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Short communication

Methods for comparing multiple digital PCR experiments

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ABSTRACT

The estimated mean copy per partition (λ) is the essential information from a digital PCR (dPCR) experiment because λ can be used to calculate the target concentration in a sample. However, little information is available how to statistically compare dPCR runs of multiple runs or reduplicates. The comparison of λ values from several runs is a multiple comparison problem, which can be solved using the binary structure of dPCR data. We propose and evaluate two novel methods based on Generalized Linear Models (GLM) and Multiple Ratio Tests (MRT) for comparison of digital PCR experiments. We enriched our MRT framework with computation of simultaneous confidence intervals suitable for comparing multiple dPCR runs. The evaluation of both statistical methods support that MRT is faster and more robust for dPCR experiments performed in large scale. Our theoretical results were confirmed by the analysis of dPCR measurements of dilution series.

Both methods were implemented in the *dpcR* package (v. 0.2) for the open source **R** statistical computing environment.

Introduction

Digital PCR (dPCR) is a PCR-based method, which enables a precise quantification of nucleic acids. The conventional PCR performs single reaction per one sample. However, in the case of dPCR the sample is separated into a large number of partitions, in which the reaction is carried out individually (clonal amplification). The partitioning allows to assess the number of amplified template molecules by detection of their presence (positive call) or absence (negative call) in particular partitions [1,2]. Since the output of these results is binary, we do not know if the positive partition contains one or more template molecules. The Poisson transformation is required to compute the average number of template molecules per partition, expressed by λ:

$$\lambda = -\log\left(1 - \frac{k}{n}\right) \quad (1)$$

where k is number of positive partitions and n is number of negative partitions.

Thanks to that, it is possible to measure precisely concentrations of nucleic acids with high sensitivity and reliability. Therefore, dPCR found common applications in amplification of DNA samples for next-generation sequencing and detection of variation in genomic sequences, e.g. point mutations and repeats [1].

In contrast to the conventional PCR, in which the number of amplification cycles ideally is proportional to the initial copy number, dPCR does not depend on the cycle number to determine the initial amount of nucleic acids in the sample. In particular, the quantitative real-time PCR is known to be demanding regarding preprocessing, quantification cycle determination and multi-plate measurements [3–6]. The dPCR methodology eliminates the dependence on the exponential shape of data to estimate the concentration of target nucleic acids and enables their absolute quantification. Therefore, this method does not need calibration curves and may even be less susceptible to inhibitors. The amplification chemistry of absolute quantification in the dPCR is orchestrated by well established methods such as analogue PCR or isothermal amplification [7,2,8–10].

Precision, sensitivity, dynamic range, number of partitions and their volume are important parameters in a dPCR system [11]. Moreover, technical replicates are affected by different intrinsic and extrinsic influences increasing the variation of obtained results. This variation needs to be assessed to make a valid statement about

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the assay performance. As all diagnostic methods, the dPCR requires tools to check consistency of obtained results. There is a growing need for statistical methods for the analysis and design of experiments using digital PCR experiments.

Previously, two methods to compute the λ value and its uncertainty were described. Dube's approach uses confidence intervals [12], whereas Bhat's method is based on the uncertainty [13]. The latter is not a confidence interval in the statistical sense, but nevertheless can be employed to compute probability coverage of the estimated λ value. The Dube's method computes binomial confidence intervals for proportion k/n using the method of normal approximation. Briefly, the binomial distribution of positive counts with the parameters $p = k/n$ and n trials is approximated by a normal distribution. Both Bhat's and Dube's methodologies do not address multiple comparisons of runs, which is a common task during the design and analysis of dPCR experiments.

Here, we propose two approaches for the comparison of multiple dPCR experiments. Both are able to simultaneously compare the λ values of multiple runs. One of them is based on Generalized Linear Models and the second one is the uniformly most powerful ratio test combined with multiple testing correction. Our findings were implemented in the **R** statistical computing environment [14], which has numerous functionalities devoted to analysis of dPCR and qPCR reactions [15].

Methods

Generalized Linear Models – GLM

Generalized Linear Models (GLM) are linear models for data in which the response variable may have a non-normal distribution (e.g. binomial distribution of positive partitions in the case of dPCR experiments). We employ a simplistic model reflecting the relationships between variables in dPCR results, given by formula:

$$\log Y = \beta^T X \tag{2}$$

where Y are counts of positive partitions, X are experiments names (categorical data) and β are coefficients for every run. Since the binomially distributed response is explained by the linear combination of parameters (in our specific case experiment names) we call such model binomial regression as described in detail elsewhere [16]. Briefly, we employed the logarithm function (function that limits values of response) and the estimated means of copies per partitions by calculating $\hat{\lambda} = \exp \beta$. Importantly, our GLM employs the quasibinomial model, which describes the binomial distribution with excessive zeros. That means that number of zeros may surpass its value predicted by the binomial distribution [17].

The GLM model used in this analysis and represented by Eq. (2) can be refined by adding further effects, such as the technical replication. This may decrease the variance within replicate experiments.

Next the differences between estimated coefficients are evaluated pairwise [18,19]. The resulting p-values require no posterior correction because the familywise error is controlled. This approach is a single-step procedure, because the decision (rejection or acceptance of null hypothesis) is not based on the decision of another hypothesis. As implied by the name, all tests are made in the single step, independently and simultaneously.

Multiple testing

The $\hat{\lambda}$ from two or more dPCR experiments may be pairwise compared using the uniformly most powerful (UMP) ratio test. Uniformly most powerful tests have the highest statistical power (probability that the test correctly rejects the false null hypothesis H_0) for all tests with the same α. The p-values are computed using the TST (twice the smaller tail) method [20,21]. Similarly to the GLM method, this is a single-step approach. However, to control the familywise error rate the pairwise comparison requires an appropriate adjustment of p-values, as the Benjamini–Hochberg correction [22]. The UMP ratio test has the following null-hypothesis:

$$H_0 : \frac{\lambda_1}{\lambda_2} = 1 \tag{3}$$

where λ_1 and λ_2 are mean numbers of template molecules per partition in two experiments. It is also possible to employ other tests (e.g., proportion test) designed to determine the probabilities of having positive partitions [23].

Instead of relying on confidence intervals (CI) computed by the UMP test, we used CIs calculated by the Wilson method. It was

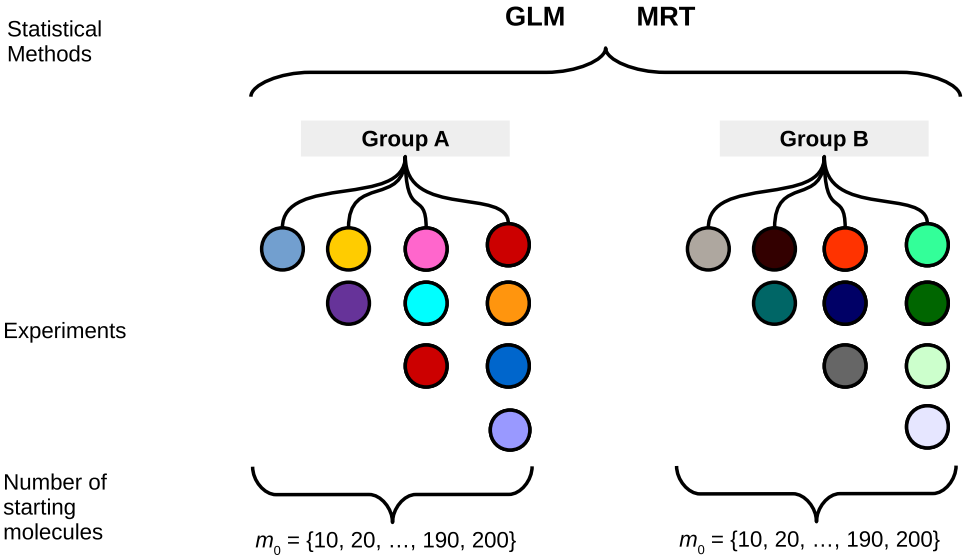

Figure 1. Scheme of the *in silico* dPCR for two approaches based on Generalized Linear Models (GLM) and Multiple Ratio Test (MRT). The groups (A and B) contain one to four runs (coloured circles). Each run within one group is generated using the same number of molecules in 1000 partitions. The number of molecules (m_0) range from 10 to 200. Equally-sized groups were finally compared by GLM and MRT methods. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)