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Editorial

Digital PCR as a Novel Technology and Its Potential Implications for Molecular Diagnostics

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The latest incarnation of PCR, digital PCR (dPCR),[2] takes 2 decades of development in enzyme chemistry and assay design and applies them with formidable precision and sensitivity. dPCR is achieved by performing a limiting dilution of DNA into a succession of individual PCR reactions (or partitions). Limiting dilution, made practical by advances in partitioning with nanofluidics and emulsion chemistries, capitalizes on the random distribution of the DNA template and the fact that Poisson statistics can be used to measure the quantities of DNA present for a given proportion of positive partitions. And what is more, it appears to work; results obtained with the technique are linear, and it is capable of detecting and quantifying miniscule amounts of template *(1, 2)*.

All of these features are achievable without the calibration curve required with almost all other molecular methods for accurately quantifying DNA. Compared with real-time quantitative PCR (qPCR), dPCR has already been heralded as more precise *(3)*, better at detecting rare genetic variants *(4)*, and less susceptible to inhibitors *(5, 6)*. Recognition of these advantages has naturally led to speculation as to the potential of dPCR for molecular diagnostics.

This issue of *Clinical Chemistry* presents the reports of 2 studies that have demonstrated the unique clinical application of dPCR for measuring circulating cell-free nucleic acids. Taly et al. *(7)* build on their group's leading research in the application of dPCR to investigating the detection of rare tumor-associated mutations in cell-free DNA (cfDNA) in the plasma of cancer patients, and Beck et al. *(8)* report that the cfDNA of transplantation patients contains detectable quantities of DNA from donor organs and that monitoring of such DNA may serve as a surrogate marker of graft injury and rejection. The articles demonstrate the clinical application of 2 aspects of dPCR, namely detection of rare mutations and nucleic acid quantification.

The detection of rare mutants, in which a variant of a single-nucleotide polymorphism is present among predominantly wild-type sequences, was a subject of the report that coined the term "digital PCR" *(9)*. The limitation of qPCR for measuring rare single-nucleotide polymorphisms is that primers/probes usually also detect the wild-type sequence (which is usually not of interest), although at a much-reduced efficiency. This limitation can lead to a specificity problem when the wild-type sequence predominates, thus limiting the analytical sensitivity of the method. Volgelstein and Kinzler demonstrated that the process of limiting dilution facilitated a reduction in the ratio of the wild-type sequence to the mutant sequence in each PCR, thus improving the sensitivity of the method *(9)*.

Taly et al. evaluated whether dPCR could measure key mutations present in solid tumors by targeting cfDNA originating from the tumor. Mutations in genes such as *KRAS*[3] (Kirsten rat sarcoma viral oncogene homolog) can predict response to therapy, yet current methods for genotyping require invasive biopsies of the tumor. Consequently, being able to perform the same analysis with a simple blood test is highly desirable. Taly et al. developed a method to measure 7 mutations via 2 reactions that applied the unique ability of some dPCR instruments to multiplex by using different concentrations of the same fluorophore. Application of this multiplexing approach, which had previously been illustrated with extracted DNA *(10)*, to actual clinical samples opens the possibility for screening multiple loci with a simple format.

Compared with singleplex reactions, multiplexing not only increases the number of targets measured in a single reaction (thereby improving assay times, costs, and so forth) but also reduces the amount of clinical material required to analyze multiple single-nucleotide polymorphisms by measuring >1 target in a single reaction. This feature is particularly relevant when dealing with plasma cfDNA, which, although notably variable, is present at approximately 1000 genome equivalents per milliliter of blood *(11)* and thus can offer a fairly dilute sample for DNA analysis. Beck et al.

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[2] Nonstandard abbreviations: dPCR, digital PCR; qPCR, real-time quantitative PCR; cfDNA, cell-free DNA; MIQE, minimum information for publication of quantitative digital PCR experiments.

[3] Human genes: *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *ERBB2*, v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (also known as *HER2*).

use an alternative approach to counter the low abundance of cfDNA in plasma by applying a preamplification step before the dPCR analysis. As in the study of Taly et al., Beck et al. also capitalize on the ability of dPCR to measure minority mutants, and they use this feature of dPCR to measure donor organ–derived DNA present in the circulation of transplantation patients. The use of cfDNA in analyses for such rare variants also offers other opportunities, including the potential for noninvasive prenatal genotyping *(12, 13)*.

Both Taly et al. and Beck et al. provide quantitative estimates of the amounts of the variants they measure. Beck et al. demonstrate that an increased amount of graft DNA is associated with acute rejection. Taly et al. also speculate that quantifying tumor-derived cfDNA via dPCR might provide a mechanism to monitor the efficacy of treatment. The application of quantitative molecular measurements to assist the management of transplantation patients or the treatment of solid tumors in a manner akin to the current monitoring of viral load could revolutionize patient care.

dPCR has novel applications that encompass more than sensitively detecting minority mutations. The quantitative ability of dPCR has already been applied to monitoring viral load *(14)* and measuring copy number variations in *HER2* (*ERBB2*, v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2) gene status in breast cancer *(15)*. The superior precision afforded by dPCR means it could also offer an improved and cheaper method for measuring fetal aneuploidy from maternal blood *(16)* for such conditions as Down syndrome. What is clear from the early application of dPCR is that it has the potential for very sensitive and precise quantitative measurements that could facilitate a variety of clinical analyses.

dPCR is also capable of absolute measurements of DNA. Not only is such a characteristic unique among molecular methods, it also simplifies both experimentation and data comparability considerably. These properties can only assist in the translation of such approaches to their impact on patient care, but now is the time for caution. The diagnostic potential of this technology is beginning to be proved, yet for dPCR to have maximum impact, additional well-designed and transparent studies—as exemplified by those of Taly et al. and Beck et al.—are needed for dPCR to have maximum impact. This endeavor was assisted with the publication of the MIQE (*M*inimum *I*nformation for publication of *Q*uantitative digital PCR *E*xperiments) guidelines in *Clinical Chemistry* earlier this year. These guidelines outline some of the considerations for performing dPCR and some key information that should be reported in such publications *(17)*.

A major reason for the MIQE guidelines is to assist in improving the reproducibility of dPCR, a key requirement for the translation of any technology to the clinic. This patently obvious statement is at odds with the reality that the practice of current research applying molecular methods does not encourage data comparability. The popularity of commercial kits—with their proprietary components—means that researchers often are not aware of all the details of the methods they are using. For qPCR methods, that fact is confounded by the nature of the output metric, the quantification cycle ("Cq," also termed "Ct" and "Cp"). Although it is very precise and capable of quantification over a large dynamic range, the difficulties with calibrating the quantification cycle create considerable potential for measurement bias. dPCR should not be as challenging to calibrate as qPCR; however, it would be unwise to assume, as some have suggested, that dPCR will be calibration free. Routine clinical quantification of rare genetic variants is a good example of an application for which calibration controls of a suitable format are likely to assist such measurements, thereby facilitating interlaboratory comparisons and adherence to clinical guidelines.

Although the application of dPCR is likely to enable unique clinical analyses, this novel technology could have wider implications for molecular diagnostics than just these new niche areas. Simpler means of achieving reproducibility could have a major impact on areas that already use molecular quantification, such as monitoring for viral load and tumor–associated transcripts such as BCR-ABL. Whether dPCR will be used to assign laboratory calibrators that will then be used for qPCR analysis or whether it will begin to replace qPCR itself remains to be seen. What is required, however, is the development of instruments that simplify analysis at a cost and turnaround time that will be comparable with those of qPCR.

The rise of dPCR comes at a time when the molecular diagnostics market is moving forward at an incredible rate. With the development of next-generation sequencing occurring concurrently with that of dPCR, there is potential for complementarity between these 2 technologies. It is probably fair to say that advanced sequencing approaches will eventually replace the PCR; however, whether that will occur in the next 5 years or in the next 50 years is difficult to know. What is certain is that dPCR takes a method about which much is known and expands what it can offer. Besides presenting cutting-edge findings, Taly et al. and Beck et al. demonstrate that it is an exciting time to push the envelope of molecular diagnostics. dPCR is likely to have much more to offer.

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