Model-based classification of rain in digital PCR as a benchmark for assay reliability

Pieter Berden[1,2,3], Willem Van Roy[1], Rodrigo S. Wiederkehr[1], Liesbet Lagae[1,2], Tim Stakenborg[1], Jan Michiels[2,3] and Maarten Fauvart[1,2,3]

[1]Imec, Leuven, Belgium

[2] KU Leuven, Leuven, Belgium, and

[3]VIB, Leuven, Belgium

ABSTRACT

Digital PCR is a widely used method for DNA quantification. However, the presence of rain makes it difficult to unambiguously classify positive and negative partitions, resulting in unreliable DNA concentration estimates. This is especially the case for large DNA vectors. Therefore, we developed an analytical model that uses single-partition real-time PCR amplification data to distinguish between intrinsic and extrinsic droplet rain, providing an indication for the reliability of the assay. We demonstrated this method by optimizing a digital droplet PCR assay for the reliable detection of a fosmid containing the biosynthetic gene cluster for the xantholysin A antibiotic.

KEYWORDS: Digital droplet PCR, rain, DNA quantification, fosmid detection

INTRODUCTION

Digital PCR estimates DNA concentrations by digitizing the end-point fluorescence (EPF) of the partitions using an arbitrary threshold. Partitions that cannot be easily classified as positive or negative, are referred to as rain [1] (Figure 1A). Rain is linked to the distribution in PCR cycle at which partition fluorescence passes a threshold (threshold cycle, Ct) (Figure 1B). A suboptimal PCR efficiency (< 2) will result in stochastic amplification at low copy numbers, leading to a distribution in Ct values defined here as intrinsic rain, described by the equation below (N = partitions associated with intrinsic rain, N_{tot} = total positive partitions, C_{offset} = cycle of the main peak, η = PCR efficiency) [W. Van Roy, *et al.* unpublished].

$$N(C_t) = \frac{N_{tot}}{0.333\sqrt{2\pi}\,(C_t - C_{offset})} \exp\left[-\frac{\left(\ln\,(C_t - C_{offset}) - \mu\right)^2}{2(0.333)^2}\right], \text{ with } \mu = 3.045 - 2.907(\eta - 1) + 2.25\exp\left[\frac{-(\eta-1)}{0.092}\right] \quad (1)$$

Partitions that do not follow this distribution are associated with additional sources of rain (i.e. extrinsic rain), e.g. inaccessibility of the template, a common source of rain in the detection of DNA vectors [2]. The proportion of extrinsic rain provides an indication of the reliability of the assay, as it depicts the proportion of partitions not showing expected PCR behaviour and can therefore not be trusted for determining the DNA concentration.

EXPERIMENTAL

The digital droplet PCR assay was performed using a glass-silicon microfluidic chip, in which the generated droplets (~3200) were stored during thermocycling [3] (Figure 1C). FluoSurf 2% in HFE-7500 was used as the continuous phase. Following PCR mix: 1x QX200 ddPCR EvaGreen Supermix, 1x ROX dye, forward primer: 5'-CTGCTGCACTTCCACCATCT-3', reverse primer: 5'-CCCTTCGTTCGCCATCAGTA-3' at 500 nM, template: pCPMG6126 fosmid vector [4], was used. Fluorescent images were taken every PCR cycle from which the Ct values per droplet were calculated. Ct distributions were fitted with eq. (1) to determine the amount of intrinsic rain.

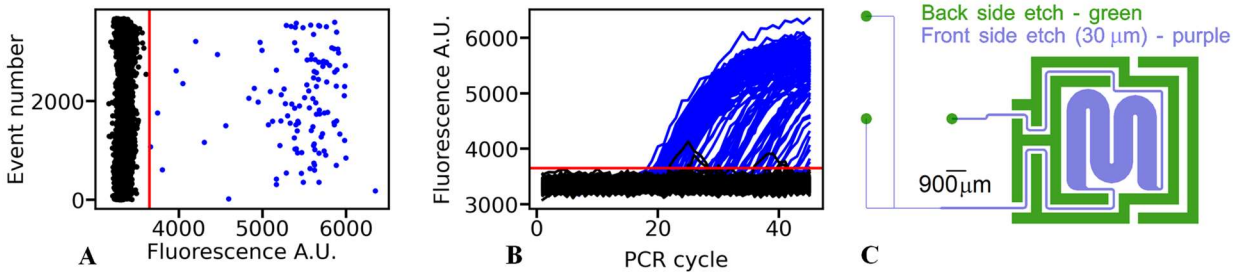

Figure 1: (A): EPF of droplets in function of event number. Intermediate values depicting rain. (B): Single-droplet amplification curves (red line = Ct threshold, • positive droplets, • negative droplets). (C):Mask design of the chip (depth 30µm).

RESU LTS AND DISCUSSION

To evaluate whether the model can distinguish intrinsic from extrinsic rain, digital droplet PCR assays were performed on the fosmid vector and on the purified target amplicon as a control. As expected, the inaccessibility of intact DNA vectors causes a high degree of extrinsic rain, reflected by the large distribution in Ct values of which only 53% can be attributed to intrinsic rain (Figure 2B). In contrast, for the amplicon, which has no target inaccessibility due to its linear form and short size (87 bp), no extrinsic rain was observed and intrinsic rain fully described the Ct distribution (Figure 2A). The number of positive droplets for the fosmid vector corresponded to $8.16*10^2$ copies/µl. However, a large amount of extrinsic rain (47%) suggests template inaccessibility and hence the result was deemed not reliable. This was confirmed by testing the same sample with addition of DMSO (8% final concentration). DMSO relaxes the circular vector, making it more accessible, which should reduce extrinsic rain. Indeed, when DMSO was added the proportion of extrinsic rain decreased to only 7% and the corresponding DNA concentration equaled $6.66*10^3$ copies/µl, an 8x difference (Figure 2C). A melting curve analysis was performed, which verified that this increase in measured DNA concentration was not caused by non-specific amplification. This clearly shows the importance of understanding and reducing extrinsic rain for obtaining a more reliable assay.

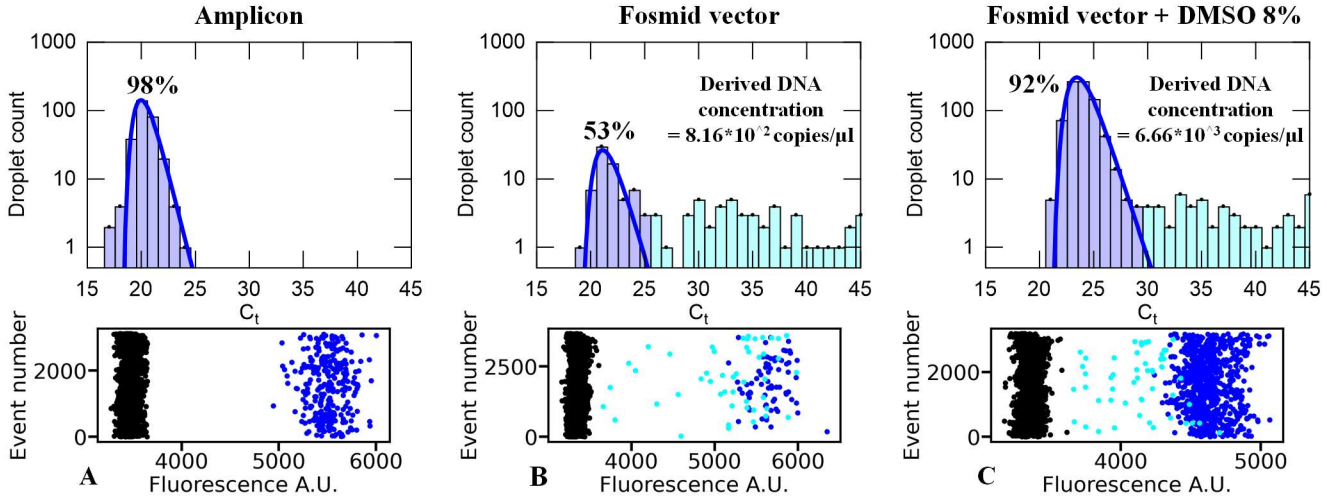

Figure 2: Top: Ct distributions with the corresponding fit (blue line) for the amplicon control (A), fosmid vector (B), and fosmid vector with DMSO (C). The partitions with Ct values that fall under the fit depict the proportion of intrinsic rain, while the partitions with Ct values not following the fit depict the proportion of extrinsic rain. Bottom: EPF values of the partitions in function of the event number, classified according to the type of rain (• intrinsic rain, • extrinsic rain, • negative droplets).

CONCLUSION

We experimentally validated a mathematical model that can distinguish the proportion of partitions that show expected digital PCR behavior from those contributing to extrinsic rain. Distinguishing intrinsic and extrinsic rain provides a measure for the reliability of digital PCR assays, while also facilitating assay optimization. We envision that this model will contribute in the optimization of digital PCR assays, especially for the detection of difficult targets like DNA vectors.

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CONTACT

* P. Berden; phone: +3216281444; pieter.berden@imec.be