applied biosystems
by life technologies™

Digital PCR for virology research—beyond C_t

Absolute answers with definitive sensitivity and precision

Nucleic acid–based testing using quantitative real-time PCR (qPCR) is a commonly used tool for virology researchers. qPCR offers sensitivity and specificity for viral detection, viral contamination, and viral load quantification—especially compared to other techniques such as ELISA, immunohistochemical, or cell culture methods.

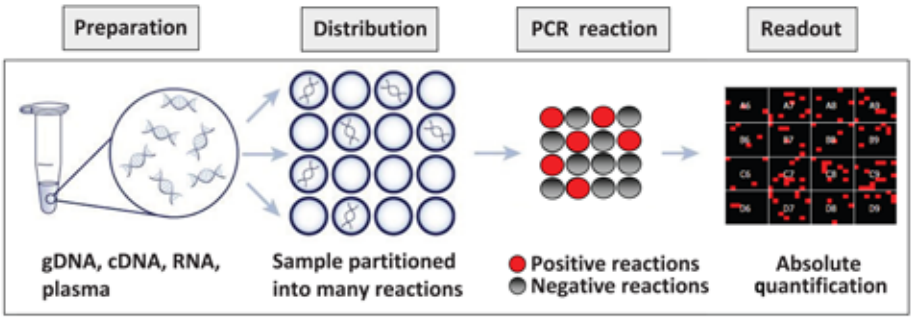


Figure 1. Digital PCR employs a simple workflow using familiar techniques.

However, there are times when qPCR results are ambiguous or inconclusive. Sensitivity or precision may be limited, for example, by extremely low levels of virus. This can cause researchers anxiety and uncertainty over the possibility of virus present but at levels too low to detect.

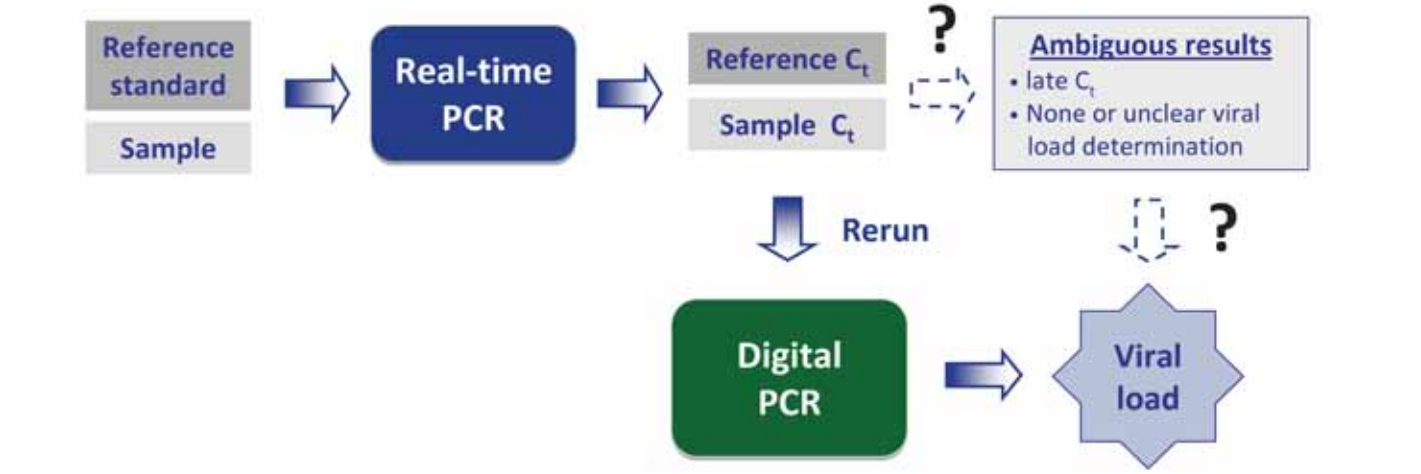
Digital PCR is a new and complementary approach for detection and quantification of viruses present at levels below the limit of detection of conventional methods, including qPCR. Digital PCR enables you to directly measure the amount of nucleic acid target in research samples, providing a more sensitive and precise method than qPCR. Another unique benefit of digital PCR is that it can provide absolute quantification of viral DNA or cDNA prepared from RNA—without reference standard curves or viral reference materials.

Figure 1 shows how digital PCR works by partitioning a sample into hundreds to thousands of nanoliter-scale individual PCR reactions; some of these reactions contain the target molecule (positive) while others do not (negative). Following PCR analysis, the fraction of negative reactions is used to calculate the number of target molecules in the sample.

This application note describes how digital PCR can be used as a complementary approach for validation and quantification of conventional qPCR results obtained from viral samples at different levels of detection, especially low viral load.

life technologies™

A. Viral detection by real-time PCR is complemented by digital PCR.



B. Digital PCR workflow for creating accurate viral reference standards.

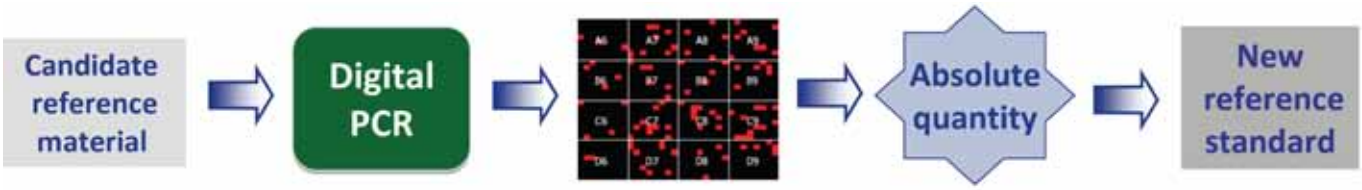


Figure 2. Digital PCR provides a solution to the problems of resolving ambiguous experimental results in molecular testing for viruses (A) and obtaining viral reference standards (B).

Digital PCR enables higher sensitivity for viral detection

One of the main benefits of digital PCR is that it offers increased sensitivity—dependent only on the sample volume used—and greater precision at low virus concentrations. These attributes can be especially useful for research samples in which virus is detected sporadically using other methods, leading to ambiguous results. In these cases, digital PCR is a more sensitive and precise alternative (Figure 2A).

As an example, Figure 3 shows detection of HIV and HCV in research samples by digital PCR using TaqMan® OpenArray® Digital PCR Kits. This experiment illustrates detection of these RNA viruses in a research sample with a high viral load (HIV), and also in a research sample with suspected HCV that is not reliably detected using conventional qPCR. In the suspected HCV research sample, digital PCR successfully detected and quantified the virus, using two different TaqMan® Assays targeting HCV, even though the virus was present at only 1–2 copies per microliter (µL) in the extracted sample (1,000–2,000 copies/mL). One advantage of digital PCR on the QuantStudio™ 12K Flex OpenArray® platform is that amplification curves can be viewed to evaluate reaction quality after thermal cycling is complete. Illustrative of this point, the amplification plots in Figure 3 show clean amplification of positive wells and only background fluorescence associated with the negative wells, providing an additional confidence check for the digital PCR experiment.

Benefits of digital PCR for virologists

- Quantify viral genomes in samples without using a standard curve—make your own viral standard if needed
- Detect virus even at extremely low levels—well beyond the detection range of conventional qPCR
- Use low amounts of sample—digital PCR requires only very small quantities of sample to create a viral standard
- Simplify comparison of results from lab to lab, instrument to instrument, and scientist to scientist, because molecules are "directly counted" rather than inferred through comparison to a standard
- Discriminate subtle differences in viral load—adjust the number of replicate reactions to obtain the precision you need
- Analyze data quickly and easily—DigitalSuite™ Software provides rapid, simple data analysis
- Take advantage of rapid nucleic acid isolation protocols—digital PCR dramatically reduces the impact of polymerase inhibitors in complex samples

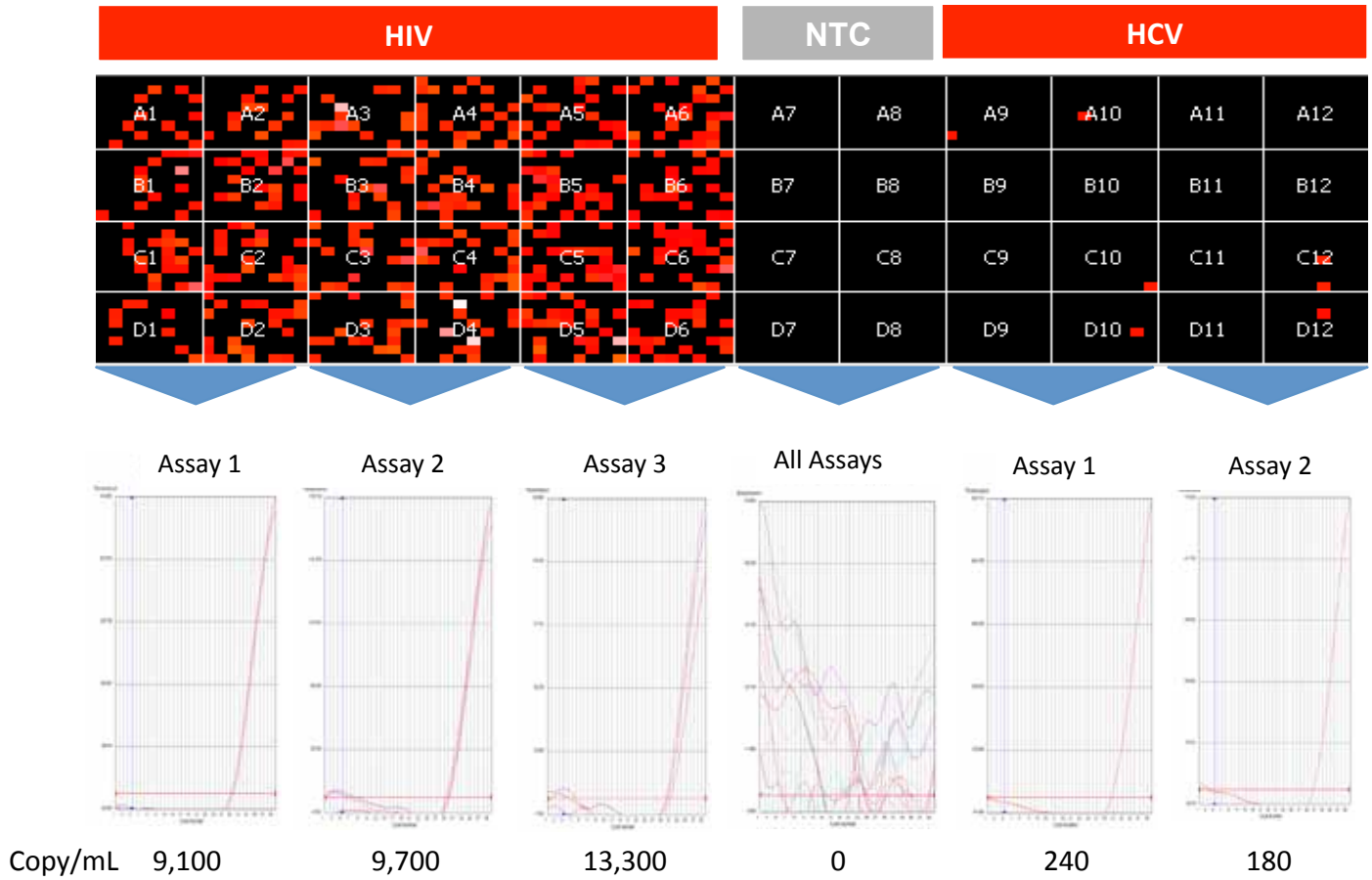


Figure 3. Detection of HIV and HCV by TaqMan® OpenArray® Digital PCR in research samples with high and low viral loads. The SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase, and three (HIV) or two (HCV) TaqMan® Assays, were used for digital PCR analysis of RNA obtained from research samples with high (HIV) and low (HCV) viral loads. The experiment was run using TaqMan® OpenArray® Digital PCR Plates. The amplification heat map (upper part of the figure) shows wells with detected (red) or undetected (black) target amplification. Real-time PCR amplification curves specific for each assay are shown below the corresponding sections of the heat map. The final digital PCR results are shown below the amplification curves as copies of HIV or HCV detected per milliliter (mL) of sample.

Make your own viral standard using digital PCR

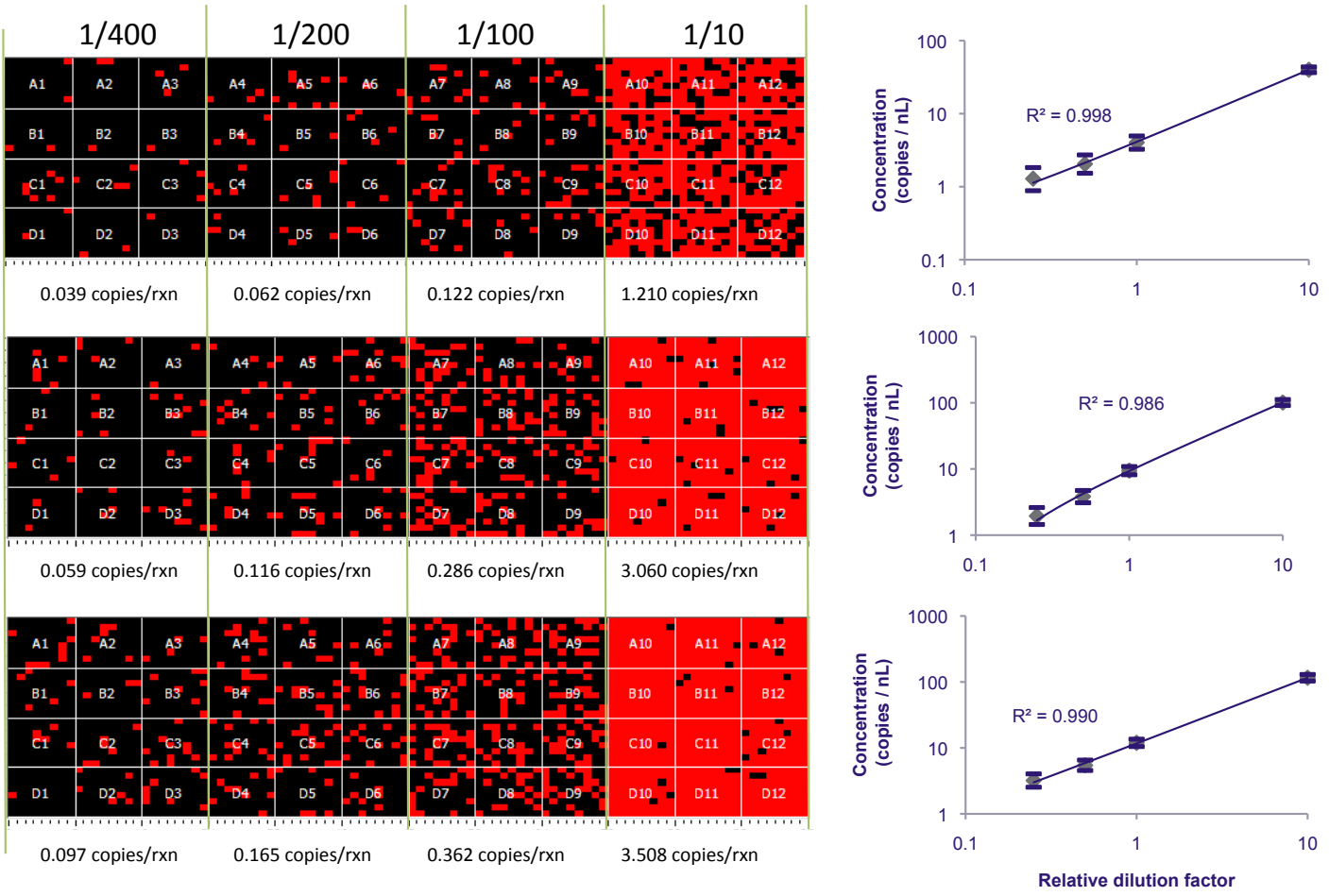
Virologists rely on nucleic acid standards or controls as benchmarks for relative quantification by qPCR (Figure 2B). In some cases, however, viral standards may not be available, either commercially or from public health agencies or academic institutions. Digital PCR is unique among viral detection and quantification methods because it can directly quantify DNA or cDNA prepared from RNA—thus, digital PCR can be used to create reference standards or controls for use in other assays. Figure 4 shows an example of how digital PCR can be used for development and validation of viral reference standards.

Target quantification is accomplished by evaluating the number of positive and negative digital PCR results in a sample that has been diluted to have a single copy of target per reaction well.

Figure 4 shows the results of experiments in which the viral titers for a set of standards were determined using digital PCR. Three different Acrometrix® viral standards were tested with this approach. Due to the high starting titer, the first step was to create a series of dilutions, diluting the standard from 1:10 to 1:400. Analysis of the digital PCR data provides absolute quantification of the three samples.

Increase the precision of your quantification with a digital answer

By its very nature, digital PCR is a highly precise method for quantifying viral nucleic acid molecules, with the level of precision achieved determined by the number of digital replicates run. To maximize throughput on a QuantStudio™ TaqMan® OpenArray® Digital PCR Plate, a research sample can be evaluated using as few as 64 replicates: one subarray on a QuantStudio™ TaqMan® OpenArray® Digital PCR Plate. With 64 replicates, each plate can be used to evaluate 48 samples. At the other extreme, when precision is of the utmost importance, a single sample can be evaluated on up to 3 plates, providing up to 9,216 replicates in a single instrument run (Figure 5).



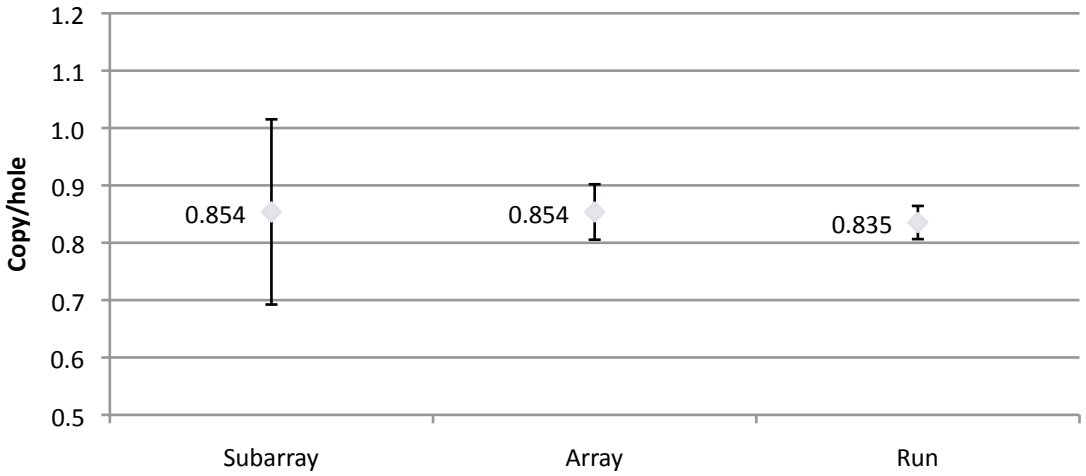
With the increase in precision that comes with increased numbers of replicates, digital PCR offers cost-effective, low- to medium-throughput capabilities with minimal pipetting, to easily accommodate large numbers of replicates. This can help you obtain the precision required for your experimental goals. Figure 5 shows the effect on the 95% confidence interval of increasing the number of replicate wells in digital PCR experiments, with samples with an average copy number of ~0.85 copies/reaction. The gray diamonds display the digitally determined, absolute target concentrations, while the error bars indicate the 95% confidence interval, or precision, of the measurements. The results clearly demonstrate that increases in the number of replicate digital PCR reactions result in commensurate increases in the precision of the quantitative measurement.

Sample	Digital PCR result (copies/mL)	95% confidence interval
EBV	3.7×10^6	±9%
CMV	8.7×10^6	±14%
BK	5.5×10^7	±13%

Figure 4. Digital PCR can be used to create accurate viral standards and controls. A set of Acrometrix® standards for several dsDNA viruses—Epstein-Barr virus (EBV); cytomegalovirus (CMV), also known as human herpesvirus 5 (HHV-5); and BK virus (BK)—were evaluated using digital PCR. The viral standards were diluted as indicated and quantified using TaqMan® OpenArray® Digital PCR Plates. For each dilution set, digital PCR reactions were run in 12 subarrays, each containing 64 through-holes, for 768 replicates per sample (heat maps on left). The graphs on the right show the linearity and precision of target detection. The table at the bottom shows the final results of the digital PCR analysis.

Conclusion

As the pace of biological discovery continues to accelerate, the techniques available to advance science are continuously evolving. Digital PCR is the next advance in nucleic acid detection and quantification. It provides virology researchers with a valuable new tool to complement qPCR and help solve some of today's challenges—and has the potential to expand opportunities for advancement of the field in the future. Digital PCR using QuantStudio™ TaqMan® OpenArray® Digital PCR research products offers virologists the following benefits:



Sample distribution	Samples	Replicates
1 subarray	48	256
1 plate	1	3,072
3 plates	1	9,216

Figure 5. Get the precision you need for your digital PCR experiments by adjusting the number of replicates. In this experiment, genomic DNA from Raji cells, corresponding to the amount that is found in a single cell, was analyzed using a TaqMan® Assay for the RNase P gene, known to be present in one copy in healthy cells. Three digital PCR experiments, with an aliquot of the same DNA, but with the indicated number of replicate reactions were run using TaqMan® OpenArray® Digital PCR Plates. In the first experiment, the sample was diluted in a series of four dilutions: 1:10, 1:100, 1:200, and 1:400. There were 64 replicates for each concentration of diluted sample for a total of 256 replicates. The second experiment contained the same dilution series; however, each dilution was analyzed using one-fourth of an entire plate, providing 3,072 replicates. The third experiment used the same dilution series run on three plates, resulting in 9,216 replicates. The results, number of DNA copies per well, are represented as an average value, with the error bars indicating the 95% confidence interval, or precision, of the measurement.

- **Lower limit of viral detection**—Get results beyond what conventional real-time PCR can provide (Table 1).
- **Ability to create viral standards/controls**—Reliable standards not available? No problem, digital PCR quantifies viral nucleic acids without the need for these.
- **Customizable precision that can be calibrated to individual needs**—Run the number of replicates needed for the precision your research requires.
- **Precise reaction size and real-time data collection**—Fabricated from stainless steel, QuantStudio™ TaqMan® OpenArray® Digital PCR Plates are designed to provide uniform, well-controlled reaction volumes for high-precision digital PCR, with measured volume variation of <10%.
- **Viewable amplification data**—DigitalSuite™ Software displays qPCR amplification plots for each reaction, providing added confidence in your digital results.
- **TaqMan® Assays and Master Mixes tailored for qPCR**—Get started faster. Order premixed PCR reagents (no additives necessary), OpenArray® plates, and validated TaqMan® Assays, developed and tested together for maximum accuracy and precision.
- **On-the-fly assay selection**—With QuantStudio™ TaqMan® OpenArray® Digital PCR products, you can run any TaqMan® Assay in your collection without up-front preparatory work either in your lab or at Life Technologies.

Be at the forefront of the next breakthrough in virology research—QuantStudio™ TaqMan® OpenArray® Digital PCR research products can help you get there.

QuantStudio™ 12K Flex OpenArray® platform technology
The QuantStudio™ 12K Flex OpenArray® platform is a broadly applicable fluidics PCR technology offering nanoliter-scale, solution-phase reactions. It enables the parallelism of microarrays with the data quality of TaqMan® Assay–based PCR.

OpenArray® plates
OpenArray® Plates are microscope slide–sized, containing 3,072 through-holes, each accommodating an individual digital PCR reaction. Proprietory coating renders the exterior surface of the plate hydrophobic, and the interiors of the through-holes hydrophilic and biocompatible. Each through-hole is 300 µm in diameter and 300 µm deep, and accommodates 33 nL of reaction mixture held in place by means of surface tension. The plates are arranged in 48 subarrays of 64 through-holes.

Digital PCR on OpenArray® plates
For digital PCR applications, plates are pretreated to accept your TaqMan® Assay or SYBR® Green dye–based reaction mixtures in your lab.

DigitalSuite™ Software
DigitalSuite™ Software enables quick and easy digital PCR data analysis and management. It can accommodate a wide variety of experimental designs, displays data for review, and lets you adjust analysis parameters to help you achieve optimal results.

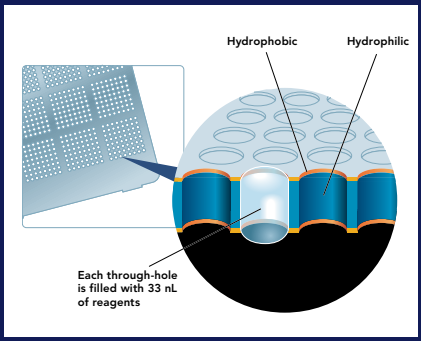


Table 1. Comparison of conventional qPCR to digital PCR.

	qPCR	Digital PCR
Output from experiment	C_t, ΔC_t, or $\Delta\Delta C_t$	Copies/mL
Quantification	Relative quantification	Absolute quantification (with no need for a reference standard)
Results can be affected by:	Choice of reference material used for standard curve	Results are not affected by any of these parameters
	Real-time instrument used	
	Amplification efficiency of PCR primers/probe	

Ordering information

Product	Description	Cat. No.
QuantStudio™ TaqMan® OpenArray® Digital PCR, 10 pack	Includes 10 QuantStudio™ 12K Flex TaqMan® OpenArray® Digital PCR Plates, 2X TaqMan® OpenArray® Digital PCR Master Mix (5 mL), QuantStudio™ 12K Flex TaqMan® OpenArray® Real-Time PCR Accessories Kit (enough to run 10 plates)	4470184
QuantStudio™ TaqMan® OpenArray® Digital PCR, 4 pack (requires additional purchase—see below)	Includes 4 QuantStudio™ 12K Flex TaqMan® OpenArray® Digital PCR Plates, 2X TaqMan® OpenArray® Digital PCR Master Mix (1.5 mL)	4470185
DigitalSuite™ Software	Stand-alone analysis package, for use in laboratory or office for analysis of digital PCR results (includes licenses for installation on up to 10 computers)	4472103

Required for use with 4-pack kit; must be purchased separately

QuantStudio™ 12K Flex OpenArray® Accessories Kit PCR Accessories Kit	Includes 10 QuantStudio™ 12K Flex OpenArray® Real-Time PCR Cases, 10 QuantStudio™ 12K Flex OpenArray® Immersion Fluids, 2 QuantStudio™ 12K Flex OpenArray® Case Sealing Glues	4469576

Contact your local Life Technologies support representative for more information on QuantStudio™ TaqMan® OpenArray® digital PCR products and sample preparation details, or go to **lifetechnologies.com/digitalpcr**

Reference

Lee PK et al. (2006) HIV-1 viral load blips are of limited clinical significance. *J Antimicrob Chemother* 57:803–805.

lifetechnologies.com

life technologies™