Statistical Considerations in Detecting Circulating Tumor DNA Using Digital PCR

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Abstract
Circulating tumor DNA (ctDNA) provides a non-invasive and effective method for the detection, monitoring and treatment of cancer. It has been suggested to be a promising biomarker for patient disease prognosis and response to treatment. Recent developments in digital PCR (dPCR) make it a sensitive technology for ctDNA detection. Under assumption of Poisson distribution, the expected count of ctDNA in a patient blood sample was estimated from dPCR data. A limit of detection (LoD) for the assay was developed to allow an accurate detection of ctDNA in a sample. The proposed LoD was consistent with the data generated from several serial dilution experiments.

Key Words: dPCR, Genetics, Detection limit

1. Introduction

Polymerase chain reaction (PCR) is a widely used technology for the quantification of nucleic acids such as DNA. Digital PCR (dPCR) was first proposed in the 1990s (Sykes et al 1992). Compared with the conventional PCR, dPCR splits the sample into multiple chambers and each has its own reaction. To a certain degree dPCR generates many technical replicates in a single reaction, thus provides a more precise quantification of the nucleic acids. The florescent signal from each chamber was scanned with a binary readout – indicating whether the target DNA is present or not. This is where the "digital" aspect of the technology comes.

Microfluidic chamber based digital PCR (cdPCR) from Fluidigm and droplet digital PCR (ddPCR) from Bio-Rad are two commonly used platforms. They differ in their methods of partitioning samples. The ddPCR technology from Bio-Rad Laboratories partitions a sample into 20000 droplets. In the estimation of the number of nucleic acid copies, both technologies assume a Poisson distribution for the copy number. For this manuscript, we will focus on the ddPCR platform, though our results can be extended to the other platform.

Cancer is a genetic disease and often caused by alteration of DNA. In cancer research, constantly monitoring tumor progression and response to treatment at molecular level is essential to provide personalized treatment. In solid tumor, it typically requires biopsy to obtain samples for genotyping. This procedure is invasive and cumbersome. Digital PCR provides a non-invasive alternative by measuring the circulating DNA that tumors shed in blood stream. It can provide a real time monitoring of tumor progression.

In the ddPCR technology, the copy number of a target DNA is calculated from the detected positive or negative sample partitions (droplets or chambers). For clinical utility, it is often desired to have a dichotomous readout – either present or absent of the target

DNA. A threshold or detection limit is required to provide such a binary outcome. In this manuscript, we define a limit of detection (LoD) for ddPCR assay from statistical consideration, for both single and replicated sample measurement.

2. Statistical Method

2.1 Droplet Digital PCR

ddPCR technology uses a combination of microfluidics and proprietary surfactant chemistries to partition input DNA into thousands of water-in-oil droplets that are subsequently PCR amplified and fluorescently labeled and scanned (Hindson et al. 2011). Compared with early conventional dPCR, ddPCR provides a simpler and more partitions of a sample thus can better quantify the target DNA.

Figure 1. Illustration of copy number distribution in droplets (a) and a scanned readout of droplets (b). (Bio-Rad publication 6407)

The Bio-Rad ddPCR system used in our experiments divides a 20 ul sample into 20000 droplets. DNA copies randomly distribute in the partitioning droplets (Figure 1a). The number of copies in each droplet is typically assumed to follow a Poisson distribution (Dube et al 2008). After PCR amplification, droplets with fluorescent labeled target DNA light up and are detected while passing through an image scanner (Figure 1b). The direct measurement of the system is the count of positive (light-up) droplets and the count of negative droplets.

2.2 Statistical Distribution

The number of target DNA copies (X) in a droplet is assumed to have a Poisson distribution

$$P(x = k) = {\lambda^k e^{-\lambda}}/{k!} \quad (1)$$

with λ= the expected number of copies in the droplet.

The probability of a negative droplet or a droplet has zero copy of the target DNA is $P(x = 0) = e^{-\lambda}$. The probability of a positive droplet is

$$\pi = 1 - e^{-\lambda}. \quad (2)$$

Let the total number of droplets in a sample be N (~20000) and the number of positive droplets be T. Since π can be estimated by $\hat{\pi} = T/N$, the expected copy number in a droplet can be estimated

$$\hat{\lambda} = -\ln(1 - \hat{\pi}) = -\ln(1 - \frac{T}{N}) \quad (3)$$

Let *Y* be a random variable of the number of positive droplets in a sample, and it follows a binomial distribution $Y \sim \text{Binom}(\pi)$. When the target DNA is very rare ($\lambda << 1$), $\pi \approx \lambda$.

The total copy number ($X=\sum x_i$) of the target DNA in a sample also follows a Poisson distribution $X \sim \text{Poi}(\lambda_s)$, with the expected total copy number $\lambda_s = N\lambda$ and it can be estimated by $\hat{\lambda}_s = -N \cdot \ln(1-T/N)$. Again when the target DNA is very rare ($T << N$), $\hat{\lambda}_s \approx T$.

2.3 Limit of Detection

The limit of detection of an instrument is typically defined by measuring negative samples with the instrument, and three standard deviations above the mean of the negative samples is commonly used as the LoD. This definition does not work well with DNA copy number as the data are discrete in nature and do not follow a normal distribution, particularly at low count level. Furthermore, the number of negative control samples is often small, and it is almost impossible to have a good estimate of the standard deviation. From the data we had, the LoD in this definition varied tremendously from experiment to experiment.

Another question associated with LoD definition is how to define it when there are technical replicates of the same sample. Should we define it using the average of the replicates and the corresponding standard error in negative samples? It is possible a sample is called as negative based on the average copy number but one replicate is positive on the individual test.

In ddPCR, the parameter of interest is the expected total copy number of target DNA in a sample λ_s which is the Poisson parameter for the observed total copy number. We know the confidence interval of λ_s can be expressed from a chi-square distribution (Johnson et al 1993)

$$\chi^2(\alpha/2, 2x)/2 < \lambda_s < \chi^2(1-\alpha/2, 2x+2)/2 \quad (4)$$

where *x* is the observed total copy number.

Table 1: 95% confidence interval for λ_s

x	*Lower limit*	*Upper limit*
0	0	3.689
1	0.025	5.571
2	0.242	7.224
3	0.619	8.766
4	1.090	10.24

Table 1 provides the 95% confidence limits for λ_s when observed 0-4 copies of target DNA.

If we define an assay to be positive as "have at least one copy of the target DNA", we can set the detection limit as the observed copy number that provides a lower limit of confidence interval for λ_s to be at least one

$$\chi^2(\alpha/2\,,2x)/2 \geq 1 \tag{5}$$

Solving this inequality for *x*, we have a LoD of 3.82, round up to 4, which provides a lower 95% CI of 1.09 from Table 1. It says if we observe at least 4 copies of the target DNA in a sample, we have 95% confidence that there is at least one copy of DNA in the sample.

Alternatively, we can define the LoD as the upper confidence limit of λ_s when no copy of the target DNA is detected ($x = 0$):

$$LoD = \chi^2(1-\alpha/2\,,2)/2 = 3.69 \tag{6}$$

Again it rounds up to 4. It says if we observe at least 4 copies of the target DNA, it is unlikely ($p \leq 0.05$) that it is false positive.

2.4 Sample with Replicate

When a sample has some, say 3, technical replicates, how can we call the sample positive/negative for the target DNA? We can consider three definitions for the threshold.

Def1: At least one copy of target DNA in all the replicates combined, $\Sigma X_i \geq 1$.
Def2: At least one copy of target DNA in any replicates, $\max(X_i) \geq 1$.
Def3: The average copy number is at least 1, $\text{ave}(Xi) \geq 1$.

Definition 1 calls sample positive if there is at least one copy of target DNA when pooling all replicates together. We can use $S=\Sigma Xi$ as our test statistic. Sample is positive when we have sufficient confidence that $S \geq 1$. As *X* has a Poisson distribution, *S* will also follow a Poisson distribution. Therefore the LoD for *S* is 4, the same as *X*. That is, if the sum of replicates has 4 copies or more of target DNA, we have a positive detection; namely, we have 95% confidence that there is at least one copy of DNA in all three replicates combined.

Definition 2 calls sample positive if there is at least one copy of target DNA in any replicates. The test statistic $S_{\max}= max(X_i)$ has a distribution that could be derived from extreme value theory. However, for practical purpose, we can use a Bonferroni adjustment for multiple comparison on the Poisson data, and control α at $0.05/k$ level with k = the number of replicates, that is

$$\chi^2(\alpha/2\,k, 2S_{max})/2 \geq 1 \tag{7}$$

For k=3 replicates, we have $S_{\max} = 4.514$. So if we observe at least one replicate with 5 or more copies of target DNA, we have a positive sample.

Definition 3 calls sample positive if the average copy number of replicates is at least 1, $\lambda_{ave} \geq 1$. The test statistic is $S_{ave}=E(X)= \Sigma X_i /k = S/k$.. We have a positive sample if $S \geq k$. Since S follows a Poisson distribution $S \sim Pois(k*\lambda_{ave})$, the LoD of λ_{ave} can be estimated using the lower limit of CI from the Poisson parameter. For example, if k=3, we need at least S =7.294 copies of total DNA for the lower limit of Poisson parameter ($k*\lambda_{ave}$) to be at least 3. Equivalently, $S_{ave} \geq 7.294/3= 2.432$. That is, if we have an average of at least 2.432 copies of DNA in the three replicates, we have a positive sample.

Definition 1 is the least conservative among the three definitions.

3. Experiment Validation

To check the validity of the LoD we proposed above, we conducted serial dilution experiments on several EGFR gene mutants in lung cancer, namely L858R, T790M and Exon 19 deletion. ddPCR assays were developed to detect these mutant DNA. In the first experiment, we used genetically engineered cell lines of these DNA mutations. In a second experiment, we applied the ddPCR assays to NCI-H1975 lung cancer cell line which harbors L858R and T790M mutation, as well as another lung cancer PC9 cell line which has exon 19 deletion. We also applied the threshold to 24 negative samples to evaluate the specificity of the proposed LoD.

3.1 Genetically Engineered Cell Lines

Genetically engineered cell line DNA were purchased from Horizon Diagnostics. Each cell line was genetically manipulated to contain either one of the three mutations of EGFR gene. DNA samples from these mutant cell lines were diluted with its isogenic wild type EGFR DNA samples, starting from 25% mutation sample with a 4 fold dilution. A total of 6-8 concentration levels plus a negative control were tested using the ddPCR assay in triplicates. The true copy number can be calculated at each dilution level, starting from 1136 (25% dilution).

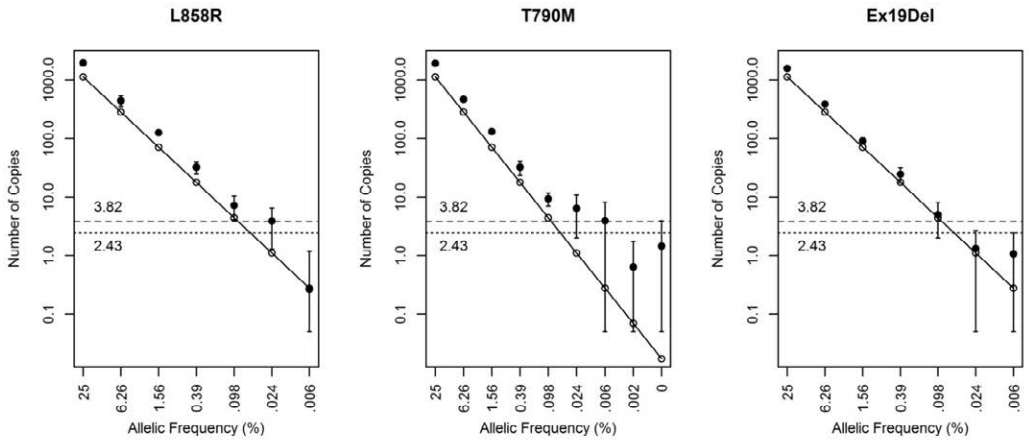

Figure 2: Measured copy number (filled dots) of mutant EGFR DNA, its standard error and the true copy number (open circles connected by straight line) in the genetically engineered cell lines. The threshold defined in single measurement (3.82) and triplicates (2.43) were shown in dash or dotted horizontal lines.

Figure 2 illustrates precision and accuracy of the mutant EGFR DNA copy number in the serial dilution experiment using the ddPCR assay. There is an upward bias in the measured copy number. For samples well above the detection limit, the measured copy number should follow a (log) linear relation with the dilution concentration, the precision should be tight. As concentration drops, the measured copy number count gets closer to the LoD, its error bar starts to increase. The experimental LoD is the concentration just before the copy number measurement starts to deviate from linearity and standard error starts to rise dramatically. In all three target mutations, this occurred at about 0.098%

dilution (5th dilution concentration), which corresponds to a total copy number of 4.44. The next concentration below (~ 1 copy) is not well measured due to its deviation from linearity and increased error bar. This suggests the experimental LoD from this study is around 4, consistent with the LoD we proposed in section 2 under Poisson distribution.

3.2 Cancer Cell Lines

In this experiment we used a lung cancer cell line NCI-H1975 that is known to harbor EGFR mutations of L858R and T790M, and another lung cancer cell line of PC9 that harbors exon 19 deletion in EGFR. Similar serial dilution was performed starting from 100% mutant cell lines (7576 copies of mutant DNA in the 20ul input sample) with 1/3 dilution for 8 levels in triplicates. For H1975 cell line experiments, we also added two negative controls.

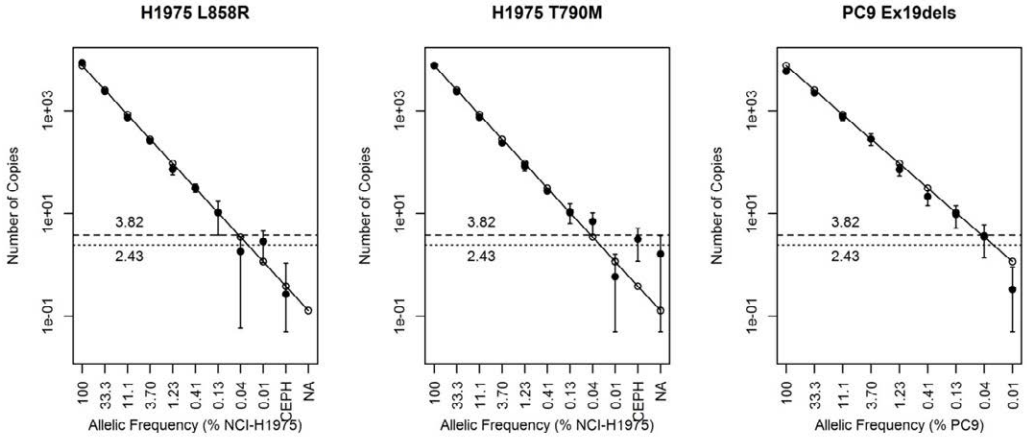

Figure 3: Measured copy number (filled dots) of mutant EGFR DNA, its standard error and the true copy number (open circles connected by straight line) in NCI-H1975 cell line and PC9 cell line. The threshold defined in single measurement (3.82) and triplicates (2.43) were also shown in dash or dotted horizontal lines.

Figure 3 shows the results for the lung cancer cell lines and it exhibit a very similar pattern to that of the genetically engineered DNA we observed in section 3.1, except this time it showed little bias in the measured copy number compared to the true copy number. At the concentration around the proposed LoD, the measured copy number starts to deviate from linearity and variability starts to increase dramatically. It again confirms the proposed LoD is consistent with the experimental LoD.

3.3 Negative Control Experiment

To evaluate the assay specificity, we applied the EGFR L858R assay to 24 normal genomic DNA samples which are known to not have any of the EGFR mutations. A single run of the assay was conducted on the samples. We expected to see zero copy number of L858R from these samples.

Figure 4 displays the measured copy number from the 24 target negative samples. While most samples had zero copy number detected, there were a few samples with non-zero copy number readout. One sample had copy number just above our proposed LoD of 4 for single measurement. Since our LoD was proposed using a 95% confidence limit, we would expect a 5% false positive result and the observed 1 out of 24 false positive sample

demonstrated the consistency between the proposed LoD and the experiment results on specificity.

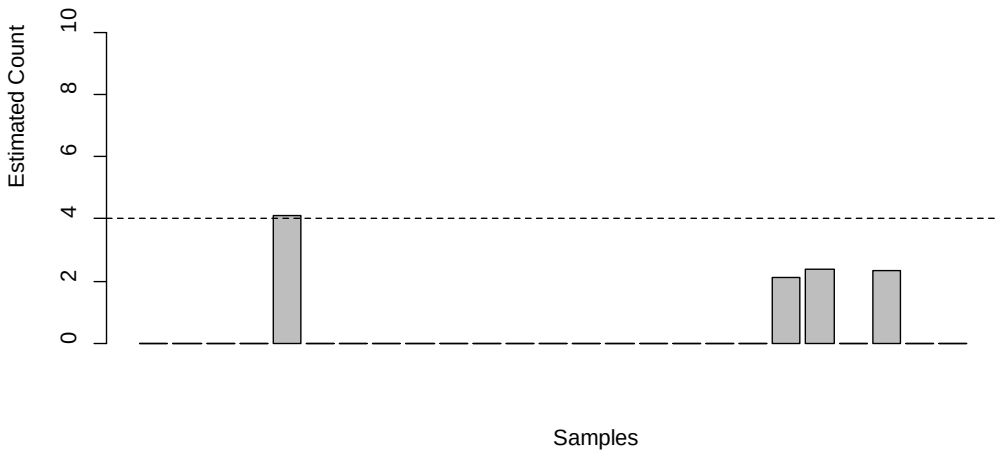

Figure 4: Measured copy number of L858R mutation on 24 normal genomic DNA samples.

4. Discussions

We have proposed a limit of detection (LoD) for a digital PCR assay in detection of a target DNA in a biological sample, based on a Poisson distributional assumption for DNA copy number. We also conducted several experiments to verify the proposed LoD is consistent with what would be observed in the experiments. The results from the experiments showed the proposed LoD for detecting DNA copy number provides good sensitivity and specificity for ddPCR to detect target tumor DNA.

The measured copy number from ddPCR was calculated using the observed count of positive droplets under Poisson distribution. We can derive a limit of detection for the count of positive droplets itself under the assumption that the number of positive droplets in a sample follows a binomial distribution with parameter π provided in eqn (2). The confidence interval of π can be expressed from a beta distribution (Clopper-Pearson interval)

$$B\left(\frac{\alpha}{2}, t, N-t+1\right) \leq \pi \leq B(1-\frac{\alpha}{2}, t+1, N-t) \quad (8)$$

with t = the observed number of positive droplets.

In a sample with small number of target DNA, particularly when the copy number is close to LoD, the number of positive droplets and the number of target DNA is almost the same as the probability a droplet has more than one copy of target DNA is extremely small (<1e-5). The binomial LoD and Poisson LoD will essentially be the same.

Acknowledgements

The authors thank Fred Immermann, Charles Tan and colleagues at Oncology Translational Research group in Pfizer for their discussions and comments.

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