Research paper

Designing and interpretation of digital assays: Concentration of target in the sample and in the source of sample

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ABSTRACT

We explain how to design classic digital assays, comprising identical partitions, in order to obtain the required precision of the estimate within a defined range of concentrations. The design, including the number and volume of partitions, depends significantly on whether the assay is to assess the concentration of the target analyte *in the sample* or *in the source of the sample* (e.g. a patient body) with a given precision. We also show how to translate the result referring to the concentration in the sample into the concentration in the source of the sample, including the significant change in the breath of the confidence intervals.

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1. Introduction

Here we discuss explicitly the significant differences in the precision of estimates provided by digital assays, depending on whether the assay addresses the concentration of the target in the sample, or in the clinical source of the sample (e.g. a human body). The two estimates enforce different statistical description of the signals in the assay and require a different design of the experimental protocol to provide the estimates with required quality.

DNA-, RNA- and immuno-diagnostics require assays that provide precise, quantitative answers and that address wide dynamic ranges of detectable concentrations. Digital assays [[1–6]; the history of digital PCR is described in a review article by Morley] introduced absolute and precise quantization. The performance of the digital analytical techniques is typically parameterized by the dynamic range of concentration within which a given standard deviation (or variance) of the estimate is guaranteed. It is not commonly appreciated that both the standard deviation of the estimate and the dynamic range of the digital assessment depend critically on the method of calculation and of the interpretation

of the result. The proper understanding of the results generated by digital assays is also important in optimization [7]. Here we explain the full analytical procedure, the sources of errors and the proper understanding of the estimate of concentration of the target marker.

Standard analytical techniques use a single 'analogue' measurement on the sample to estimate the concentration of the analyte via a comparison to a calibration curve (of e.g. absorbance of light by reference samples). Digital analytical assays, first proposed by McCrady in 1915 for quantification of bacteria and later developed in 1990s for PCR by Sykes [5], and Vogelstein and Kinzler [2], split the sample into a large number of partitions, each later separately amplified for signal. The initial concentration of the target analyte is estimated from the fraction of positive end-point signals. Positive signals typically reflect the presence of at least one molecule of the target analyte. (Fig. 1).

Digital assays have a number of attractive characteristics. Most importantly, they provide absolute estimate of concentration, increasing accuracy and alleviating the need to use reference standards. Digital methods can also be highly sensitive (from 1 molecule/assay) and highly precise. These features prompt the widening use of digital techniques, especially in oncology and in the detection of very minute quantities of diagnostic markers but also progressively as standard quantitative assays for DNA markers [8]. Moreover, digital techniques, and therefore the methodology we presented here, may find use in quantitative identification of viral and microbial pathogens in physiological samples in assays pre-
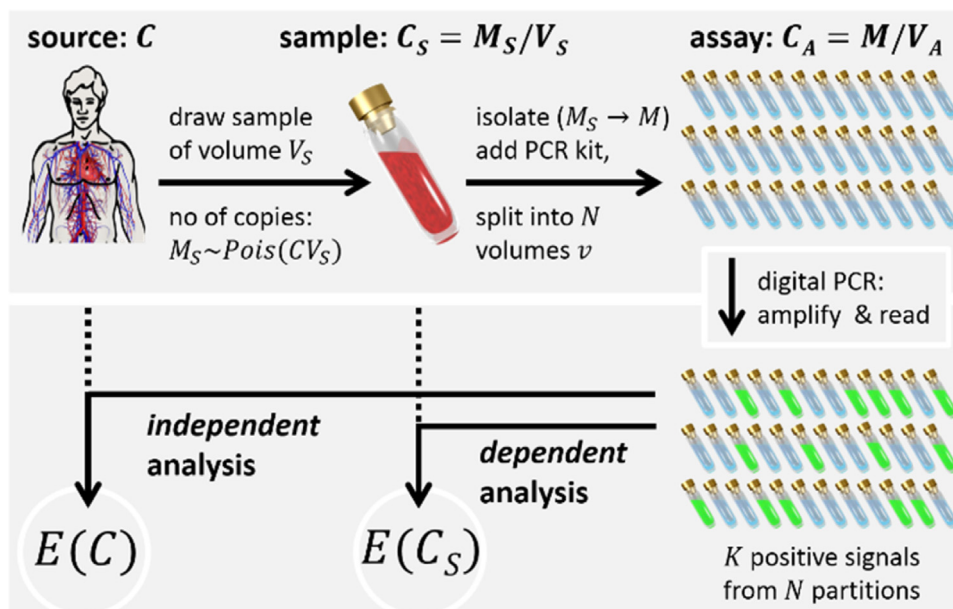

Fig. 1. The complete protocol for a digital assay. The upper shaded area shows the medical and analytical procedure. The source (e.g. a human body) presents an unknown concentration C of the analyte. A small sample of volume V_S drawn from the source contains M_S molecules. The concentration $C_S = M_S/V_S$ of target in the sample is — especially for small V_S and C — a *stochastic variable* of C. The sample then undergoes a procedure of isolation of nucleic acids and the elute (containing $M \leq M_S$ molecules) is mixed with reagents for PCR to a final volume of the assay V_A. The PCR-ready mixture is then divided into N partitions, with the molecules of the target randomly distributed between them. Amplification via PCR yields the raw result: K positive signals from N partitions. This result can be then translated via analysis (bottom shaded area) — either within a *'dependent'* scheme to yield $E_D(C_A)$ corresponding to the estimate $E(C_S)$ of concentration in the sample, or, via the *'independent'* analysis to yield $E_I(C_A)$ corresponding to the estimate $E(C)$ of the concentration of target in the original source of sample. Each of these estimates has a different confidence interval. The schematic picture of the human body is based on graphics available at: http://cliparts.co/, and https://commons.wikimedia.org/wiki/ File:Vein_art_near.png.

pared for the point-of-care formats. Looking further, finding new chemically specific chain- and avalanche reactions that could serve for amplification of the presence of small counts of molecules of analyte could become an important direction in the development of new analytical methods in chemistry, biochemistry and medicine [9].

The analytical procedure usually focuses on determining the concentration C of the target analyte in the source (e.q. human body). It begins with drawing a sample of volume V_S that contains a stochastic number M_S of molecules of the target. The number of molecules is a Poisson random variable with average value equal $C \times V_S$. Therefore, $M_S \sim Pois(CV_S) = \frac{\exp(-CV_S) \times (CV_S)^{M_S}}{M_S!}$. The concentration C_S in the sample is thus a stochastic variable of C and of V_S: $C_S = M_S/V_S \sim \frac{1}{V_s} \times Pois(CV_s) = \frac{1}{V_s} \times \frac{\exp(-CV_s) \times (CV_s)^{M_S}}{M_S!}$. The sample is then purified and mixed with the reagents for the PCR assay. This results in a volume of the assay $V_A = V_{elution} + V_{PCR}$. The volume of the assay is then split into N compartments, each of volume v, $Nv = V_A$. After the PCR reaction on all of the individual compartments, we read the raw result, i.e. K positive signals from N partitions. This raw result can be then translated into the estimate of the initial concentration of the analyte in the assay $E(C_A)$ that is provided with a finite relative standard deviation $\sigma(C_A)$, which can also be treated as the precision of the estimate.

As we show below, the analysis can be performed via two schemes. In the first, one assumes that the probability of finding a molecule of target in any partition depends on the placement of target analyte in all other compartments in the assay. This corresponds to the actual situation of drawing the molecules from a finite pool containing only M target molecules. In this context, the finite pool is the volume of an assay. This (*dependent*) scheme can be translated into the estimate of the concentration of target in the sample: $E(C_S) = \alpha E_D(C_A)$, and $\sigma(C_S) = \sigma_D(C_A)$, where α is a numerical factor reflecting the change in the volume between the sample and

the assay and the efficiency of isolation $\eta = M/M_S$: $\alpha = (V_A/V_S)/\eta$. However, this estimate is limited only to the volume of the sample, which is usually very small compared to its source, i.e. human body. Therefore, the precision of the estimate provided by the test, which is typically given by the producer of a dPCR system, might be misleading if the concentration in the human body is of interest.

In the second (*independent*) scheme, the estimate is provided via an analysis based on the superficial assumption that the probability of finding a molecule of target in any particular partition is independent of all the other ones (just as if the material for each partition was drawn directly from large reservoir — a source of the sample, i.e. human body). Via this analysis we can obtain the estimate of the concentration of the target in the

Source: $E(C) = \alpha E_I(C_A)$, and $\sigma(C) = \sigma_I(C_A)$. Therefore, the precision of the estimate, calculated in this approach, provided by the test concerns the concentration in the human body.

Below we show how the two methods of analysis of digital estimates can be conducted, how different are the relative standard deviations σ_D and σ_I, and how to design digital assays for the required precision of assessment of the concentration in the sample or in the source over a required range of concentrations.

2. Results

In a classic digital assay [2,10,11] the sample is equally divided between a number N of identical partitions. All the partitions are then amplified via PCR after which the binary signal is read from each partition. Once the signals are collected, they must be translated into the estimate of the (a priori unknown) concentration of the analyte in the sample, and concentration of the analyte in the source of the sample (e.g. in a human body). (Fig. 2).

Current mathematical procedures used to analyze the outcome of the assay are based either on the Most Probable Number (MPN)
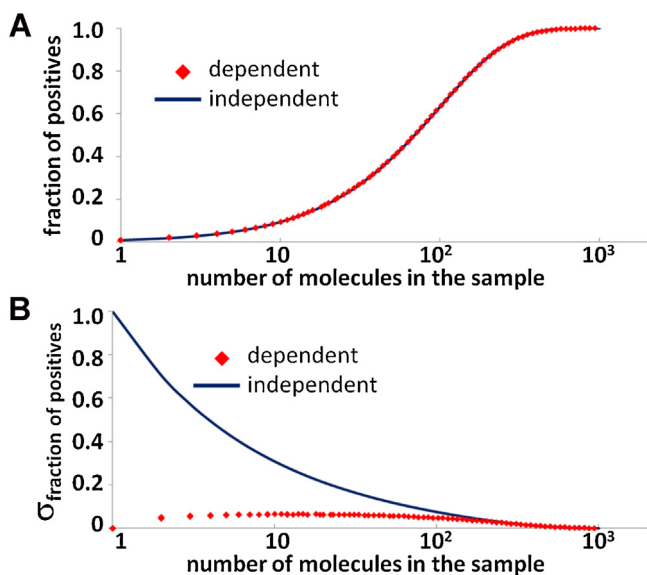
Fig. 2. (a) Average (most probable) response of a classic digital assay to a given concentration in the source (*independent* approach — blue line, calculated analytically) or concentration in the sample (*dependent* approach — red points, each point represents an average from 10,000 MC simulations). The assay comprises $N = 100$ identical partitions. (b) Although the average response of the assay is the same regardless of the statistical formalism, the confidence intervals for the results are. For small concentrations corresponding to a low number of molecules in the sample, the standard deviation of the estimate based on independent analysis reaches 100% of number of signals. In a sharp contrast, the number of positive signals vs. the concentration in the sample has vanishing standard deviation in the limit of one target molecule.

Fig. 3. The difference in the standard deviation of the estimate of the concentration of analyte for the two approaches. Standard deviation $\sigma_I(C_A)$ of the estimate of $E_I(C_A)$ in the *independent* approach (equal to the standard deviation $\sigma(C)$ of the estimate $E(C)$ of concentration in the source, blue points) is almost always significantly higher than the standard deviation $\sigma_D(C_A)$ of the estimate of $E_D(C_A)$ (equal to the standard deviation $\sigma(C_S)$ of the estimate $E(C_S)$ of concentration in the sample, red points).

algorithm [12–18] or Bayes' formalism [8,19]. Both methods are applicable either for the assessment of the concentration in the source C, or concentration in the sample C_S (or alternatively, the number of molecules in the sample M_S). The MPN method estimates the initial concentration of the analyte in the sample as a concentration that yields the highest probability of obtaining the particular recorded outcome of the assay (i.e. ratio of positive and negative partitions). Bayes' formalism inverts the concentration dependent probability of the recorded result to yield the probability distribution of a concentration having caused the result. Such obtained probability distribution provides the estimate of concentration together with its standard deviation. Here we focus on the Bayes' formalism as it explicitly uses *all* the information contained in the outcome of the assay. Still, the conclusions are valid also for the MPN formalism.

The most important distinction reflecting the difference between calculating the estimate of the concentration of the analyte in the sample and in the source is reflected in the statistical description of the individual partitions in the assay. As we show below, digital assays can be designed and analyzed in two different ways to provide either the estimate of the concentration of the analyte in the sample, or in the source of the sample.

The most commonly used approach (that we call 'dependent random variables') focuses on the number of particles in the sample. The number of particles M available for distribution between compartments in the digital assay is fixed. The act of splitting the volume of the assay into partitions only randomizes the placement of these particles between compartments. Thus, the occupancies of the partitions by particles of analyte are *not* independent of each other. For example, if there was only one molecule ($C_A = 1/V_A$) in the assay divided between hundred compartments, and one compartment already had the particle, for all other compartments the probability of finding a particl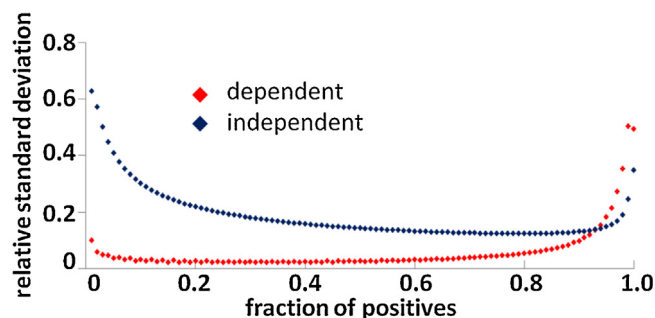e is exactly zero. Generally, the use of dependent variables in the analysis allows one to estimate the concentration of the analyte in the sample, and *not* in the source of the sample.

In the second approach one is interested in the concentration of the analyte in the source of the sample. Even though the procedure is the same (i.e. the partitions are drawn from the sample), one assumes that each of the compartments is as being drawn independently from the source. Then, the probability p of finding particles in a compartment of volume v is independent of the presence of particles in any of the other compartments and is only a function of C_A: $p = 1 - e^{-C_A v}$. This analysis is superficial. For example, it does not exclude an impossible situation in which for $C_A = 1/V_A$, two or more compartments yield a positive signal. Nonetheless this scheme of analysis provides correct estimates, yet with higher standard deviations corresponding to the concentration of particles in the source C and not just in the sample C_S.

The difference between these two approaches to the analysis of the result (i.e. of the number of positive signals from an assay) can be best observed in the standard deviations of the estimates of concentration. In the 'dependent' scheme, the probability of observing exactly K positive compartments while there are $M = \alpha \times C_S \times V_S = C_A \times V_A$ molecules in the assay is

$$p(K|M,N) = \frac{\binom{N}{K} \sum_{i=0}^{K-1} \left[(-1)^i \binom{K}{K-i} (K-i)^M \right]}{N^M} .$$

In contrast, in the 'independent' scheme, the probability of observing exactly K positive compartments given the concentration of the molecules in the assay is C_A is:

$$p(K|C_A,N) = \binom{N}{K} (1 - e^{-C_A v})^K (e^{-C_A v})^{N-K} .$$

As a result, the 'dependent' algorithm yields a much smaller standard deviation of the estimate of $E_D(C_A)$ and $E(C_S)$, than the standard deviation of the estimate of $E_I(C_A)$ and $E(C)$ provided by the 'independent' algorithm (Fig. 3). In other words, and quite intuitively, the estimate of the concentration of the target in the sample can be given with tighter confidence intervals than the estimate of the concentration in the source of the sample.

There is a way to translate between the two estimates — i.e. once having the estimate for the concentration of particles in the sample, and knowing the volume of the sample, one can calculate the estimate of the concentration of particles in the body (with a higher standard deviation).

The translation of the results from the *dependent* approach i.e. the distribution of concentration in the sample into the distribution
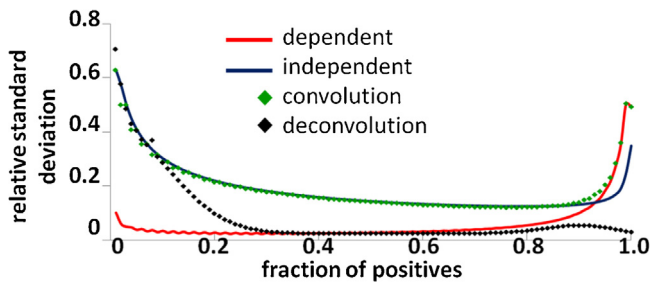
Fig. 4. Convolution as way to translate *dependent* (i.e. relating to the concentration in the sample) to *independent* (concentration in the source of sample) analysis.

of concentration in the source (*independent* approach) is done by the mathematical procedure of convolution. In essence, the probability that one state of the assay (fraction of positive compartments) was caused by a given concentration C (in the source), must take into account the conditional probability that one observed K signals provided there were M molecules in the sample *and* the probability that there were M molecules in the sample provided the concentration in the source was C. Formally, the probability of registering K signals provided concentration in the source was C is equal to the following sum: $p(K|C) = \sum_{M=0}^{\infty} [p(K|M) \times p(M|C)]$, where $p(K|M)$ is the outcome from *dependent* analysis, and $p(M|C)$ is the probability that at concentration C there were M particles in the sample, given by the Poisson distribution with expected value $\lambda = CV_S$. It can be easily shown, that the outcome of this procedure is equal to the outcome of *independent* analysis (Fig. 4, green points and blue line, respectively). The assessment of the concentration directly via *independent* analysis is less complicated mathematically than via *dependent* analysis and convolution.

Mathematically, the process of convolution (marked with the symbol $*$) employs the integral transformation of two functions, one of which is shifted. By definition, it is given by the following:

$$f(x) = (g \times h)(x) = \int_{-\infty}^{\infty} g(\tau) h(x - \tau) d\tau .$$

In our case, we can replace f with $p(K|C)$, g with $p(K|M)$ and h with Poisson distribution $p(M|C) = Pois(M, CV_S)$. Also, the discrete, non-negative integer character of number of molecules in the sample M has to be taken into account. Thus, the convolution for the case of digital assays can be given as:

$$p(K|C) = \sum_{\tau=0}^{\infty} p(\tau|C) p(K|\tau) .$$

The symbol of integration was replaced by summation due to integer character of the number of molecules M and τ, and the limits of integral are changed because this number cannot be negative.

There is also a possibility to translate the concentration in the source (*independent* analysis) into the concentration in the sample (*dependent*). Provided the distribution $p(M|C)$ (i.e. probability that there are M molecules in the sample provided concentration in the source is C) is known, one can recalculate the distribution $p(K|C)$ to $p(K|M)$ with the procedure of deconvolution using $p(M|C)$ [ESI]. However, this translation does not always reproduce the distribution $p(K|M)$ perfectly, especially for very small (close to zero) and very large (close to unity) fractions of positives and therefore it is advisable to use *dependent* analysis if the concentration in the sample is wanted (Fig. 4, red line).
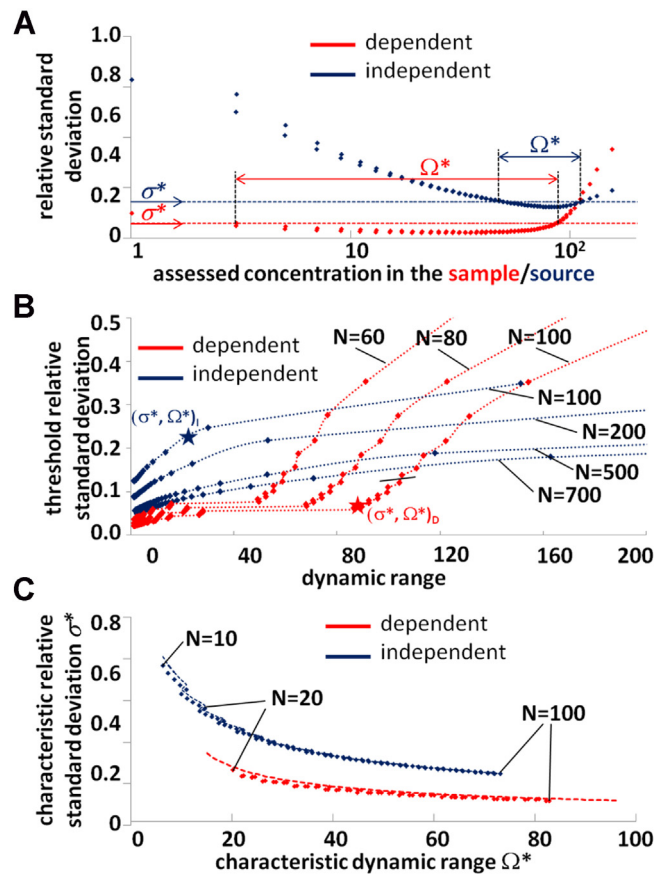

Fig. 5. (a) Standard deviation of the estimate of concentration in the source based on 'independent' approach (blue dots) and the concentration in the sample based on 'dependent' approach (red dots). Dotted lines show the threshold values of the relative standard deviation of the estimate ($\sigma_{threshold}$) for which one can univocally determine the dynamic range $\Omega = C^+/C^-$, where C^- is the minimum and C^+ is the maximum concentration. (b) Then, for any assay, it is possible to draw a function $\Omega(\sigma_{threshold})$. The deflection point (red and blue stars) of this function defines (Ω^*, σ^*) that corresponds to an objective, reasonable trade-off between the breadth of dynamic range and tightness of standard deviation of the estimate. (c) Dependence of $\Omega^*(\sigma^*)$ shows how these most important characteristics of a digital assay are bound, as a function of the number N of compartments in the assay. The dashed lines represent best fits to the numerical results (blue represents for $\Omega^*(\sigma^*) = 0.9925 \times (\sigma^*)^{-2.065}$ and red represents $\Omega^*(\sigma^*) = 0.1697 \times (\sigma^*)^{-2.214}$).

The proper understanding of the response of an assay, i.e. the functions $p(K|C)$ and $p(K|M)$ allows to correctly interpret the assessment of the concentration of the analyte. In this analysis, we determine the precision of the assessment as the relative standard deviation of the estimate of initial concentration/number of molecules of the analyte. We use the calculated standard deviations from the dependent and independent assays to derive equations that allow to design the digital assays that address the requested dynamic range of concentrations with the required standard deviation of the estimate.

We start with introducing an objective definition (or measure) of the dynamic range of the assay. Fig. 5a shows that although the standard deviation of the estimates calculated via the dependent and independent algorithms are very different, they share the property of having a minimum, a relative plateau around the minimum, and that they increase sharply at the low and at high concentrations of the analyte. In general we would like the assay to provide an estimate with a standard deviation not larger than σ^* in a dynamic range $\Omega^* = C^+/C^-$ (where C^- and C^+ are the lower and upper limit of the dynamic range, respectively). As can be seen

in Fig. 5b, broadening the range enforces lessening the requirement on precision and vice versa. We propose a reasonable and objective definition of the dynamic range by the deflection point $d^2\Omega/d\sigma^2 = 0 \rightarrow (\Omega^*, \sigma^*)$. For $\sigma < \sigma^*$, loosening the requirements on precision (i.e. increase of σ) rapidly increases Ω. Above this threshold a the gain in breath of dynamic range does not justify the decrease of precision of the assay. Although the choice of the deflection points as bounds of the dynamic range is arbitrary, other definitions, based e.g. on Shannon entropy or other measures of information will yield similar results.

The definition allows us to calculate Ω^* and σ^* as a function of the size of the assay N (Fig. 5c). As indicated earlier, the estimates of the concentration in the sample (*dependent* analysis) almost always (i.e. at small and intermediate concentrations) yield more precise results (Fig. 5a). This translates into the fact, that reaching the same precision requires much larger assays for estimating the concentration in the source (e.g. a human body).

As shown before, the standard deviation and dynamic range of the classic digital assay are bounded together (Fig. 5c) and can only be changed by tuning the number of compartments in the assay. Indeed, after calculating the performance of several digital assays via Monte Carlo simulations, we have found that the standard deviation σ of the estimate is provided by an assay comprising at least N compartments only if they follow a simple conditional formula $N \geq a\sigma^{-b}$ (Fig. 5). This formula was determined from a close algebraic fit to the numerical results.

This behavior of digital assays is qualitatively the same in both schemes (*dependent* and *independent* analysis). However, they differ quantitatively because the assay responses in the independent scheme show more fluctuations. Still, the relationship of the characteristic parameters (σ^*, Ω^*) can be drawn from best fits to the results. For independent scheme, one gets $\Omega^*(\sigma^*) = 0.9925 \times (\sigma^*)^{-2.065}$, while for dependent analysis it is equal to: $\Omega^*(\sigma^*) = 0.1697 \times (\sigma^*)^{-2.214}$.

Knowing the relation between the dynamic range and the standard deviation of the estimate for a classic digital assay containing identical partitions of the sample allows us to derive an explicit prescription for the number of compartments N required to provide an assessment with the required precision over the required range of concentrations.

The 'design' equations listed below use the required dynamic range $\Omega = C^+/C^-$ and the relative standard deviation of the estimate σ as an input. For the *independent* scheme the values of C^- and C^+ can be chosen freely, with the only obvious condition that $C^- < C^+$:

$$N = max(N_\Omega \times N_\sigma)$$

$$N_\Omega = 1.447 \times \Omega^{0.985}$$

$$N_\sigma = 1.436 \times \sigma^{-2.033}.$$

For *dependent* scheme, we assumed that C^- is equal to one molecule in the assay (i.e. $C^- = 1/V_A$). C^+ corresponds to the maximum number of molecules M_{max} expected to be found in the volume of the assay. For the dependent analysis: $N = max(N_\Omega; N_\sigma)$

$$N_\Omega = 2.154 \times \Omega^{0.857}$$

$$N_\sigma = 0.454 \times \sigma^{-1.912}.$$

N_Ω and N_σ are the minimum numbers of compartments needed to provide assessment with the required dynamic range or the required standard deviation respectively. The assay should comprise at least the higher number (of the two) of compartments in order to satisfy both the requirement on precision and on the breath

of the dynamic range. However, in some dPCR systems, the number of compartments cannot be hard-defied. In this case, the design equations can be inverted to give the value of the relative standard deviation of the estimate and the dynamic range the system provides [ESI].

Surprisingly, in the i*ndependent* analysis, the assay reaches its optimum mode (i.e. reaches the plateau of standard deviation of the estimate, shown in Fig. 5a) where about 20% of its compartments are positive [ESI], which provides the condition for the volume of a single compartment of the assay. Knowing C^- and N, the volume v of each of the compartments should be: $v = \frac{\ln(1.25)}{C^-}$, yielding the total volume of the assay $V_A = Nv = N \times \ln(1.25)/C^-$. Please note that by the volume of the assay V_A we understand the volume of the PCR mix of the eluate and reagents, ready to be split into compartments for PCR amplification. The volume of the actual sample V_S drawn from the source needs to be calculated for each particular purification and isolation protocol and the composition of the PCR kits.

As described above, the analysis of the result needs to be done through numerical procedures based either on the Most Probable Number method and Bayes' formalism. Providing explicit analytical equations for translating the raw result (K positive signals from N compartments) should in general be possible, yet has not yet been demonstrated. This is because the mathematics behind especially the dependent scheme is quite involved. Here we can offer only the formula for the estimate of initial concentration in the independent scheme, based on Most Probable Number method [ESI]:

$$E_I(C_A) = v^{-1} \ln \frac{N}{N-K} \qquad ,$$

where v is the volume of a single compartment, N is the number of compartments in the assay and K is the number of compartments that yielded positive signal.

We verified our methodology numerically using canonical and grand canonical Monte Carlo simulations. The digital assays were designed using the abovementioned expressions to deliver assessment in the dynamic range (i) $\Omega_1 = 10^2$, (ii) $\Omega_2 = 5 \times 10^2$ and (iii) $\Omega_3 = 10^3$, which required $N_1 = 135$, $N_2 = 660$ and $N_3 = 1305$ compartments in *independent* scheme and $N_1 = 112$, $N_2 = 443$ and $N_3 = 802$ compartments in *dependent* scheme. The assays described satisfy the given requirements (Fig. 6).

3. Discussion

We have discussed explicitly two possible ways of the interpretation of the result of a digital PCR assay that lead to the assessment of the concentration of the analyte either in the sample or in the source of the sample. The differences in the mathematical procedures, including the use of dependent and independent random variables, reflect the qualitative difference between these physical quantities (i.e. the number of molecules, or concentration of the analyte in the sample, and the concentration in the source).

We have shown how the precision and dynamic range of digital assays with respect to the estimates of concentration in the sample and in the source depend on each other and on the number of partitions in the assay.

These characteristics allowed us to provide explicit formulas for designing single-volume digital assays that provide assessment of the initial concentration of the analyte within the requested dynamic range and with required standard deviation of the estimate, both in *dependent* and *independent* scheme. The mathematical algorithm presented is simple and could be used to tailor digital tests for various applications.
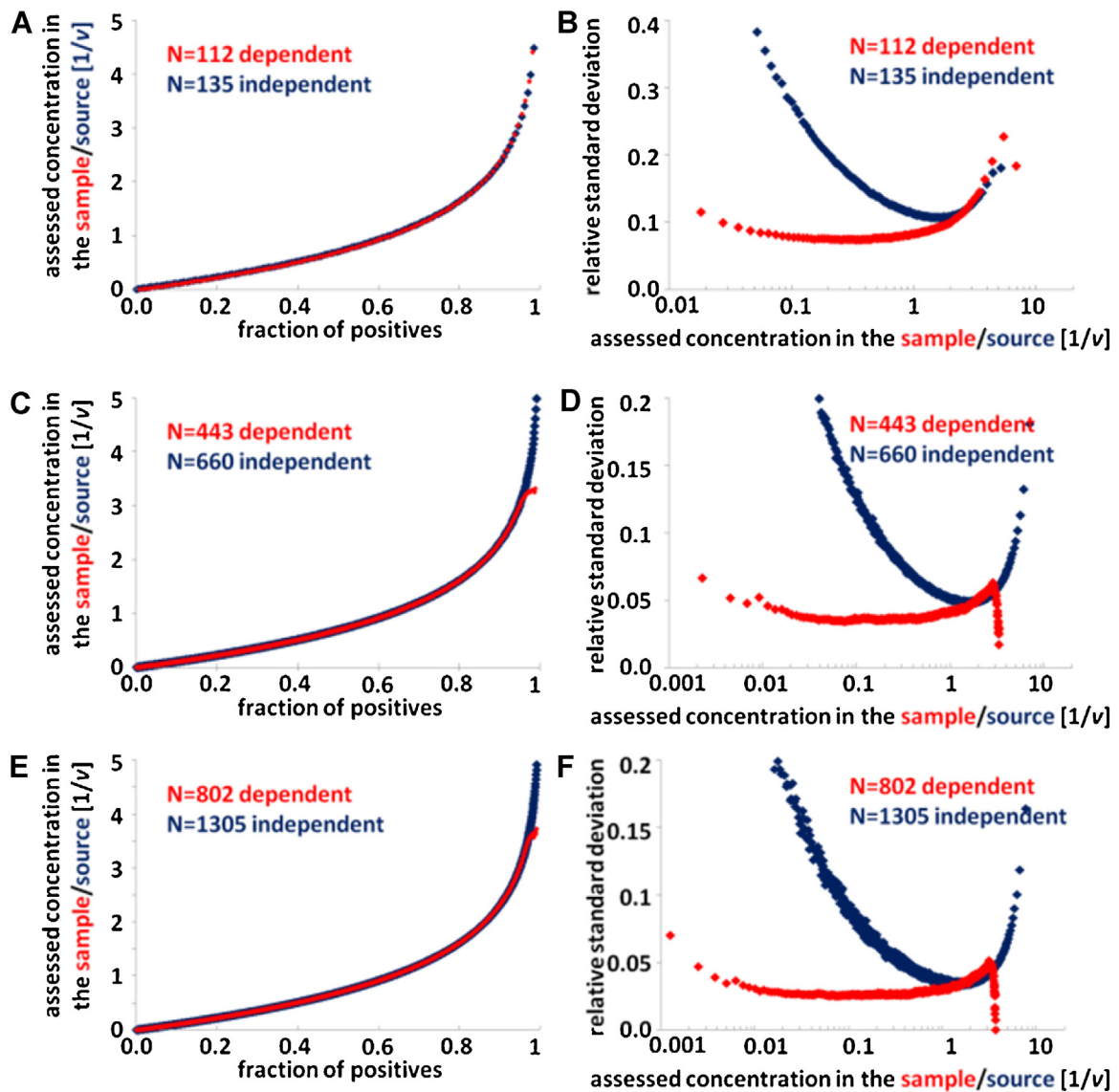
Fig. 6. Comparison of the performance of assays working in *dependent* (red) and *independent* scheme (blue). The test was performed by means of Monte Carlo simulations. The assays are paired by the dynamic range (a,b show assays that cover dynamic range $\Omega = 10^2$, c,d show assays covering $\Omega = 5 \cdot 10^2$, and e,f show assays covering $\Omega = 10^3$). (a–e) The estimate of the concentration in the sample (equal to the number of molecules in the sample — *dependent* scheme) or in the source (*independent* scheme) as a function of fraction of positive compartments. The concentration is given in units $[1/v]$. (b–d) The relative standard deviation of the estimated concentration. In each case, the assay working in *independent* scheme assesses concentration with higher standard deviation, which results the randomness of the actual of number molecules found in the sample for a given concentration in the source.

Author contributions

PRD and PG developed the algorithms, analyzed data and wrote the manuscript.

Conflict of interest

PRD and PG declare competing financial interest in possession of equity in Curiosity Diagnostics Sp. z o.o.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bdq.2016.04.002.

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[18] It is worth mentioning that Dorazio and Hunter also provide the design of an assay for specific applications, however, their approach is less general and do not consider dependent scheme. Majdumar, Wessel and Marks also focus on independent scheme, and hard-fix the precision at the value of 10%. Corbisier, et al. present a thorough systematic study of one commercially available dPCR system and use log-log model to calculate the initial concentration.
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