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Review Article

Digital PCR: A brief history

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ABSTRACT

Digital PCR for quantification of a target of interest has been independently developed several times, being described in 1990 and 1991 using the term "limiting dilution PCR" and in 1999 using the term "digital PCR". It came into use in the decade following its first development but its use was cut short by the description of real-time PCR in 1996. However digital PCR has now had a renaissance due to the recent development of new instruments and chemistry which have made it a much simpler and more practical technique.

The term "digital PCR" was first used in the 1999 paper by Kinzler and Vogelstein [1] in which they described the quantitation of *ras* mutations in a sample by partitioning the sample in order to perform a series of PCRs in 384 well microplates. The term "digital PCR" was very apposite as it captured both the nature of the reaction and the spirit of the times and it immediately became established. However the method that they described was not new as it had been used over the previous decade under the terms "single molecule PCR" or "limiting dilution PCR". They referenced single molecule PCR but not quantitation by limiting dilution PCR.

In broad terms, classical PCR can be used for a qualitative or a quantitative purpose, either to study the properties of a target molecule or to determine the number of a target molecule. Digital PCR is used similarly, the difference being that in digital PCR the sample is partitioned to the level of single molecules, PCR amplification is then performed, an all-or-none, i.e. digital, signal is obtained and either the nature of the target molecule is analysed or the number of the target molecule is calculated using the Poisson distribution.

To my knowledge, Saiki et al. [2], in an important early study of PCR published in 1988, were the first to use this approach. They limit diluted a sample of genomes containing B-globin genes in a sample of genomes from which the β-globin gene had been deleted, and showed that single β-globin molecules could be amplified and detected. The frequency of positive amplifications when analysed by the Poisson distribution suggested that virtually every β-globin molecule was amplifiable by the PCR. They were thus the first to use PCR to isolate and analyse a single molecule but they did not conceptualise in the reverse direction and use the frequency of detection of single molecules as a tool for quantification.

The ability of PCR to amplify a single molecule for analysis was soon recognised and exploited. In 1990 Jeffreys et al. [3] published on the use of single molecule PCR to study minisatellite evolution and Ruano et al. [4] published on the use of single molecule PCR to analyse haplotyping. Single molecule PCR continues to be a useful approach to study a target of interest. I searched Medline and Google Scholar using the search terms of "single molecule" and "PCR", and found 4–10 publications annually in subsequent years. "Single molecule PCR" is probably a more descriptive term than "digital PCR" when referring to the process of PCR cloning of a target molecule in order to perform qualitative analysis, as it refers to the target molecule rather than to the signal.

The first publication on the use of digital PCR to quantify a target of interest, in this case HIV, was that by Simmonds et al. [5] in 1990 and I am indebted to Professor Simmonds for information on the background. The group was interested in determining the genetic diversity of HIV populations infecting lymphocytes in blood samples from HIV-positive individuals but recognised that study of bulk samples would prevent study of sequence differences between individual proviral molecules. Limiting dilution followed by PCR of replicates and sequencing of positives was performed (another early example of single molecule PCR). It soon became evident that the frequency of positive amplifications followed the Poisson distribution and that, conversely, the number of target HIV provirus molecules in the original sample could be calculated from the degree of dilution and the frequency of negative (or positive) amplifications. The original publication described the limiting dilution of both mononuclear cells and provirus molecules and in this way documented the number of cells carrying HIV provirus and the number of provirus molecules per infected cell. In subsequent

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years the group continued to use limiting dilution PCR in a number of follow-up studies of HIV and HCV.

Limiting dilution PCR was developed contemporaneously and independently by our own group. It involved the conjunction of two lines of research. In one line of research we had been using genetic selection and lymphocyte cloning to study human somatic mutations at the X-linked HPRT and autosomal HLA loci. Cell cloning was used to amplify the rare mutant cells, Poisson statistics was used to quantify their number and DNA sequencing was used to analyse the nature of individual mutated genes. The second line of research was our development of a PCR-based method to identify and sequence the rearranged and mutated immunoglobulin heavy chain (IGH) genes which can serve as clonal markers for neoplastic lymphocyte clones in leukaemia. It was a natural step to merge these two lines of research and to isolate and sequence the rearranged IGH gene molecule at the time of diagnosis in a patient with leukaemia, synthesise primers specific for the rearrangement, and to use these primers and limiting dilution PCR to quantify the marker IGH rearrangement and hence quantify the leukaemic cells in samples obtained during treatment. A publication briefly mentioning this method appeared in 1991 [6]. However, recognizing the general utility of this method for quantification of DNA targets, we published a definitive study of the method in 1992 [7] in a general biological journal. We continued to use limiting dilution PCR to study various aspects of treatment and biology of acute lymphoblastic leukaemia (ALL). The most important study was a 1994 paper in the Lancet which showed that outcome in childhood ALL could be predicted by the level of leukaemia after one month of therapy [8], and treatment decisions based on this form of assessment have now become part of routine management of childhood ALL.

Limiting dilution PCR was an improvement over previous techniques, such as competitive PCR, for quantification of PCR targets. It was precise, had a wide dynamic range, and could detect and quantify rare target molecules. However it had two disadvantages. Firstly, it was an open system and had the potential for contamination of the environment by amplified PCR product. Secondly, it was a manual system and quite laborious. Our protocol was to perform an initial series of PCRs involving three replicates at tenfold dilutions of the sample in order to approximately determine the limit of dilution and then to perform a definitive series of PCRs involving 5–10 replicates at each of a series of threefold dilutions around the limit of dilution. The endpoint of all-or-none amplification was assessed by electrophoresis.

Search of Medline and Google Scholar using the terms “limiting dilution” and “PCR” showed that the number of publications using limiting dilution PCR increased to a peak of 12 publications per year in 1999. The publications were chiefly, but not entirely, in the areas of virology and lymphoid biology and neoplasia, presumably because workers in these two fields were more likely to have become aware of the technique. However between 2000 and 2002 the annual publication rate fell steeply, and publications using limiting dilution PCR virtually disappeared thereafter. This was undoubtedly due to publication of the method of real-time quantitative PCR by Heid et al. [9]. Real-time PCR is a closed method and is technically simple and these features overcome the two disadvantages of limiting dilution PCR. Our group gratefully switched from limiting dilution PCR to real-time PCR as soon as we became aware of real time PCR.

The method for digital PCR as described by Vogelstein and Kinzler used fluorescence as the endpoint, obviating electrophoresis. It thus had an advantage over the method as previously performed. However it was still somewhat laborious and, as it was competing against real-time PCR, it did not come into widespread use. Search of Medline and Google Scholar using the term “digital PCR” showed that the publication rate remained low, at several publications per year, until 2007. However from that year on there has been a rapid and exponential increase in the number of publications referring to digital PCR. At first these publications were predominantly in engineering and microfluidics journals but during the last few years there has been an increasing number of publications in biological and medical journals. This rapid rise in publication rate is obviously due to the development of new instrumentation which makes digital PCR a relatively simple and practical method.

The history of the development of digital PCR has several general lessons. Firstly, it shows the value of using a title that is both descriptive and catchy. Both “limiting dilution” and “digital” are descriptive – “limiting dilution” describes the process of arriving at single molecules, “digital” describes the nature of the signal – but “digital” is in accord with the electronic nature of the times. Secondly, the method was invented several times by workers unaware of its existence in another field, despite the availability of searchable electronic databases. Presumably this lack of knowledge resulted from the magnitude of the scientific literature and the publication of methods in specialty journals. A corollary of these general factors is that workers in one field may be quite disadvantaged if they are not aware of a useful method in another field and, conversely, cross-communication between workers in different fields can be very fruitful. Thirdly, the history of digital PCR shows that a method may in large part be “ahead of its time” and may require advances in other fields such as engineering and chemistry to allow its full flowering.

Finally, what will be the future history of digital PCR? Will its use continue to expand exponentially? Will considerations of cost and throughput be overcome? What will be its place relative to real-time PCR and next-generation sequencing? Will other digital or non-digital non-PCR methods for detection be developed? Already digital PCR for rare targets can sometimes be performed using current instruments for real-time PCR, and next-generation sequencing can be used both for single molecule analysis and target quantification. Undoubtedly, the coming years will be both interesting and productive.

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