ddPCR-based Analytics

Applications and Key Parameters in Developing ddPCR-Based Analytical Methods for Cell a nd Gene Therapies

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Digital PCR (dPCR) is becoming an increasingly important tool in the development and quality control of cell and gene therapies. The technology is used for a variety of applications in cell-based therapies, including quantifying viral vectors in gene therapy products, monitoring gene-edited cell therapies, and measuring transgene expression levels in gene-modified cells.

Introduction

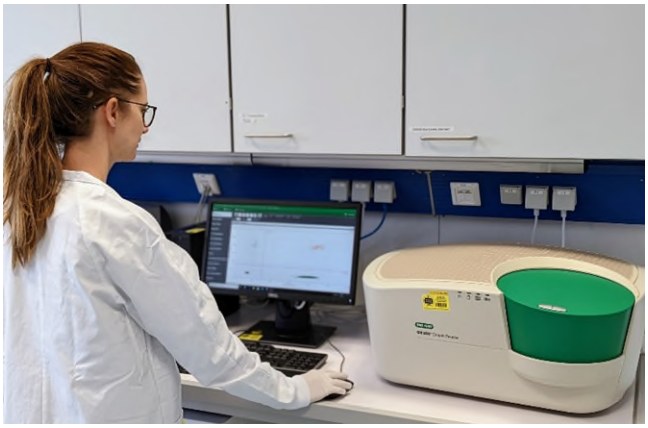
dPCR is a form of polymerase chain reaction (PCR) that partitions the starting material for the PCR into tens of thousands of small reaction units, allowing for the detection and absolute quantification of genetic targets. Popular forms of dPCR are either digital droplet PCR (ddPCR), where the reaction units consist of oil droplets, or dPCR, with microchambers that function as separate reaction vessels. The DNA amplified in these reaction units, which is fluorescently labelled by a probe or a non-specific, double-stranded DNA-binding dye, is then analyzed with an optical fluorescence detector. The data output provides a binary (digital) result for each reaction unit (droplet or microchamber) regarding the presence or absence of target DNA.

Since the distribution of DNA within the reaction units is random, the data can be fit with a Poisson distribution applied to the digital output. For this to work reliably, having a suitable number of reaction units is critical. A high number of reaction units provides a large sample size, which allows a more accurate estimate of the true concentration. Moreover, the template concentration plays an important role. The concentration should be low enough to prevent a large share of reaction units from containing multiple target molecules removing the prerequisites for using the Poisson correction. If the concentration is too low, a precise determination will also not be possible and will increase the susceptibility to experimental variability.

Since ddPCR and microchamber-based dPCR share the same fundamental principle of partitioning, fluorescence detection, and data analysis, only ddPCR will be discussed below.

ddPCR offers advantages over qPCR

The digital form of PCR is highly versatile and adaptable and has several important advantages over the well-established and widely used qPCR method (quantitative polymerase chain reaction). Whereas qPCR provides absolute quantification only when using standards, the partitioning step within ddPCR allows for direct absolute quantification, eliminating the need for standard curves. The partitioning also minimizes competition among molecules during amplification. This form of PCR is therefore more robust to inhibitors and rare targets are more likely to be captured in individual reaction units, enabling their detection even in the presence of high background noise. Additionally, the digital output of ddPCR allows for a straightforward data analysis, eliminating the need for cycle threshold (Ct) values or relative quantification calculations used in qPCR. This also simplifies the data comparison across different laboratories and studies. In addition, ddPCR can also be automated, which streamlines the analysis process and reduces the potential for human error.



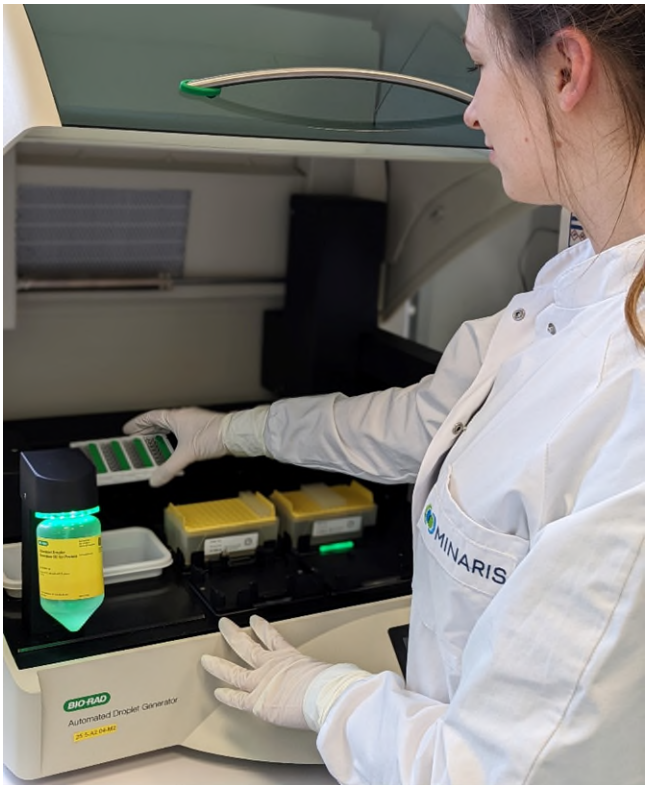
Method transfer to ddPCR is straightforward

The above summarized inherent advantages of ddPCR clearly indicate that it is ideally suited for the development of analytical PCR-based methods and should even lead to consideration of a potential transfer of existing qPCR-based methods to the ddPCR platform. In our laboratory at Minaris Regenerative Medicine Germany, we developed a straightforward process for the transfer of qPCR assays to the ddPCR platform. In most cases, setting the concentrations of primers and probes to the recommended values for ddPCR (900 nM for primers and 250 nM for probes) suffices. To determine the appropriate annealing temperature for ddPCR, a gradient PCR covering a range of 2-5 degrees below the melting temperature of the probe is conducted on reference material. If available, the gradient feature of the ddPCR device can be utilized to test a range of temperatures simultaneously, hence the annealing temperature can be optimized in a single experiment. The optimal temperature is selected based on the clearest separation between positive and negative droplets after droplet reading. Once the optimal temperature is identified, control experiments are performed comparing the ddPCR results with the qPCR data to ensure consistency. Appropriate positive and negative controls, as well as samples previously analyzed by qPCR, should be included to verify the performance of the ddPCR assay. The transition of qPCR to ddPCR is particularly beneficial during the initial stages of method development for cell and gene therapies when the qPCR assay has not yet been validated. Otherwise, method comparability needs to be demonstrated under GMP (Good Manufacturing Practice) conditions.

ddPCR applications for cell and gene therapies

Overall, ddPCR is a useful technology for cell and gene therapies especially for gene-modified therapies. At Minaris Regenerative Medicine we successfully used this technology to assess the vector copy number, for example. It allowed us to optimize the manufacturing process by providing reliable detection of vector copy numbers to correctly evaluate parameter changes during process development. Furthermore, we established a whole-cell ddPCR procedure to evaluate if a cell was transduced or not on a single-cell level, thereby determining the overall transduction efficiency. Of note is that this method does not require surface expression or cell staining, simplifying the experimental process. A key feature of this method is the encapsulation of whole intact cells into droplets, instead of DNA molecules. Moreover, in order to assess biodistribution of transduced cells in an animal background, Minaris Regenerative Medicine successfully developed a ddPCR assay capable of detecting rare events with a remarkably high sensitivity of 0.002%, even when dealing with challenging sample material such as animal-fixed PFA tissue with uncertain storage conditions. In addition to measuring the integration of the vector construct into the genomic DNA, ddPCR can also assess the successful expression of RNA from the construct's promoter. This analysis can be directed towards either the transgene itself or, if distinguishing it from endogenous mRNA is challenging, the regulatory elements of the vector construct under the same promoter as the transgene, if present. For these applications, prior to ddPCR, the RNA needs to undergo transcription into complementary DNA (cDNA) using a reverse transcriptase (RT) enzyme. This can be performed in an independent RT reaction, or as part of a combined 1-step RT-ddPCR.

In summary, the ddPCR based methods developed at Minaris Regenerative Medicine allow for evaluation of safety and efficacy aspects of cell and gene therapies. It provides a powerful tool to optimize process protocols, and monitor potential risks associated with an excessive or insufficient vector dose.



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Minaris Regenerative Medicine is a global contract development and manufacturing organization (CDMO) for cell and gene therapies. We offer our clients high value clinical and commercial manufacturing services, development solutions, and technologies. We are pioneers in the field with more than 20 years' experience providing outstanding quality and reliability. Our facilities in North America, Europe, and Asia allow us to supply patients globally with life-changing therapies.

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