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Review Article

Digital PCR strategies in the development and analysis of molecular biomarkers for personalized medicine

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ABSTRACT

The efficient delivery of personalized medicine is a key goal of healthcare over the next decade. It is likely that PCR strategies will play an important role in the delivery of this goal. Digital PCR has certain advantages over more traditional PCR protocols. In this article we will discuss the current status of digital PCR, highlighting its advantages and focusing on how it can be utilized in biomarker development and analysis, including the use of individualized biomarkers. We will explore recent developments in this field including examples of how digital PCR may integrate with next generation sequencing to deliver truly personalized medicine.

1. Introduction

There is currently a great emphasis in both academia and the biotechnology industry on the development of meaningful molecular biomarkers to assist in the appropriate clinical management of patients. Such biomarkers will assist the rational matching of patients to effective therapies that should benefit them, and will facilitate the use of molecular stratification to inform prognosis and clinical decision-making.

There are significant biological, clinical, logistical and economic complexities in the delivery of high quality personalized medicine. However, it is clear that defining and validating molecular biomarkers is central to the process. Although many biological substrates can be used to derive biomarkers much work to date has concentrated on detecting and quantitating nucleic acids – RNA and DNA. In this review we focus on the potential of digital PCR as a platform to analyse nucleic acid biomarkers. Digital PCR is both conceptually simple and extremely robust in terms of assay performance; it has a number of specific attributes that may make it particularly applicable to biomarker assay in clinical scenarios.

2. Principles of digital PCR

The term "digital PCR" was coined by Kinzler and Vogelstein in 1999 [1], although the conceptual framework of limiting dilution of DNA and single molecule detection was laid out in prior reports [2,3]. The origins and principles of digital PCR have been extensively reviewed [1,4–6], but the concept remains relatively poorly understood and we will briefly review the principles here.

Digital PCR depends on the ability of PCR to detect a single molecule of a target locus. The sample is greatly diluted and divided into a large number of aliquots, so that some aliquots receive at least one molecule of the target ("positive" aliquots), whilst others do not. The number of positive aliquots, as determined by PCR, then reflects the abundance of the target locus in the sample Fig. 1.

If the sample is sufficiently dilute, only a few of the aliquots will be positive, and each of these positive aliquots can be assumed to have contained only a single target molecule. In this case, the process equates to a direct and simple counting of molecules – the "digital" in "digital PCR".

More often, though, the sample is not diluted quite so far. Then many (but not all) of the aliquots will be found to be positive, and some of these positive aliquots will probably (and unbeknownst to the experimenter) have contained two, three or more target molecules. Therefore, simply counting positive aliquots will underestimate the true number of molecules. This can be corrected by using the Poisson equation, a simple statistical tool which calculates the *average* number of molecules per aliquot from the observed proportion of positive aliquots. (The equation is $A = -\log_e(1-P)$, where A is the average number of molecules per aliquot, and P is the proportion of positive aliquots.)

In this way, it is easy to calculate the absolute abundance of the target sequence in the sample [1,3]. More commonly, though, the abundance of the target sequence is compared to that of a reference sequence analysed in the same way, to determine the target's

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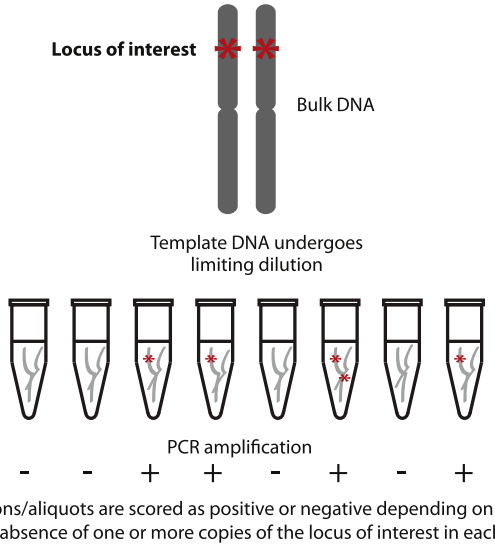

Fig. 1. Limiting dilution PCR. The principles underpinning digital PCR are very simple. DNA undergoes limiting dilution. PCR is then used to probe each aliquot for the presence (+) or absence (−) of a locus of interest.

relative abundance [1,7]. The reference sequence is usually chosen to be one whose abundance is known – for example, one which is present in two copies per diploid cell.

Besides being able to accurately quantify target sequences, digital PCR can also be used to identify rare variants, such as mutations present in only a small minority of the cells from which the DNA is isolated. Digital PCR experiments can be designed so that each positive aliquot is the result of a single or a few template molecules being amplified. In those aliquots in which a rare variant is present, it's detection is not swamped by the more common variant, as it would be if bulk DNA were amplified [8,9]. In practice, rare variant detection requires well-designed experimental protocols with marker validation and non-template controls, but here the point is to emphasize the potential of digital PCR in terms of sensitivity and quantitation of rare variants.

The precision (reproducibility) of digital PCR-based quantitation and its capacity to detect very rare variants depends on the total number of aliquots that are interrogated – the precision and sensitivity increase as more aliquots are analysed.

The degree of dilution of sample is also important: if it is too dilute, then very few aliquots will be positive, and the data will be unreliable. If it is not dilute enough, then all the aliquots will be positive, and no quantitative information can be obtained. A number of authors have investigated how linear the response is to DNA concentration using various platforms [10–12]. In a recent study using a droplet digital PCR system (ddPCR) and interrogating 20,000 partitioned reactions (microdroplets), a linear response to DNA concentration was obtained in droplet saturation in the interval 0.16–99.6% [11]. However, the relative uncertainty in DNA concentration varied across this dynamic range – in particular at the lower end of this range the impact of stochastic events on the estimated copy-number increased. Similar observations were made in a study using a microfluidic based approach [12].

The uniformity of partition volume is also a critical determinant of the accuracy of copy-number estimation and becomes particularly important when the number of partitions exceeds one thousand [11].

Finally, in digital PCR as opposed to QPCR, the efficiency of reactions only has to reach a threshold at which a product will be detected if present. Therefore, it may be unimportant if one reaction is more efficient than another as long as both are sufficiently efficient to amplify a molecule if present. This will potentially reduce the number of primer design 'failures' when biomarker assays are designed.

There are a number of clinical circumstances in which the accuracy and precision of quantitation of potential biomarkers that can be delivered by digital PCR may be very attractive.

3. Digital PCR – attributes

3.1. Rare variant detection

There is a move towards using molecular biomarkers obtained from peripheral blood sampling to detect specific mutations and monitor disease progression, recurrence and stability [13–15]. The assay needs to be able to detect a low proportion of mutant alleles in a huge excess of wild type alleles. Digital PCR can readily achieve this aim. There is also increasing evidence that each individual's cancer may have diverse subclonal populations [16,17]. The clinical relevance of this is that subclones may harbor specific mutations that confer resistance to currently available cancer therapeutics. Examples of this are discussed in more detail in Section 5.

3.2. Estimating copy-number variation

The clinical implications of very precise estimates of germ-line or somatic copy number variants (CNVs) are unclear and will vary depending on the clinical scenario. However, CNVs do alter gene expression [18] and therefore may well be of clinical importance.

The attributes of digital PCR discussed above facilitate the accurate and precise discrimination of the number of copies of specific loci. Assuming reference diploid loci have been validated and therefore have a relative copy-number of two, it is possible to distinguish between one (indicating allelic loss) and two copies, and also between higher integers, for example, five and six copies [9,19,20]. Digital PCR performs better in this regard than other currently available methods including QPCR [9,10,21].

3.3. Minimal template requirements

A key advantage of the digital PCR strategies is that template requirements are generally low. This is of particular importance in some clinical scenarios when tissue samples may be limited in size and/or heterogeneous, or when extracted nucleic acids are degraded as a result of processing [22]. In many genomic analyses (array CGH, next generation sequencing) of limited clinical material a pre-amplification step has been used with the intention of increasing the abundance of all sequences of interest, without altering their *relative* abundances. In practice, however, unbiased pre-amplification is very difficult to achieve, and has been shown to introduce bias in digital PCR [10] and other genomic platforms [23,24]. The importance of this bias will depend on the specific application.

With respect to digital PCR, the low template requirements mean pre-amplification should generally be unnecessary - therefore the data generated will not be subject to pre-amplification bias.

3.4. Ease of analysis

The digital nature of the results means that data handling is relatively straightforward. Some platforms have automated thermocycling, data capture and analysis meaning that the generation of results can be streamlined. A basic analysis of the results for relative quantitation that would probably be sufficient for most readers purposes is relatively straightforward, requiring only the

application of the Poisson equation (to convert the proportion of positive aliquots into an abundance), and the normalization of the abundance of the query sequence to that of one or more reference loci. Those readers interested in more detailed discussions on the analysis of digital PCR data and the potential sources of error in quantitation of loci are referred to the following publications and the references therein [10–12,21].

3.5. Integration with next generation sequencing protocols

Arguably the greatest biotechnology achievements of the last few years have been in the domain of sequencing. The potential for clinical benefit is huge although much more work is required to define the best use of next generation sequencing (NGS) in the clinic and the balance between whole genome and more targeted approaches. Digital PCR may be a very useful complementary technology. Examples of this have already emerged, notably in the use of digital PCR to detect individualized biomarkers in patients whose tumours have undergone paired-end NGS analysis [14,25]; but also in the quantitation and preparation of NGS libraries [26]. It remains unclear what corroboration of NGS results will be deemed clinically necessary – at present anomalies detected by next generation screens are often confirmed using standard Sanger sequencing [27], so that there may be a role for PCR-based confirmation of NGS mutations.

4. Digital PCR – other issues

4.1. Familiarity

The profile of digital PCR remains low, although there are signs that this is changing, with the entry of more biotechnology companies and new platforms into the market.

4.2. Contamination

In any PCR protocol, appropriate systems and controls are critical to ensure that contamination does not occur. This is particularly true for digital PCR, as the assay is sensitive to even a single contaminant molecule. The systems necessary to avoid contamination include a reliable supply of clean reagents, a dedicated PCR suite and controls on the concentration of template DNA permitted in the laboratory.

4.3. Standardization/sources of bias

A huge effort has been made to produce the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines for the standardization of experimental design, analysis and reporting in quantitative PCR (QPCR) [28]. Many of the remaining issues for this technique concern the pre-analytical handling of samples. Such efforts at standardization afford confidence to laboratories considering using biomarkers validated using QPCR.

The reproducibility of digital PCR has recently been shown to be superior to QPCR [10]. However digital PCR is a relatively young technology and there are potential sources of bias that need to be identified and minimized. As with QPCR, DNA fragment size can significantly impact upon digital PCR results [10,12]. Similarly, results can be affected by sustained template exposure to high temperatures and variation in partition volumes [12,29].

When the sources of variation in a digital PCR experiment are better understood, an international effort to develop digital PCR guidelines similar to the MIQE guidelines would be warranted.

4.4. Multiplexing/throughput

It will be clinically important to measure a number of separate clinical biomarkers on individual patient samples. There are two issues which affect the ability to achieve this – first, whether the experimental platform lends itself to multiplex analysis, the second the quality and quantity of nucleic acids that are available from the clinical specimen. The choice of platform will be discussed in Section 6.

In many clinical scenarios, tissue (blood) and therefore DNA/RNA is plentiful and does not limit analysis. However, in many other cases diagnostic tissue samples are heterogeneous, limited in size and fixed in formalin to preserve histological integrity. Therefore, a key goal will be to deliver a system that facilitates the routine analysis of multiple clinically relevant biomarkers using limited template. This is an aim that we have been particularly focused on in the past [22,30,31], albeit using a relatively low throughput platform. The challenge will be to deliver, on an automated high-throughput platform, a truly multiplexed digital PCR system capable of the parallel analysis of sequence mutations and copy-number variations (CNVs) using limited quantities of template derived from diagnostic specimens.

As well as the number of specific biomarkers that can be interrogated on a given sample the throughput potential and demands on laboratory staff with respect to "hands-on" time are critical issues for any clinical biomarker assay.

5. Application of digital PCR to biomarker detection

Digital PCR strategies have already been successfully applied to measure biomarkers in a range of clinical scenarios. We will review a number of these examples, in an attempt to illustrate the potential for broad application of this technology. Although we emphasize the potential application in oncology, the principles discussed may be applied to many aspects of clinical medicine.

5.1. Mutation/rare variant detection

Since the successful development of imatinib for the treatment of chronic myeloid leukemia with the pathological bcr–abl fusion [32] there has been intense interest in developing biological therapies that target specific gene-products. For example, it is increasingly common for patients to be stratified to targeted therapies on the basis of the presence or absence of specific mutations. Solid organ tumours are now routinely screened for mutations in oncogenes such as *EGFR, PIK3CA* and *KRAS* since they predict response to specific and traditional chemotherapeutic regimes [33,34]. Digital PCR strategies have been used to detect EGFR mutations directly in tumour samples, and importantly, the frequency of EGFR mutant alleles can also be accurately estimated using this approach [35]. The importance of the mutant allele frequency has been touched on before [36,37], but is not assessed in current practice. Indeed, it could be argued that the use of very sensitive screens for specific mutations that do not inform the user of the mutant allele frequency may be misleading, and result in the targeted treatment of what is, in effect, a minority subclone (Fig. 2).

It is therefore plausible that two important parameters – (1) the percentage of cancer cells that carry a specific druggable mutation; and (2) whether a specific allele is amplified as well as mutated – may have a major impact on the response to specific therapies. Clearly, this mandates the analysis of as pure population of cancer cells as is feasible with negligible contamination from surrounding stroma cells. This is less challenging than it may previously have been with newer semi-automated laser capture microdissection.

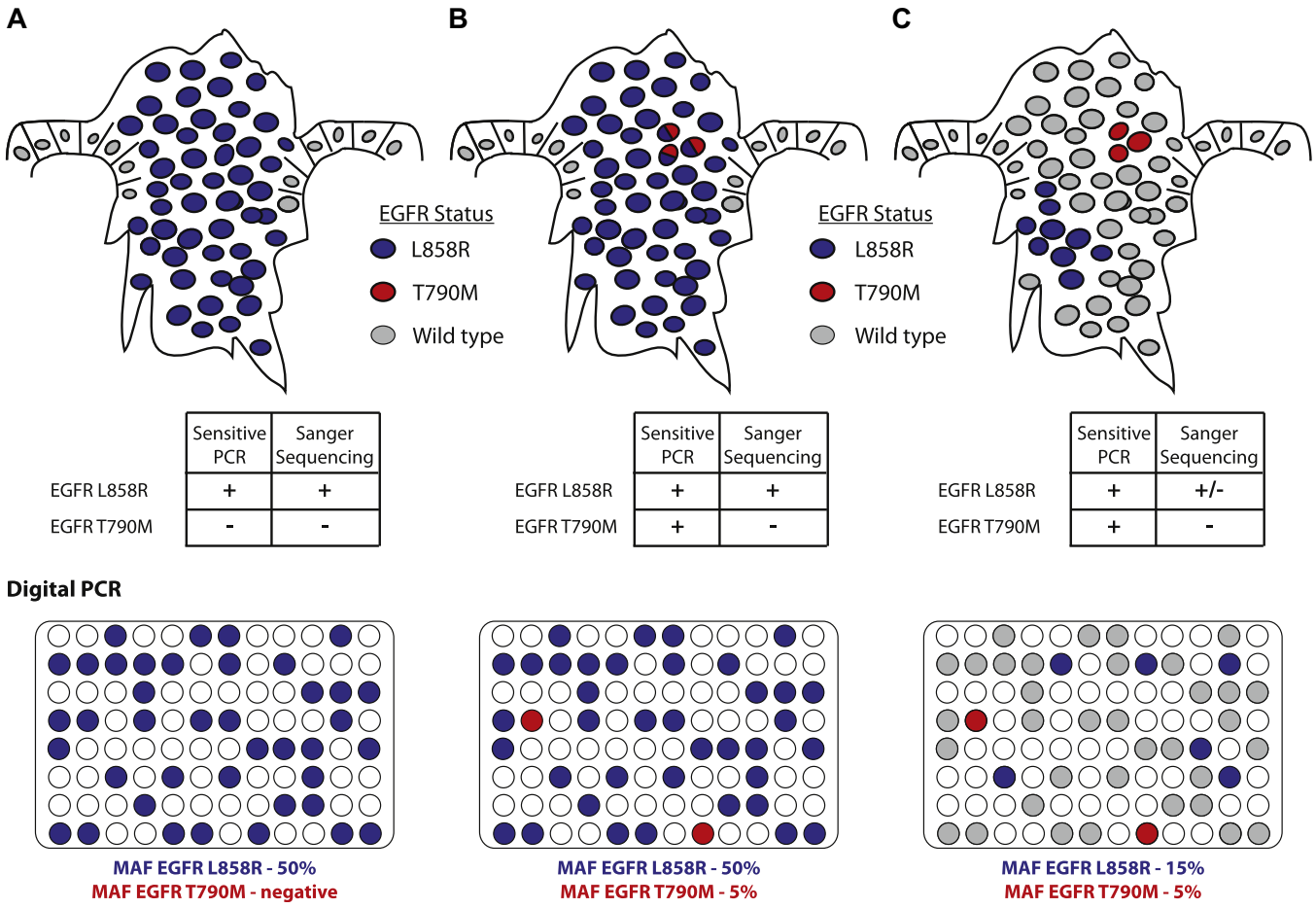

Fig. 2. Digital PCR, mutant allele frequency (MAF) and test sensitivity. The issue of sensitivity and mutant allele frequency in biopsy material has rarely been addressed but may have a significant impact on the interpretation of molecular biomarkers and the delivery of personalized cancer medicine. Currently available detection strategies (probe-based, COLD-PCR) report a sensitivity of approximately 1% for mutations. However the mutant allele frequency is not estimated. In this figure there are cartoons depicting three potential genetic scenarios in lung tumours that are morphologically identical. In each case the tumours are tested for the common mutations in the epidermal growth factor receptor (EGFR) including the activating mutation L858R that generally confers sensitivity to specific tyrosine kinase inhibitors (TKIs) and the T790M mutation (which confers resistance to the same TKIs). In scenario A, all cancer cells harbor the L858R mutation but none have T790M. Traditional (Sanger) sequencing on "bulk DNA" can detect variants to a lower MAF threshold of 20–30%. It would therefore capture this mutation, as would the more sensitive protocols available now. Recent work (Section 5.1) has shown that there is often a subclone of cells that carry the T790M mutation as is suggested in B. This rare mutation would be detected by sensitive protocols but not traditional sequencing. A further possible scenario is C in which three subclones exist, but the dominant clone does not have any mutation in EGFR. Currently, all three tumours would be treated in the same way, but are likely to have very different responses to EGFR TKIs. The potential advantages of digital PCR are that it can detect rare variants but also estimate the absolute frequency of each mutant allele. Therefore, tumours B and C that would now be genotyped as equivalent by sensitive sequencing, could be recognized from the outset to be significantly different from a biological perspective. We could then start to tease out the impact of MAF on outcome or response to therapy.

To emphasize this point, it has recently been confirmed that cancers often consist of multiple subclones [17,38]. This presents multiple challenges to successful targeted therapy, especially if a pre-existent subclone harbors a mutation that confers resistance to a drug under consideration [16,39]. Such drug resistant subclones can exist at the start of treatment and their pretreatment detection may be useful to identify patients who are likely to relapse early after therapy [40] or those who may benefit from combination targeted therapy. This proven tumour heterogeneity presents a direct challenge to the notion of personalized medicine.

A recent study has illustrated the potential importance of digital PCR in evaluating the presence of rare variants/subclonal populations. In this NGS study of hepatocellular carcinoma a mutation in a key tumour suppressor gene was not detected in a whole genome screen with 30× coverage, but was detected in a paired exome resequencing screen with 76× coverage [37]. The same mutation was then sought by traditional capillary sequencing and was difficult to call with certainty. However, a digital PCR analysis both detected the mutation easily, and precisely quantified the mutant allele frequency at 13.2% [37]. Therefore platform sensitivity and ability to accurately call mutant allele frequency may be a major focus of future research.

A potentially very important application of the ability of digital PCR to detect rare variants is the use of routine blood samples for the analysis of nucleic acids originating from solid tumours – either as a result of metastasizing cells or the leakage of DNA from solid tumours into the peripheral circulation [41]. This would facilitate the (repeated) analysis of molecular biomarkers from peripheral blood with a minimum of inconvenience for the patient [42]. One obvious application would be monitoring a patient's response to chemotherapy. For example, digital PCR has been shown to be effective at detecting residual copies of the bcr–abl fusion transcript in patients with chronic myeloid leukemia [25,43]. PCR detection of residual disease in CML has been used clinically for some time. This recent head-to-head comparison of digital and more traditional PCR approaches demonstrated that digital PCR

may offer better sensitivity without the need for a pre-amplification stage [25]. In patients with lung cancer circulating DNA has been successfully analysed for EGFR mutations using digital PCR [42]. Encouragingly, using a microdroplet system the ability to detect a mutant allele at a ratio of 1:100,000 was recently demonstrated [9,20]. Again, the clinical relevance of such low frequency circulating mutant alleles in solid organ tumours has not yet been clarified, although it suggest the lack of a complete response to treatment: this is an issue that needs to be addressed in future studies.

A non-oncological application for the detection of rare variant DNA in peripheral blood has been demonstrated in analysing fetal DNA in maternal peripheral blood samples. The proof of principle for this type of approach has been established in Trisomy 21 [44]; however it may be clinically superseded by the use of NGS platforms to detect fetal trisomy [45,46].

In a related, and very exciting potential application in the field of transplant medicine, digital PCR was recently used to quantify cell-free donor-specific DNA molecules in the peripheral blood of heart transplant recipients [47]. On the premise that the quantity of donor DNA in the circulation would reflect cellular rejection of the graft, the quantity of donor specific loci was used as a molecular biomarker that predicted graft rejection. Again, digital PCR was not used to identify the biomarker (performed by shotgun NGS), but it was the method of choice for quantitation of specific loci in consecutive clinical samples.

5.2. Pharmacogenetics

In addition to the detection of somatic variants that inform the choice of treatment there is evidence that germline variation in certain genes can profoundly affect the individual's response to particular therapies [48,49]. In some cases these are point mutations or SNPs that may affect drug metabolism; in other cases copy-number variants at specific loci may be predictive of how an individual will handle a drug. The added precision of digital PCR may become useful in the future when more data emerges on the role of germline CNVs in pharmacogenetics.

5.3. Gene expression analysis

As well as the analysis of genomic DNA, gene expression analysis using cDNA as template is theoretically and technically straightforward using digital PCR protocols. The dynamic range required to analyse variably expressed genes and reference/housekeeping genes may be more suited to QPCR, particularly when comparing common highly expressed reference loci to rarer transcipts. The dynamic range afforded by newer digital PCR platforms could support this application using well chosen reference loci.

An important potential niche for digital PCR may be the profiling of transcripts from single cells or small numbers of cells. For the reasons discussed earlier, digital PCR is particularly well suited to samples in which material is limited. Single cell transcript analysis has been much discussed in the recent literature and in particular the greater cell-to-cell variability in RNA levels and the potential for bias in a preamplification step prior to QPCR [50]. With respect to dynamic range, most digital PCR platforms (assuming good experimental design and choice of reference transcript) will have sufficient partitioned reactions to deliver precise absolute quantitation of specific transcripts from single cells. However, it remains to be seen whether single cell expression analyses, even if optimized, could be exploited for developing or assaying biomarkers in the clinic.

There has been an explosion of interest in the biology of non-coding RNA (ncRNA) in the last few years. The role of a subgroup of ncRNA–microRNAs – in human pathology has been a subject of particularly high research activity [51,52]. A microfluidic system has been successfully used for miRNA analysis in a RT-PCR protocol with pre-amplification [53]. In principle, microRNA analysis using digital PCR will be feasible, as it has been used to quantitate other transcripts, and has the potential to complement discovery platforms (NGS) in the validation and analysis of ncRNAs.

5.4. Methylation-specific digital PCR

The epigenetic control of gene expression is altered in multiple disease states, in particular cancer. For many years there has been interest in exploiting this to derive molecular biomarkers for both prediction and prognosis. To some extent progress has been disappointing in terms of FDA-approved diagnostics, although there is evidence to support the use of specific methylated loci in some diseases [54]. Methylation-specific PCR has been the cornerstone of these efforts. Digital PCR protocols have also been optimized for the analysis of methylated loci and again, have the potential to afford some advantages over standard techniques [55]. Examples include the use of digital protocols to quantitatively assess methylation at specific loci in colorectal cancer specimens and in plasma obtained from patients with breast cancer [56]. Others have developed digital protocols that allow the single molecule capture of both methylation and histone modifications [57].

6. Choice of platform

There are now numerous available platforms for digital PCR. A significant distinction is whether a platform incorporating a microfluidic chip is chosen [20,58–61], or if the PCR is performed in microdroplets [9], [62,63]. Attributes of the currently available systems are outlined in Table 1. Choice of platform depends on a user's specific experimental/assay requirements and it would be advisable to contact the companies involved as platforms are constantly being refined.

The issues on which to base a decision will be weighed by the degree of precision required (number of partitions, availability of template DNA), the cost of the platform, the cost per assay and the throughput required. Further important considerations will include whether applications other than digital PCR are desired, whether measurement of absolute or relative quantitation is more relevant, whether there is potential to integrate the platform with other technology such as NGS and whether a laboratory would wish to run low volume QPCR experiments on the same platform.

7. Role of digital PCR in the era of next-generation/whole genome sequencing

There has been a huge increase in the use and breadth of applications of next generation sequencing technology in the last three years. NGS has huge experimental capacity and facilitates the parallel analysis of massively multiplexed bar-coded samples. The number of reads per locus affords the potential for data from targeted resequencing protocols to be used to estimate copy-number variation [64]. This means that strong cases need to be made for any proposal to analyse nucleic acids in the future using a non-NGS platform such as digital PCR. The relative advantages of digital PCR remain the accuracy of quantitation, the reproducibility of the data and the ability to analyse very small samples. Furthermore, as discussed in Section 5, there is great potential for the integration of the two protocols in terms of targeted resequencing, the development and use of individualized biomarkers and monitoring the response to chemotherapy in peripheral blood samples.

Table 1
Summary characteristics of the major platforms currently available for digital PCR.

Platform	Description	Number of reactions	Aliquot volume	Analysis	Published sensitivity for rare variants	Integrated thermocycling and analysis	Commercial availability	Published applications
Microdroplets								
ddPCR, BioRad	Microdroplets are generated in an emulsion and transferred to 96 well plates for cycling then to the custom analysis unit (QX100 droplet reader). The reader unpacks the emulsion to single droplets for analysis.	20,000 per 20 μl sample	1 nL	Automated droplet flow cytometer (two colors) with Taqman probes	0.001%	No	Yes QX100 Droplet digital PCR System	Genotyping absolute quantification [9,11]
RainDrop, raindance technologies	Microdroplets, are generated in an emulsion, collected and transferred for thermocycled. The emulsion is then injected onto a microfluidic device and each droplet is analysed	Continuous flow	9 pL	End point analysis with TaqMan probes	0.0005%	No	Yes RainDrop digital PCR system	Genotyping[65] Absolute quantification[63]
BEAMing (beads, emulsion, amplification, magnetics)	Microdroplets containing magnetic beads are generated in an emulsion and transferred to 96 well plates for thermocycling. The emulsion is dispersed and the beads separated. A circularizable probe is hybridized to the sequences on the beads and the changes of interest are labeled with fluorescently labeled dideoxynucleotide terminators	5×10^7 beads	9 μm diameter	Labeled beads are analyzed by flow cytometry	0.01%	No	No	Genotyping Absolute quantification [13]
Microfluidic chambers								
MegaPixel digital PCR	Surface tension based sample partitioning creates aliquots that are thermocycled and analyzed on the device. Fluorescent probes are annealing during thermocycling to enable analysis	1×10^6	10 pL	Microarray scanner	0.001%	Yes	No	Genotyping [20]
Spinning disk platform	Aliquots are generated by passive compartmentalization through centrifugation. These are thermocycled and analysed on the device	1,000	33 nL	CCD camera – end point melting curve analysis	–	Yes	No	Copy number variation and absolute quantification [61]
OpenArray Life technologies/ ABI	Microfluidc reaction chambers are loaded, thermocycled and analysed using the OpenArray system. Chambers may be preloaded with the assay of choice	3,072	33 nL	CCD camera – real time PCR end point melting curve analysis	–	Yes	Yes OpenArray Real-Time PCR platform	
Digital array chip, fluidigm	Microfluidic reaction chambers are loaded, thermocycled and analysed using the BioMark system	9,180 (12×765) Prototype $2 \times 100{,}893$	6 nL	CCD camera – real time PCR end point melting curve analysis		Yes	Yes BioMark HD system	microRNA expression [53] Single cell gene expression [66] Genotyping [35] Targeted resequencing [67] Copy number variation[19,21,60] Absolute quantification [25]

8. Summary

Despite the important caveats expressed in Section 5.1 about personalized medicine in oncology, it is definitely here to stay. Digital PCR is a quantitative method that combines a robust and well-validated technique (PCR) with unrivalled accuracy and precision of quantitation. It is likely that digital PCR will continue to be a very useful tool for those searching for and validating nucleic acid molecular biomarkers for clinical application.

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