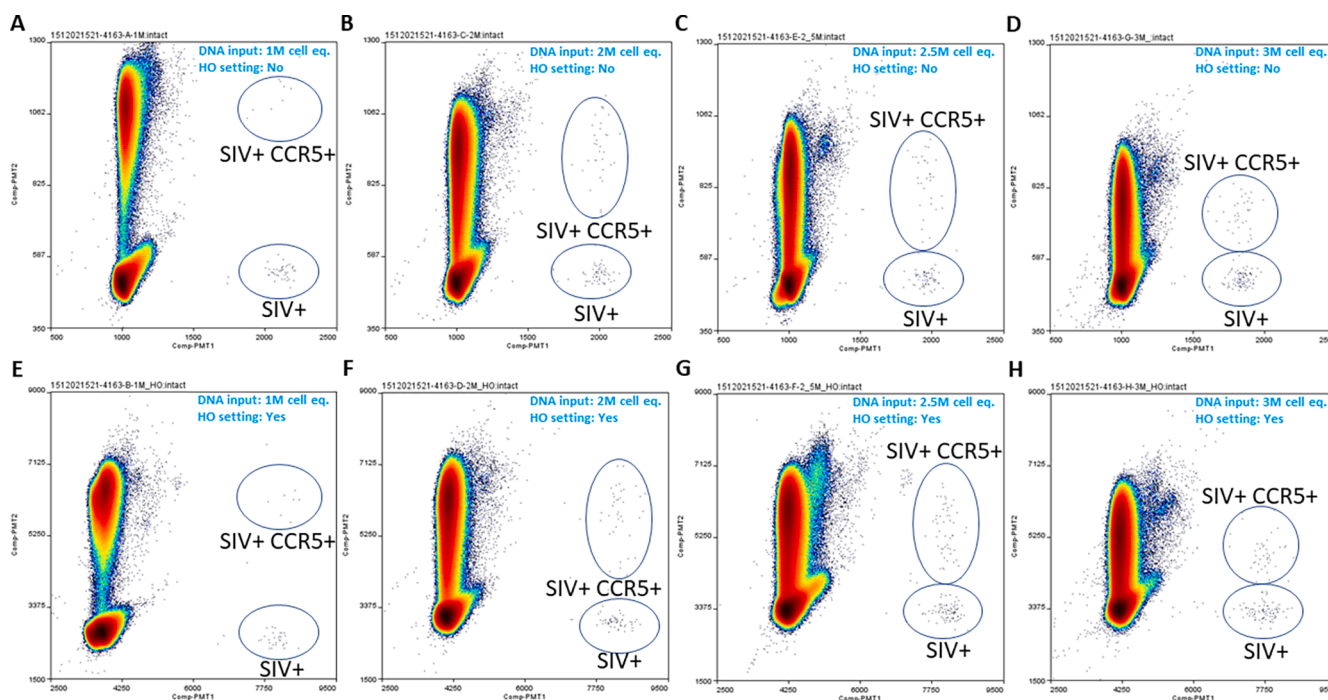


Fig. 2. The effect of high occupancy setting on cluster separation. The high occupancy (HO) setting during the “sense” step was used to alleviate the “cluster-squeeze” issue caused by large template DNA input in Raindance ddPCR reactions (A–D, no HO setting; E–H, HO setting). Note that in the 1 million cell to 3 million cell equivalent DNA input range tested, the HO setting did not lead to appreciably improved cluster separation.

glycogen as a carrier [118].

4.2. RNA integrity

Another issue related to RNA extraction/purification is RNA quality. Isolation of high-quality RNA and preservation of such RNA's integrity is key to the accuracy and comparability of quantitation data. Tissue samples (as frequently encountered in cell or tissue associated viral quantitation studies) from which RNA is derived, can be extremely diverse in characteristics such as size, matrix, preservation method, storage history etc. It is critical that during the extraction procedure, all endogenous and exogenous RNases are properly inhibited, as RNA is extremely susceptible to ribonuclease degradation. Literature from especially retrospective clinical studies aiming to correlate molecular findings with patients' treatment response and clinical outcome reported that RNA extracted from most FFPE archive samples can be quantified by qRT-PCR assays [119,120], due to the fact that most quantitative RT-PCR's amplicons are shorter or in the same length range as the average size of the degraded RNA from archival samples (i.e. 200–250 nucleotides). However, significant discrepancy was observed when comparing gene expression profiles generated from FFPE samples and those from the corresponding frozen samples [121,122] in that results from FFPE samples underestimate or report misleading changes in gene expression patterns. Although viral load determination from FFPE samples has been rare and thus far has mainly focused on detecting a limited number of virus types [123,124], the Bikikova et al results [121,122] nevertheless highlight the importance of performing quantitation on intact RNA templates, if possible, regardless of whether FFPE samples are involved.

4.3. Assay background

Critical to ultrasensitive detection of viral RNA molecule is a clean background in viral signal region on the 2-D plotting space when no viral RNA is present (i.e. in no template control reactions). This is particularly noteworthy in HIV cure research, where it is extremely important to differentiate between a highly suppressed, low viral signal and a blank signal (i.e. indicating eradication). We previously demonstrated the significant role of reverse transcriptases (RTs) in ddPCR background signal generation [46,61]. One possible cause of background signals in RNA quantification is off-target priming that can occur during reaction set up and ramping up from room temperature to the annealing/polymerization temperature. Background signals produced due to off-target priming can theoretically be eliminated by combining a high temperature cDNA synthesis step (i.e. using RTs with elevated thermostability (such as the SuperScript IV RT enzyme, which has 100% activity up to 56 °C and 90% activity at 60 °C) and a hot-start qPCR step (i.e. employing a hot-start Taq polymerase). On the Raindance ddPCR platform, priming method also appears to influence assay background, as demonstrated in a recent study [61] (also see below).

As quantification of RNA is a two-step process, background signals generated at the digital PCR step can also contribute to the overall test background. To minimize or eliminate background signals at this step, the ddPCR assay primer and amplicon quality and specificity need to be tested. For example, at the in-silico design stage, the sequence of the amplicon can be examined using a web-based tool such as NCBI BLAST to identify any potential non-target region (including pseudogenes) of high homology that may exist in the nucleic acid template mixture. Primers can then be tested independently (without probes) to ensure amplification of a clean amplicon product (as visualized on the Agilent BioAnalyzer or an agarose gel). At this step, a standard gradient PCR may be needed to identify the most appropriate annealing temperature to achieve the highest quality amplicon. As probe degradation can contribute to background signal, probe quality should be assessed through standard qPCR reactions which are analyzed without the baseline subtraction [59]. Primers and probes can then be tested in a

qPCR setting with a no-template control sample (but preferably with the same genetic background and complexity as test samples) to ensure high probe specificity with a simple gradient analysis of the annealing temperature (typically between 50 °C and 65 °C). The optimal annealing temperature thus identified in a qPCR setting also needs to be empirically tested using dPCR evaluation using a traditional three-step PCR cycling program in which both the annealing step and the extension step can occur under optimal conditions. In addition to reducing non-specific probe hydrolysis, annealing temperature optimization often can improve amplification efficiency as well, although in many applications assay specificity (especially in the nucleic acid background from test samples) is typically more critical than the end point fluorescence intensity due to the assay lower limit of detection consideration. Carefully minimizing or eliminating background signals at the digital PCR step is also a major consideration that applies to viral DNA quantitation, especially under low viral load testing scenarios.

4.4. Reverse transcription

The reverse transcription step, in which RNA is converted to a DNA template by a reverse transcriptase, can potentially introduce variability and ambiguity to eventual digital PCR quantitation data. cDNA synthesis from RNA can be performed using a variety of primers such as random primers (including hexamers, octamers, or nonamers), oligo(dT) (typically 13–18mers), a mixture of both, or target/gene specific primers. Priming choice can cause a significant difference in measured RNA quantity and therefore variations when comparing the results from different priming methods.

Among the different priming options, random priming yields the highest amounts of cDNA. Due to the fact that priming initiates from multiple points along the template, more than one cDNA molecule is produced from each original template. Because of the random priming nature of the reactions, the majority of cDNA generated (if total RNA is used as template) will be from ribosomal RNA and may compete with low level targets. Additionally, the random primers have low Tms and are not compatible with thermostable RT enzymes without a low-temperature pre-incubation step. Further, in our experience, random hexamer priming often led to under-quantification of viral RNA signals (compared to input signal amount) on the Raindance ddPCR platform.

Oligo(dT) priming is more specific than random priming in that it will not prime from ribosomal RNA. This is an ideal option to copy the entire mRNA pool, although any RNA species that lacks a polyadenylated tail will not be primed. In addition, oligo(dT) priming is a suboptimal choice for priming RNA that contains fragmented fractions as it requires full-length template. If RNA secondary structures exist or if the downstream assay's primer/probe-binding site is at the 5' end of a long mRNA, the reverse transcriptase can fail to reach the primer/probe-binding site. Random primers and oligo(dT) are frequently mixed together; however, this may complicate the quantification process due to potential variability introduced by oligo(dT) priming as described above.

Target/gene-specific priming leads to the most specific cDNA. A previous study comparing hexamer priming, oligo(dT) priming and gene-specific priming found that the latter provided the most sensitive method of quantification [127]. The main disadvantage associated with target/gene-specific priming is that it requires separate priming reactions for each target, which will effectively reduce each assay's sensitivity if a limited amount of RNA needs to be divided among several reverse transcription reactions. On the Raindance ddPCR platform, gene-specific priming, when combined with a suitable reverse transcriptase, yielded clean target region background signal, distinct target positive signal clusters, and good agreement between the ddPCR reading counts with template inputs, allowing confident detection and quantification of low level, genuine signals [46,61].

A second important factor to consider is the reverse transcriptase's enzyme processivity. Processivity is defined as a polymerase's ability to

continue to copy the template rather than falling off, even in the presence of inhibitor molecules [46]. Consistent with the importance of the reverse transcriptase's processivity to overcome inhibition at the RT step, when SuperScript III, a lower processivity RT enzyme, and SuperScript IV, a high processivity enzyme, were compared using a severely inhibited RNA sample as the starting material, about half of the input RNA templates were detected in the final Raindance ddPCR quantitation reaction when SuperScript III was used compared to quantitative detection when SuperScript IV was used at the RT step (also see section 2).

4.5. One step vs. two step RT-dPCR

One main procedural/workflow decision a researcher needs to make when performing a reverse transcription digital PCR (RT-dPCR) experiment is the choice between one-step and two-step RT-dPCR. The "one-step" RT-dPCR nomenclature by definition refers to the RT step being performed in the same reaction compartment (i.e. tube, microwell, or droplet) as the PCR step. In this scenario, all reagents, primers and probe (s) and RNA templates required for the droplet generation step, the RT reaction and the PCR step are mixed together, and immediately followed by dropletization. The collection of emulsification-generated droplets is then incubated on a thermocycler which is compatible with a required low ramping speed, which benefits temperature equilibration across all droplets due to the fact that heat transfer is slower in emulsified samples compared to bulk qRT-PCR reactions. Both the RT step and the PCR step are completed in individual droplets. In the "two-step" RT-dPCR scenario, the RT step and the PCR step are separated into two reaction vessels. First, the RT enzyme is mixed with other components required for the RT reaction, and the cDNA synthesis is performed in bulk. The RT reaction product is then supplemented with components required for the PCR step and droplet generation, dropletized, and PCR cycled on the low ramping speed-compatible thermocycler to end point. The end-point PCR products from both one-step RT-dPCR and two-step RT-dPCR are then analyzed for droplet counts and fluorescent intensity reading. The resulting data are then analyzed with appropriate software to generate graph and statistical data.

The one-step procedure and the two-step procedure each has advantages. For example, due to its obvious simpler workflow, the one-step procedure has reduced contamination risk, lower sample-to-sample variation, and greater automation potential (see 4.6). Also the fact that the entire population of cDNA synthesized is used in the downstream PCR can contribute to increased detection sensitivity, compared to the two-step procedure, which often only allows a fraction of the cDNA synthesized to be used as template in the PCR step due to inhibition concerns. On the other hand, the two-step procedure allows separate optimization of both the RT and PCR steps, and provides flexibility regarding priming methods and enzyme choices. One main advantage of the two-step procedure is the ability to immediately transcribe precious RNA samples into more stable cDNA to allow potential long-term storage. In our experience, the main issue associated with the one-step procedure when performed with Raindance ddPCR system was background signals in target region when there was no target present, which prevented the utility of the one-step procedure in quantifying low level viruses. With the two-step procedure, both the reverse transcriptase and the priming methods (see 4.4) can affect the data quality such as background signals and quantification accuracy. Therefore, different combinations of RT enzymes, priming methods, and master mixes should be explored to identify the best performing assay condition, the specification of which may vary among different applications.

4.6. Automation

The variability of PCR results obtained from identical samples that are assayed in different laboratories (even with identical lots of reagents

and polymerases) points to another major source of data variability, the operator carrying out the experiment. This is not unexpected especially in tissue nucleic acid analysis due to the many steps involved leading to a quantitative result. The availability of robots with the capacity to extract nucleic acids from tissue samples, and to perform liquid handling of very small volumes can potentially address the variation during template preparation and reagent dispensing. In addition, automated processes can also minimize potential contamination [128]. One potential drawback of automated nucleic acid extraction platforms and procedures is that depending on the extraction methods, the nucleic acid yield can often be compromised and no longer provides quantitative recovery [129], which may be critical in certain high sensitivity detection applications (see above). In addition, even with an automated platform, there may be manual pre-platform processing steps that are required, especially for complex materials such as tissue samples. In the context of Raindance ddPCR, we routinely perform manual nucleic acid extraction to ensure quantitative recovery, but rely on the Raindance source and sense machines to perform the downstream automation leading up to quantitation.

5. Conclusions

Sensitive detection and accurate quantification of pathogen nucleic acids are critical in infectious disease research, diagnosis and treatment. For example, in the field of HIV cure research, it has become increasingly apparent that accurate and sensitive quantification of viral reservoirs is a key methodology that has yet to be refined. It is difficult to determine whether attempts at decreasing the size of the latent reservoir are successful, and consequently, which interventions should be prioritized without optimized methodologies. Similarly, the current COVID-19 pandemic highlights the urgent need for the highest sensitivity assay obtainable in the setting of clinical screening and patient disease monitoring. As described in [46,47,59,61] and this review, ddPCR assays performed on the Raindance platform have the potential to address some of the main roadblocks that befall traditional attempts at ultralow-level viral load quantification, namely, the large nucleic acid input that is required for low viral copy detection, and the presence of inhibitors that are often present and difficult to remove. The most significant and well-characterized advantage of the Raindance-based ddPCR assay is the amount of genomic DNA that can be tolerated in a single reaction without compromising droplet formation, integrity or leading to reaction inhibition [46], thus making assays performed on this platform ideally suited for the detection of rare events, i.e. low level viral nucleic acids. In addition, side-by-side comparisons of the RainDance ddPCR assay with standard qRT-PCR for viral RNA quantification showed that the presence of PCR inhibitor(s) does not interfere with viral nucleic acid detection on the RainDance platform, when a suitable reverse transcriptase is used to enhance the RT step processivity.

In this review, factors that can influence or contribute to digital PCR assay sensitivity were summarized, partly based on the Raindance ddPCR platform. While some of the factors are specific for one type of viral nucleic acid application (e.g. reverse transcription for RNA quantification), most of the factors influence both DNA and RNA quantification. For example, as digital PCR measurement of cDNA is a follow-up step to reverse transcription in RNA quantification, almost all factors that affect DNA quantification (such as inhibitors, PCR assay design, PCR reaction condition including reagent sufficiency, assay background and dead/lost volumes) would also contribute to the sensitivity and accuracy of RNA quantification. Due to the capacity to accommodate a large quantity of nucleic acid input and the ability to overcome inhibition, the RainDance platform-enabled ddPCR assays are well-positioned to detect low (e.g. single digit) level cell- and tissue-derived viral nucleic acids. In conclusion, ddPCR assays as performed on this platform has many potential applications that can benefit the broad infectious disease field as well as HIV reservoir/cure studies.

CRedit authorship contribution statement

Samuel Long: Conceptualization, Writing - review & editing, Resources, Project administration.

Declaration of competing interest

The author declares no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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Ethics approval statement

All Rhesus macaques (*Macaca mulatta*) animal procedures were performed in accordance with protocols approved by the Emory University Institutional Animal Care and Use Committee.

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