CHAPTER

# Virology: The Next Generation from Digital PCR to Single Virion Genomics

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## 1 INTRODUCTION

The first written record of the term 'Virus', was coined by the Roman encyclopaedist Aulus Cornelius Celsus in ca. 50 A.D, in association with the rabies virus in dogs (Creager, 2002). The term 'Virus' is of Latin origin and means poison, venom, or slime, in direct association with a disease that cannot be confirmed to be bacteriological. Virology has come a long way from the days of 'unfilterable agents', of Ivanoviskii's and Beijerinck's Tobacco Mosaic virus, to Hershey and Chase's experiments leading to the discovery that genes are composed of DNA (Creager, 2002; Hershey & Chase, 1952). Studies of bacterial viruses dominated until the 1980s, until they were discovered in freshwater and marine systems in the 1990s. Virology, mainly phage biology, has given insight into genetic code, transcription, and genetic exchange, leading to the molecular revolution (Brenner, Jacob, & Meselson, 1961; Crick, Barnett, Brenner, & Watts-Tobin, 1961; Hershey & Chase, 1952).

Just as the Middle Ages led into the Renaissance Age, so too the molecular revolution led to the biotechnology industry, leaving its footprints in virology. Viral genes and proteins are so commonly used in genetic engineering that the work to establish them has long been forgotten. From the genes themselves (e.g. phage/viral promoters: T7/cytomegalovirus), to novel enzymes which catalyse molecular reactions (T4-ligase or Moloney murine leukaemia virus reverse transcriptase), have led to advancements in everything from the genomics of human health, to understanding the biosphere. The discovery of viruses has led to advancements in technology needed to study them, which has had a broader impact on science and society as a whole. Viruses were first identified by electron microscopy and genomic sequencing of DNA and RNA by Sanger sequencing. MS2 (RNA phage) and phiX (DNA phage) were the first RNA and DNA genomes sequenced, but widely used techniques

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such as SDS-PAGE gels and whole-genome amplification with multiple displacement amplification (MDA), have been key technologies allowing the study of other life forms and the biosphere at large (Dean et al., 2002; Dean, Nelson, Giesler, & Lasken, 2001; Fiers et al., 1976; Luria, Delbruck, & Anderson, 1943; Sanger et al., 1977; Weber & Osborn, 1969).

It is generally accepted that viruses are the most abundant biological organism on the planet. Their estimated population of $10^{31}$ is at least an order of magnitude higher than the $10^{30}$ estimate for bacteria (Suttle, 2005). Furthermore, the clinical impact of viral infection is indisputable. Viral infections manifest as a broad range of diseases, ranging from asymptomatic carriage to severe, fulminant, and sometimes fatal processes. While diagnostic tools used to detect and characterise infection were initially based on culture, electron microscopy, and antigen detection, these have been increasingly supplanted by molecular techniques, largely endpoint and real-time PCR (Ko et al., 2015; Lipkin & Anthony, 2015; Pang & Lee, 2015). Quantitative applications of these techniques have become integral to clinical care (Dioverti & Razonable, 2015; Tan, Waggoner, & Pinsky, 2015).

## 2 DIGITAL PCR

Digital PCR (dPCR) is a departure from previous quantitative PCR (qPCR) methodologies, in that it relies on neither rate-based measurements (cycle threshold values) nor calibration curves. Originally developed to detect rare mutations, dPCR was an approach designed to overcome detection difficulties encountered by DNA sequencing, and the difficulty of quantitating a small fraction of mutant molecules within a starting population (Sidransky et al., 1992; Vogelstein & Kinzler, 1999). Further work has demonstrated its potential role in clinical diagnostic virology. dPCR builds on traditional endpoint PCR amplification and fluorescent probe-based detection methods, with the option of adding an RT step for analysis of RNA-based targets. The basic premise of dPCR is centred on limiting dilution, in which single DNA molecules are amplified by PCR in reaction partitions created by methods of separation, including microfluidic chambers, capillaries, or small emulsion droplets. This physical partitioning and separation allows for positive PCR amplifications to be counted directly as the number of positive microreactions (positive partitions) at the reaction endpoint. The limiting dilution factor is chosen such that a high proportion of single reaction partitions (up to 35%) contain no template molecules per partition, giving a '0' (negative) result, with the balance producing a '1' (positive) result, indicating one molecule per positive well (White, Blainey, Fan, & Quake, 2009; White, Quake, & Curr, 2012).

Limiting dilution PCR, the forefather of dPCR, was first described by Sykes et al. (1992) for the quantitation of rearranged immunoglobulin heavy chain (IgH) in leukaemic and non-leukaemic cells, by a limiting dilution-based nested PCR. The Kalinina et al. paper was the next major advancement leading to dPCR. This paper coupled real-time PCR quantitation using 5′ exonuclease (TaqMan) chemistry and

scaled the limiting dilution down to a manageable nanolitre level (Kalinina, Lebedeva, Brown, & Silver, 1997). This facilitated massively parallel reactions by setting the partitioning of these reactions in a capillary (Kalinina et al., 1997). The term 'digital PCR' was coined by Vogelstein and Kinzler (1999), in a study quantifying mutation frequency in colorectal cancer cell lines. dPCR has since been used in a wide variety of biological applications, including maize genotyping and quantitation of copy number in genetically modified corn (Corbisier, Bhat, Partis, Xie, & Emslie, 2010), quantitation of next-generation sequencing libraries (White et al., 2009, 2012), foetal diagnosis of Down's syndrome (Fan, Blumenfeld, Chitkara, Hudgins, & Quake, 2008), diagnosis of organ transplant rejection (Snyder, Khush, Valantine, & Quake, 2011), determination of copy number variation in autism (Sanders, Ercan-Sencicek, et al., 2011), to predict relapse in leukaemia (Mori et al., 2015), response to anti-EGFR therapies in colon cancer (Laurent-Puig et al., 2015), and many more applications too numerous to mention.

The workflow for dPCR is similar to most other PCR methods and is illustrated in Figure 1 (Mazaika & Homsy, 2014).

A typical real-time PCR mastermix, consisting of buffer, deoxynucleotide triphosphate solution mix, primers, DNA polymerase, and DNA template material, is made, with TaqMan probes used for detection of amplified product. Usually, one primer probe is specific for the region of interest, while another is targeted to

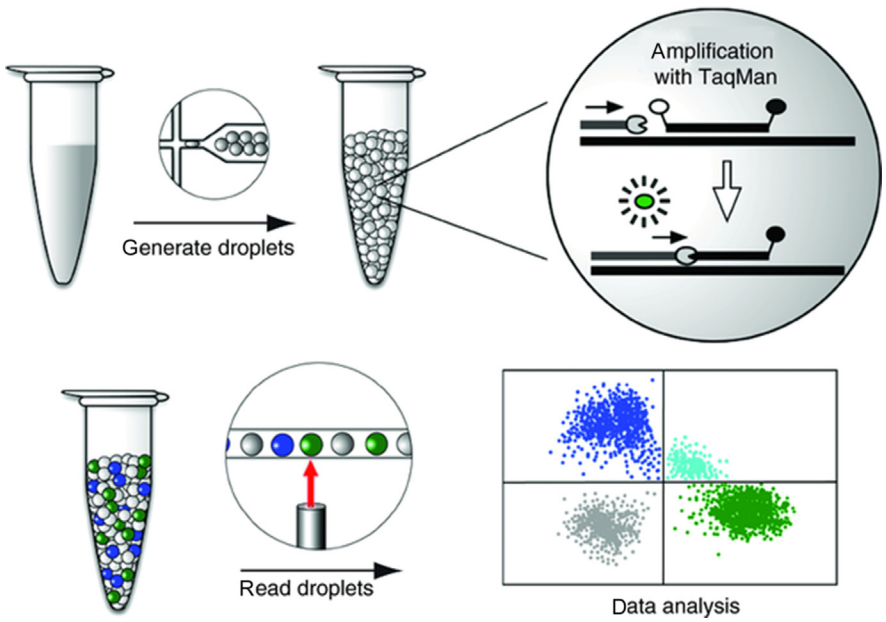


**FIGURE 1**

Overview of digital PCR workflow.

*From Mazaika and Homsy (2014).*

a standard reference (internal control). The reaction is partitioned into many separate reactions by either droplet emulsion or physical separation, each partition containing a separate amplification. After endpoint amplification, fluorescent signal in each partition is read and those containing primary target or control will fluoresce in their corresponding channel, whereas those without target will not. The number of positive versus negative reactions is counted, and Poisson statistics are used to directly calculate the number of DNA molecules in the original sample. Because some reactions may contain more than one target molecule, simply counting may lead to an underestimation of the actual concentration of the sample—this is corrected using the Poisson equation, which is used to calculate the average number of molecules per reaction from the observed proportion of positive reactions within the sample (Mazaika & Homsy, 2014; Sedlak & Jerome, 2013).

Currently, a handful of different dPCR platforms are commercially available, differing primarily by their method of partitioning and the number of partitions produced. The plate format, a microfluidics-based system utilised by Fluidigm and Life Technologies, physically separates the reactions into individual reaction wells. These systems allow for up to several thousand partitions per chip or plate. The droplet format, utilised by Bio-Rad Laboratories and RainDance, creates an oil-in-water emulsion in which each droplet represents a single reaction. The droplet format systems allow for tens of thousands of droplets per sample to over a million droplets per sample, for Bio-Rad and RainDance, respectively (Sedlak & Jerome, 2013). Currently, most dPCR systems only allow for two colour multiplexing. Despite limitations in fluorophore number and choice, some groups have been able to create multiplexed assays. By manipulating the concentration of the fluorogenic probes, it is possible to differentiate multiple targets based on resulting fluorescence intensity (Zhong et al., 2011). With the rapid rate of technological advances, an accurate comparison of dPCR systems with regard to pricing and dynamic range is impossible to discuss. There are pros and cons to each platform, and the choice in platform should be inherently linked to the type of research and study design for which it is purchased. With that statement, the growing interest and use in dPCR has led to the introduction of the minimum information for publication of quantitative digital PCR experiments guidelines, in the hope that it will assist researchers in independent evaluation of experimental data and proper reporting of dPCR data (Huggett et al., 2013). Several items specific to dPCR experiments should be included in the publication, namely, mean copies per partition, partition number, template structural information, individual partition volume, total volume of the partitions measured, comprehensive details and appropriate use of controls, examples of positive and negative experimental results as supplemental data, and experimental variance or CI (Huggett et al., 2013). Regardless of the platform used in the experiments, the addition of these guidelines will allow for more reproducible data and reliable scientific reporting.

As stated before, dPCR has been used in a wide variety of biological applications including oncology, genetics, and environmental testing (Corbisier et al., 2010; Fan et al., 2008; Sanders, Huggett, et al., 2011; Snyder et al., 2011; White et al., 2009, 2012), and it has more recently been gaining traction in the field of clinical virology.

An early report described environmental viruses in single bacterial cells, with a subsequent study illustrating quantitation of a cryptic occult human virus known as GB virus C (GBV-C) (Tadmor, Ottesen, Leadbetter, & Phillips, 2011; White et al., 2009). An important discovery was the demonstration that single bacterial cell hosts could be linked to corresponding infectious phage particles, previously a major challenge in environmental virology (Clokie, Millard, Letarov, & Heaphy, 2011; Wagner & Waldor, 2002; Williams, 2013). Classical phage studies have used a susceptible host to probe for new viruses (Tadmor et al., 2011). A remaining weakness for this application is that universal markers for phages do not exist (Tadmor et al., 2011). A given degenerate viral primer may not necessarily detect targets representative of recent infection of the host, but remnants of previous viral infection(s) in which virus integrated into the host genome. White et al. (2009) demonstrated application of dPCR for the quantification of human RNA virus, with potential clinical relevance for HIV-1-positive patients. The GBV-C virus used in this study had been associated with inference of the HIV-1 viral cycle in co-infected patients (Simons, Desai, & Mushahwar, 2000). This study showed a detection limit of three viral genome equivalents in GBV-C infected native peripheral blood mononuclear cells (PBMCs) without the use of a standard curve (White et al., 2009). Further studies might be designed to show dynamic changes in viral load in relation to HIV-1/GBV-C co-infections in cell lines, PBMCs, or in patient samples, or to quantitate quasi-species formed during GBV-C viral replication (White et al., 2009).

These studies represent some of the first steps in the application of this technology in the field of virology. dPCR technology may also prove very useful in understanding environmental virology and clinical pathophysiologic aspects of viral infection in humans. In particular, dPCR has a benefit over qPCR in that it has been shown to be more resistant to PCR inhibition (Coudray-Meunier et al., 2015; Sedlak, Kuypers, & Jerome, 2014). In a clinical setting, many sample types have high concentrations of PCR-interfering substances, rendering PCR inhibition a continuing problem. In a study targeting DNA viruses associated with gastrointestinal disease, it was shown that dPCR was more resistant to PCR inhibitors compared to qPCR (Sedlak, Kuypers, et al., 2014). Additional advantages of dPCR include rare variant detection and the potential to perform precise, low-level quantification in relation to a high background of cellular nucleic acid. It also offers absolute quantification without the need for calibration curves. However, dPCR is still influenced by variance and bias, and study design and optimisation is of utmost importance, particularly for applications involving RNA viruses. Reviews on the advantages and potential pitfalls of dPCR as a molecular diagnostic tool are well summarised elsewhere (Gullett & Nolte, 2015; Hall Sedlak & Jerome, 2014; Huggett, Cowen, & Foy, 2015; Sedlak & Jerome, 2013).

Because of its ability to detect low levels of viral nucleic acid, dPCR has been used extensively in detection of various viral infections, including hepatitis B (Boizeau et al., 2014; Huang et al., 2015), hepatitis C (Mukaide et al., 2014), HIV (Kiselinova et al., 2014; Malatinkova et al., 2014, 2015; Ruelle, Yfantis,

Duquenne, & Goubau, 2014), human Herpesvirus 6A and 6B (Leibovitch et al., 2014; Sedlak, Cook, Huang, et al., 2014), Enterovirus 71 (Lui & Tan, 2014), and cytomegalovirus (CMV) (Hayden et al., 2013, 2015; Nixon et al., 2014; Sedlak, Cook, Cheng, Magaret, & Jerome, 2014), among others. Many of these studies compare dPCR to qPCR, and with varying results. In a study comparing dPCR to real-time PCR for the detection of low levels of hepatitis B virus DNA, it was determined that dPCR detected fewer positive samples and seemed to under-report the mean viral load range, compared to both a commercial HBV real-time assay and a laboratory-developed qPCR assay (Boizeau et al., 2014). A second study using dPCR for quantitative detection of foodborne enteric viruses, such as norovirus and hepatitis A virus, echoed these findings, showing that the number of genomic copies of hepatitis A virus detected by dPCR was lower than the expected numbers calculated from qPCR (Coudray-Meunier et al., 2015). Some, however, have shown that dPCR has linearity and good quantitative correlation with real-time methods when used to test CMV standard material (Hall Sedlak & Jerome, 2014; Hayden et al., 2013; Huggett et al., 2015; Sedlak & Jerome, 2013), while others have found it to be an accurate method for measuring HIV DNA targets in clinical specimens (Strain et al., 2013). In the latter case, existing qPCR assays were used without modification or optimisation for the study, and dPCR was found to have improved accuracy and precision compared to qPCR (Strain et al., 2013).

## 3 VIRAL LOAD TESTING

Arguably, one of the most common clinical applications of qPCR in the field of infectious diseases is for viral load testing. For many illnesses, even low levels of viraemia are clinically relevant, and changes in viral load can have significance in prognosis and outcome (Sedlak & Jerome, 2013). Additionally, quantitative values are used to determine the efficacy of antiviral therapies or as a trigger for pre-emptive treatment prior to symptomatic infection (Hayden et al., 2013). Studies on CMV have indicated that while both digital and qPCR provide accurate viral load data over a wide dynamic range, dPCR may have a reduced variability (Hayden et al., 2013). Other studies have indicated that increased precision in CMV detection by dPCR compared to qPCR may not always be clinically relevant. For instance, one study found increased precision at viral loads greater than 4 $\log_{10}$, however, that increased precision did not hold true for lower viral loads. As CMV monitoring is important in a transplant setting, where CMV viral loads are often below 3 $\log_{10}$, the clinical relevance of the increased precision in dPCR over qPCR may not be as useful (Sedlak, Cook, Cheng, et al., 2014). Evaluations of dPCR for HIV viral load testing have focused on efforts to characterise latent HIV reservoirs and eradication interventions (Ruelle et al., 2014; Strain et al., 2013). One study reports a limit of quantification of 7 copies/mL of plasma for a HIV-2 viral load assay using dPCR, with increased sensitivity and reproducibility compared to qPCR (Ruelle et al., 2014). Studies of Enterovirus 71 viral load also show a good correlation to qPCR within a range of $2.5 \times 10^0$ to $2.5 \times 10^3$ copies and show promising use of dPCR for investigation

of low viral count diagnostics (Lui & Tan, 2014). The demonstrated use of dPCR in the analysis of common quantitative standards may also serve to improve agreement among qPCR viral load tests. By its use as a reference standard, particularly for DNA viral quantitation, there may be significant value in facilitating normalisation among qPCR calibration materials; dPCR may become critical to increasing uniformity across platforms and laboratories (Hayden et al., 2015).

## 4 SINGLE VIRION GENOMICS

The use of dPCR to screen infected cells could be coupled to single cell genomics, then to high-throughput sequencing, to show whether the viral genome is a remnant of a previous infection or a new infection. dPCR may also be used to measure virulence factors in the environment, relating to both human infection and infections that affect the biosphere at large, such as massive fish die offs or viral lysis within the phytoplankton community. In general, dPCR is a novel technology that could be useful in the characterisation, classification, and the biological role that viruses have on the human host and the biosphere.

The link between dPCR and single virion genomics starts with a new application to dPCR, which is the random amplification of high-molecular-weight DNA, driven by Φ29 DNA polymerase (DNAP), referred to as MDA (Blainey & Quake, 2011; Dean et al., 2001). MDA allows circular DNA templates to be amplified over 10,000-fold within a few hours and is the most common method of whole-genome amplification used in single cell genomics (Blainey & Quake, 2011; Dean et al., 2001). Digital MDA (dMDA) combines traditional MDA methods with a digital platform for increased sensitivity in quantifying nucleic acid fragments of an unknown sequence. Using dMDA, rapid whole-genome amplification of single cells or possibly single virions could be accomplished. In theory, dMDA could be used to amplify the viral genome for sequencing on a high-throughput platform.

Currently, the study of single virion genomics has many technological challenges not shared by single cell genomics. Viruses are much smaller than their host, meaning that trapping and capturing unknown members is very challenging. Random amplification methods such as MDA have problems for both single cell genomics and single virion genomics, in that DNA contamination (from mixes) and chimeric genome amplification bias further weaken the possibility of single virion genomics as common place as the study of single cell genomics (Mazaika & Homsy, 2014). In some studies, up to 50% of cloned MDA products were chimeric inserts prior to enzymatic treatments, and up to 6% remained after treatment (Binga, Lasken, & Neufeld, 2008; Lasken & Stockwell, 2007; Zhang et al., 2006). As viruses in nature have chimeric genomes due to random recombination, higher mutation rates, and genetic transfer from host to host, the addition of chimeric genome amplification bias through MDA makes the possibility of single virion genomics a tough prospect (Marine et al., 2014).

Only one paper illustrates a hypothetical design for single virion genomics, linking flow cytometry attempts to sort single virions into low melting agarose and then

amplifying the genome with Φ29-based MDA (Allen et al., 2011). In principle, this method illustrates that a single virion could be isolated and the most difficult part of this method is ensuring that MDA provides enough coverage of the single virus to *de novo* assemble the whole genome. This has been a limitation in single cell genomics as well. Additionally, this method does not include a nuclease step to eliminate any viral DNA that could have been mobilised in the agarose prior to the MDA amplification, and was not checked by any quantitation of genome copy number to validate whether it is a problem or not. The authors also state that 75% of agarose reaction wells had a range of viral particles from 1 to >1 (as high as five particles), once again another major problem that plagues this technique (Allen et al., 2011). However, depending on the number of virions that are sorted per well, if the genetic richness is low or high, *de novo* assembly should be relatively easy due to the higher throughput of Illumina sequencing and Sanger read lengths (>700 bp), as long as MDA can provide uniform coverage of the genome without fusing genes/genomes together through the ramp effects of amplification (Lasken & Stockwell, 2007).

Single virion genomics as a technique is still in its infancy, but may be used to define new viral pathogens, catalogue the genetic potential of viruses, and possibly provide insight into their roles in the environment, genetic transfer, and novel host–viral interactions. The cost of sequencing unknown viral genomes is significantly lower compared to bacteria or the human genome. However, with Illumina and other platforms promising a complete human genome at $1000 USD or less, the human genome sequencing push will provide virology with fresh insights that were previously unthinkable 10 years earlier.

## CONCLUSIONS

dPCR and single virion genomics are both novel technologies that can be seen as a full circle effect on virology. These methodologies can be used to catalogue and classify the unknown genetic pool that has affected biology from the very beginning. Additionally, their usefulness in the clinical realm, including molecular diagnostics and viral load testing, has already been shown through many studies and across several virus types. While dPCR is still considered a 'new' technique, the implications for its use in viral load testing could have significant impact on treatment, prognosis, and outcome for many patients. The coupling of next-generation sequencing with dPCR and single virion genomics could be the next generation of virology, leading discoveries in all fields, as these novel biological identities have done in the past.

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