

MODEL-BASED CLUSTERING OF DIGITAL PCR DROPLETS USING EXPECTATION MAXIMIZATION

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Abstract

The digital PCR (dPCR) is a method to quantify the DNA copies of known strains related to diseases. As a new approach to the gold-standard RT-PCR, further research is required to assess the quality and accuracy of this method. One particular area of dPCR is its novel step in droplet classification that distinguishes it from RT-PCR. As of writing, few droplet classifiers exist in literature as well as the assessment of these methods. This thesis reviews the classification methods of current dPCR quantification tools in literature, and proposes the Expectation Maximization Clustering method in aims to improve the accuracy of the final estimated DNA concentration.

Keywords: Quantitative PCR, Droplet Digital PCR, Expectation Maximization Clustering

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Chapter 1

Research Description

1.1 Introduction

1.2 Background of the Study

Quantification of Nucleic acids (NA) is a developing research field in molecular biology for the detection and quantification expression levels of genes (Huggett, O’Grady, & Bustin, 2015). These NA molecules are found in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), which carries genetic information and is used as biomarkers for the detection of diseases. Additionally, along with the rise of bioinformatics tools, NA quantification methods are also utilized in rare mutation detection, copy number variation detection, single-cell gene and microRNA expression analysis, and next-generation sequencing (Quan, Sauzade, & Brouzes, 2018). Outside the scope of molecular biology, its application has also found its way in forensic research (Whale, Cowen, Foy, & Huggett, 2013), medical diagnosis, environmental monitoring, and food safety analysis (Cao et al., 2017).

To be able to determine the concentration of target NAs, NA detection is naturally a pre-requisite. There are, however, NAs of interests that have very low concentrations to the point that it becomes undetectable in existing detection technologies. This problem is solved by amplifying the NA sequences using Polymerase Chain Reaction (PCR), a widely-used method for NA amplification since its invention in the 1980s (Cao et al., 2017). PCR can multiply specific NA sequences in DNA or RNA from low concentrations to millions of copies. This method exposes the NA sequences mixed with chemical components in a series of 20 to 40 temperature cycles. In each cycle, PCR doubles the NA molecule;

theoretically producing 2^n molecules after n cycles (Quan et al., 2018).

After PCR amplification, absolute NA quantification is achieved using digital Polymerase Chain Reaction (dPCR) technique. This equally divides the NA samples into thousands of partitions; each of these partitions is evaluated as either off or on, or in this context, labeled as positive or negative, hence the term "digital" (Cao et al., 2017).

The dPCR workflow, as illustrated in Figure 1.1, is usually a sequential procedure of extracting the sample from an organism, concocting the sample with several chemical components into a reaction mix, distributing the reaction mix to equal partitions, amplifying and detecting the target molecules using PCR, and the concentration is then finally estimated using a Poisson correction factor. In (Jacobs, Goetghebeur, & Clement, 2014), it was emphasized that every step of the dPCR workflow inevitably allows for the introduction of different sources of variation. These variance components within the dPCR workflow is shown in Figure 1.2.

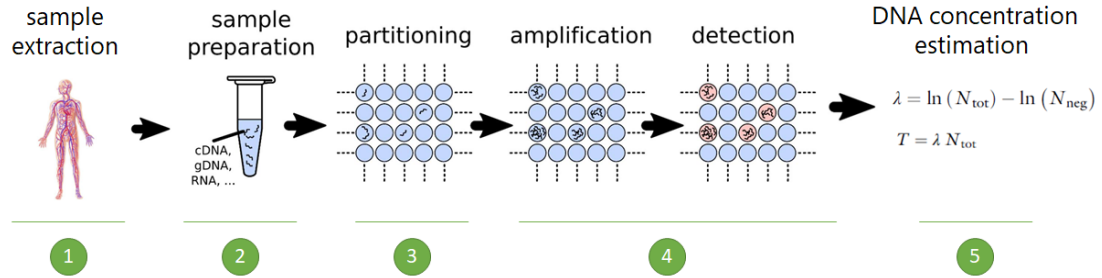


Figure 1.1: The dPCR workflow

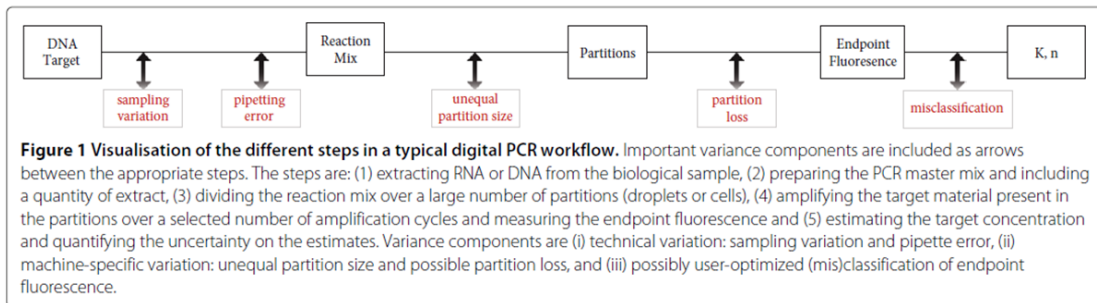


Figure 1.2: Potential variation components between steps of the dPCR workflow

Sampling variation stems from the fact that only a small sample of the organism is extracted; and although there is an expected number of target molecules per liters of a sample, drawing equally sized samples will result in different target molecules that are more or less near the average. Tzonev (2018) demonstrates the

number of target molecules that can be drawn from extraction is distributed as Poisson. Besides the sampling error, samples may also exhibit imperfections, and thus have inhibited amplification.

Preparing the reaction mix is a delicate process that strictly requires the accuracy of the pipetting volume; and yet, technical variation still occurs that results in pipetting errors. The next variation components are the possibility of the distributed partitions to be of unequal volumes and the loss of partitions due to physical interventions. Finally, upon PCR amplification, each droplet partition emits an endpoint fluorescence that would be used to classify the partition as positive or negative. However, some partitions are difficult to classify due to inhibition, delayed reactions, primer depletion, and other biological factors.

Each variance component accumulates to the bias and variance of the final estimated target molecule concentration, and thus, this gives rise to the importance of providing solutions that would increase precision in every step. To increase the sensitivity and specificity of the estimate, the misclassification of droplet partition should be minimized as much as possible. A high presence of false-negative droplets reduces sensitivity, while specificity is lowered for high false-positive count. Due to the variance contributed by misclassification, Tzonev (2018) recommends reporting the rates of false positives (FPR) and false negatives (FPN) per partition. More importance is given to either of the two depending on the kind of test being performed. If the total negative partition is expected to be large, then there is more chance that a true negative partition may turn into a false positive reading; which in this case, FPR may be of more interest than FNR.

The primary problem in misclassification lies in 'rain' droplets; these are partitions that emits an intermediate fluorescence signal that is difficult to classify as positive or negative. Figures 1.3 and 1.4 demonstrates two different DNA targets with the former showing a visually clearer distinction of the positive and negative population than the latter, of which is possessing multiple rain droplets. The data used in these figures are sourced from the publicly available dataset from the study of Lievens et al. (2016).

The estimate for DNA target concentration highly depends on the positive and negative classifications. For experiments with low copy DNA targets, the focus is on maximizing the sensitivity of the test for these very small number of positive droplets. Low sensitivity translates to failure in detecting lower concentrations.

For assays with large differences in the distance between the two fluorescence groups (positive and negative droplets), most quantification tools estimate the target concentrations with high sensitivity. As exhibited in an optimized assay experiment of *E. amylovora* (Dreo et al., 2014), slight differences of thresholds

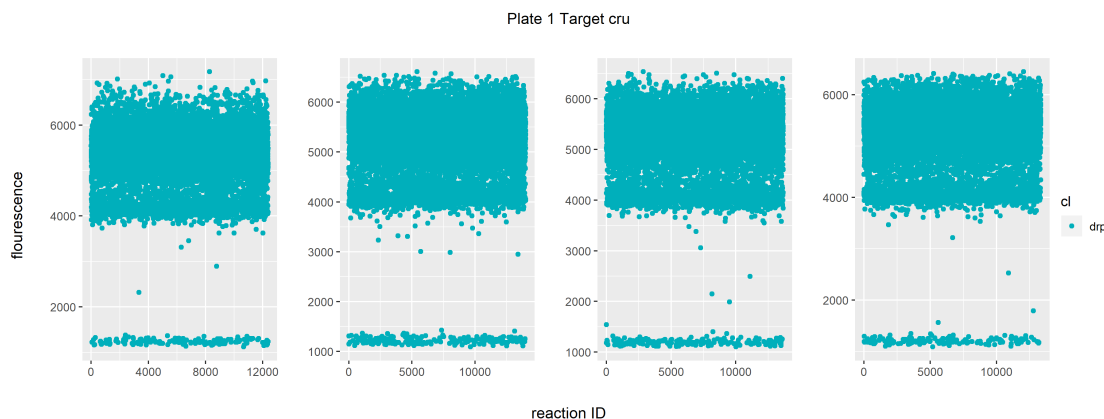


Figure 1.3: Fluorescence readings of 4 repetitions of DNA target cru

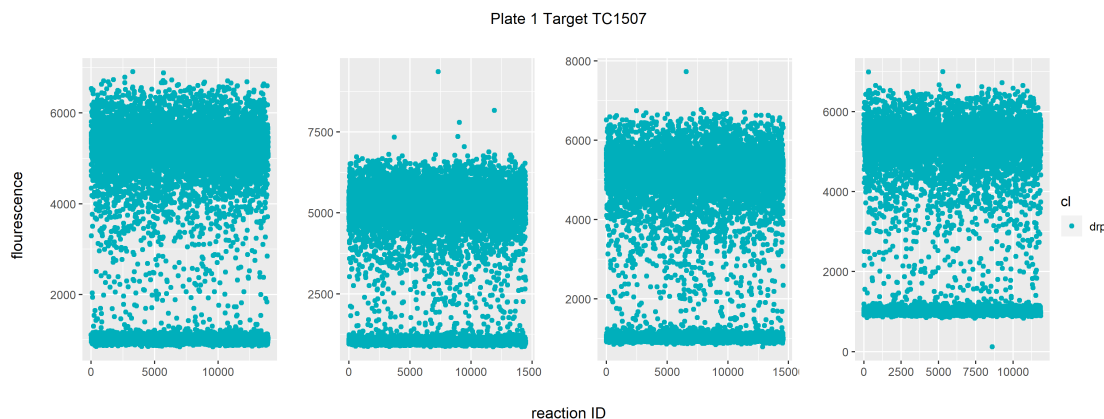


Figure 1.4: Fluorescence readings of 4 repetitions of DNA target TC1507

calculated from different tools had little effect on the final estimated concentration. However, for *R. solanacearum*, which is observed to manifest false-positive signals in qPCR experiments, produces unsatisfactory analytical sensitivity of the concentration estimates.

The danger of false rates due to misclassification is expounded at the clinical level, where false rates lead to the misdiagnosis of patients (Tzonev, 2018). One such case is the prenatal screening test for Down Syndrome; this test is expected to mostly result in normal pregnancies. However, even for a small FPR, many pregnancies are still falsely reported as positive for Down Syndrome. False negatives also risk the overall health of the patient that truly possesses the genetic disorder.

1.3 Statement of the Problem

This thesis aims to classify dPCR droplet partitions into positive or negative by exploring Model-Based clustering with Expectation Maximization. The specific objectives of this study are to:

1. Fit two-component finite mixture densities on the datasets with varying amounts of rain and increasing target DNA concentration;
2. Classify partitions using the fitted models for each experiment and estimate DNA concentration using the standard formula;
3. Evaluate and compare the precision and bias of the estimates amongst the existing classification methods.

1.4 Significance of the Study

Quantification of target concentrations for pathogenic bacteria, gene expression of diseases, cancer diagnostic, and other health-related applications strongly demand estimators with high sensitivity and precision, as lives are put to risk for false-positives. A modern approach to DNA and RNA target quantification is through the dPCR method. In one of the steps of the dPCR workflow, the classification of droplet fluorescence still has areas of improvement.

The most prominent problem in classification lies in experiments exhibiting a high frequency of rain, or intermediate fluorescence values. These are experiments that have not yet been optimized. As different DNA target samples exhibit distinct structures (Lievens et al., 2016), an optimized setup for one DNA target may not be applicable for other targets. Additionally, for samples with low concentration, the total count of detected positive droplets dramatically changes the final concentration estimate, due to the greater impact of false positives in the proportion of detected over the number of true positives. The following are some tools and methodologies proposed for droplet classification: Quantasoft propriety software, definetherain (Jones et al., 2014a), manual global threshold (Dreo et al., 2014), cloudy (Lievens et al., 2016), and Umbrella (Jacobs et al., 2017). Most of the aforementioned droplet classifying tools rely strongly on how representative reference samples are. According to Dreo et al. (2014), such approaches are sensitive to significant shifts in amplitude for previously unobserved factors, such as cross-reactions or the influence of inhibitors.

In an attempt to prevent the problem of representation, this study will explore the feasibility of estimating target concentrations without a reference sample. Similar to Umbrella, this study also aims to use model-based clustering for the droplet classification but with relaxing the assumptions using the Expectation-Maximization algorithm. The significance of the study will be useful in quantifying concentrations in targets that have not yet been optimized for dPCR experiments and also for quantifying targets of low concentrations.

1.5 Scope and Limitations

This study solely relied on publicly available fluorescence datasets from published research papers. Only two were found and will be used for statistical analysis, namely from Lievens et al. (2016) and Jones et al. (2014a). The former dataset contains twelve DNA targets from food and feed samples ran on nine different settings by controlling for experimental factors; the latter dataset is a serial dilution of the Albumin DNA ranging from 10^0 to 10^5 copies.

The droplet classification method in this study uses model-based clustering, or the use of finite mixture models to perform clustering. However, the identification of the distribution of the mixture densities will be dependent on the observed available dataset. As a consequence of the limited dataset, the paper’s methodology described here needs more study for other experimental setting and nucleic acid targets.

Lastly, statistical results presented may lack biological explanations which could be useful for explaining the variances of the droplet fluorescence. Such information may be utilized to further improve the estimation process.

Chapter 2

Review of Related Literature

2.1 Current dPCR Droplet Classification Methods

2.1.1 Droplet dPCR (ddPCR) System

The most common method in classifying positive and negative droplets is by enforcing a hard threshold. Generally, all droplets with a fluorescence amplitude greater than this threshold are then classified as positive, and negative otherwise.

One popular tool incorporated with automatic thresholding is the QuantaSoft software. The QuantaSoft software is the dPCR analysis tool that comes with the Bio-Rad droplet dPCR (ddPCR) System package. It allows for the setting up of sample and experiments, running and controlling the instrument, and finally, the analysis of the NA concentration (Bio-Rad, 2019). According to the Bio-Rad Laboratories website (<https://www.bio-rad.com>), it has been a leading product developer for 65 years in the research fields of life science and clinical diagnostics. Among its popular focus areas, dPCR is one of its most featured technology, providing ddPCR instruments; kits, reagents and assays; and other consumables. Several studies in hospitals (López et al., 2016; D. F. Chen, Zhang, Tan, & Jing, 2018; Abed, Carbonneau, L’Huillier, Kaiser, & Boivin, 2017; Tagliapietra et al., 2020), public health (Hussain & Bowers, 2017a; Nystrand, Ghanima, Waage, & Jonassen, 2018), food safety (J. Chen et al., 2020; Capobianco et al., 2020; Basanisi et al., 2020), upto environmental quality (Hamaguchi et al., 2018; Jahne et al., 2020; Dobnik, Štebih, Blejec, Morisset, & Žel, 2016; Mauvisseau et al., 2019) have found the Bio-Rad QuantaSoft dPCR systems useful for their

analyses.

Of all the QuantaSoft software features, the focus of this section is on its threshold setting. By default, QuantaSoft sets an automatic threshold to the single-well or multiple-well amplitude data; a demonstration is shown in Figure 2.1. As with other automated tools, its documentation recommends reviewing this threshold to make changes if needed; and thus, manually setting the threshold is also allowed. Unfortunately, the calculation of the automatic threshold is not publicly available.

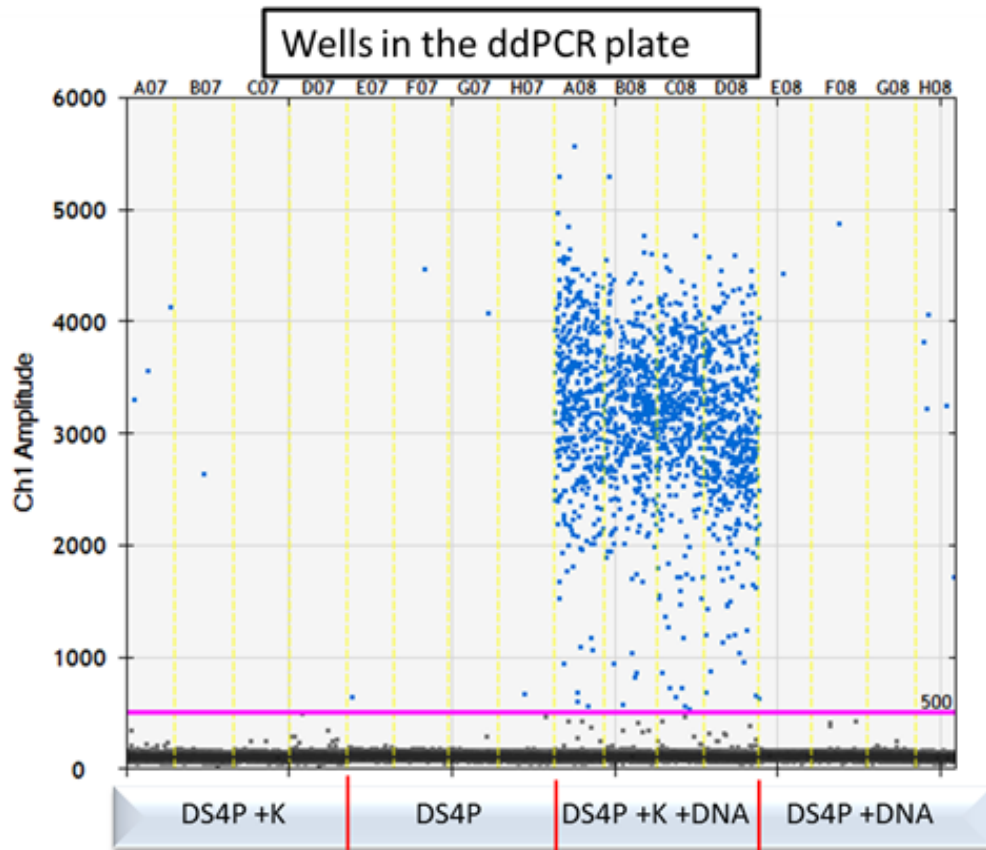


Figure 2.1: QuantaSoft threshold in pink line from (Hussain & Bowers, 2017b)

The evaluation of the QuantaSoft software show exemplary results from the food safety study of Basanisi et al. (2020), whereby nine pure meat samples were discriminated with 100% diagnostic accuracy, sensitivity, and specificity. However, upon checking the authenticity of twenty commercially available meat products, twelve samples were said to contain DNA traces of other animals not declared. Among the several reasons for this high detection, Basanisi et al. (2020) suggests

the need for a highly sensitive and specific test in the molecular level. Observation of NA molecules can be done through their droplet fluorescence in a dPCR assay.

Assessing the QuantaSoft software’s ability to classify droplets, it was shown that for samples exhibiting a substantial amount of intermediate fluorescence, the system fails to determine a threshold (outputs "No call"). This was the case in the two bacteria study of (Dreo et al., 2014), that were ran on very high concentrations. In the case of low bacteria concentrations, droplets near the negative droplets were classified as positives. This study concluded that the QuantaSoft software requires a well-optimized assay with good discrimination of positive and negative droplets for its threshold to be reliable.

2.1.2 Manual Global Threshold (MTg)

As opposed to the automatic threshold, Dreo et al. (2014) proposes setting a manual global threshold (MTg) determined by no template control (NTC) samples. This takes into consideration that individual assays behave differently, and could require expert intervention. For example, the threshold for a well-optimised assay was defined as the NTC mean + 6 standard deviations; on the other hand, a noisy assay had its threshold set above the highest value in NTC samples. It is expected in the latter example that the sensitivity would be lower, due to its high threshold. However, the paper claims that this resulted in high analytical sensitivity for that assay. A major disadvantage in this approach is the clear definition or guidelines in setting the MTg; this consequently will cause reproducibility issues for succeeding experiments and external researchers.

2.1.3 Kernel Density Estimation (KDE)

The positive and negative droplet classification can be framed as a clustering problem. One solution is by using the popular theory of kernel density estimation (KDE); whereby each data point influence is modeled using a kernel function — frequently Gaussian distributed, and the sum of all the kernel functions would be the overall density of the data. Clusters can then be derived from the estimated densities depending on its application (Hinneburg & Keim, 2003).

KDE has been particularly helpful in spatial analysis. Clusters were detected in areas with diabetes in Berlin (Kauhl, Schweikart, Krafft, Keste, & Moskwyn, 2016), HCV hepatitis C virus in Massachusetts (Stopka et al., 2017), and crime hotspots in Brazil (Junior, Da Silva, De Queiroz Neto, De MacÊdo, & Porcino,

2019). For other data types, KDE clustering has been improved in an algorithm developed by Rodriguez and Laio (2014), called clustering by fast search and find of density peaks. This technique has piqued the interests of many researchers; thereby further improving the original limitations via DNA genetic algorithm (Zang, Ren, Zhang, & Liu, 2017), heat diffusion (Mehmood, Zhang, Bie, Dawood, & Ahmad, 2016), and entropy of data field (Wang, Wang, Li, Li, & Ding, 2016).

Although not named in the dPCR optimization paper of Lievens et al. (2016), cloudy is the name of the function in their supplementary source code file that calculates the threshold from the clusters found by using Gaussian KDE. The following steps explain this in more detail:

1. Estimate the density function of the fluorescence using a Gaussian kernel density with a minimum bandwidth of 50
2. Identify density peaks using a sliding window approach. The subsequent steps will differ according to if one, two, or more than three peaks were found. But generally, the proceeding steps are followed.
3. For each population found through the peaks, its location and spread is initially estimated using the median $\hat{\mu}$ and $\hat{\sigma}$. Assuming normality, the latter is estimated as half the peak width at 60-65% of its maximum height.
4. Refine the estimates using a reiterative method, first initialized with $a = 4$.
5. Re-estimate $\hat{\mu}$ and $\hat{\sigma}$ using only the observations within $\hat{\mu} \pm (a \cdot \hat{\sigma})$.
6. Recalculate $a = 4.55 + 0.35 \cdot \log(k) + 0.045 \cdot \log(k)^2$; where k is the kurtosis of the distribution
7. Repeat steps 5-6 until stabilization.
8. After stabilizing the estimates for all the population, the last step is different when either including or excluding rain in the final categorization.
 - (a) If rain is included as a category, observations within $\hat{\mu} \pm (a \cdot \hat{\sigma})$ are then classified as members of that population; observations not falling within any population are classified as rain.
 - (b) If rain categorization is not of interest, then a threshold $\theta = \hat{\mu}_n + 1.5 \cdot a_n + \hat{\sigma}_n$ is calculated; where n is a population.

In summary, the cloudy algorithm uses the Gaussian kernel density and normality assumptions to detect peaks, which are then considered as populations.

From then population density parameters are estimated based on specified formulas. A range or a single threshold is then calculated for classifying droplets as positive, negative, or optionally, rain.

Special cases such as overlapping population boundaries and threshold placement problems are also checked in their source code. It is worth noting that in step 6, the formula for re-calculating a for density parameter estimation is based on the analysis of their in-house data, and should be used with caution when implementing for other unobserved NA targets. The rain classification rule in step 8(a) also poses a problem for fluorescence densities that are heavily skewed. Figure 2.2 left panel reveals the distribution of negative droplets to be heavily skewed to the right, thereby causing their exclusion to be labeled as negative due to the symmetry of the categorization rule (right panel).

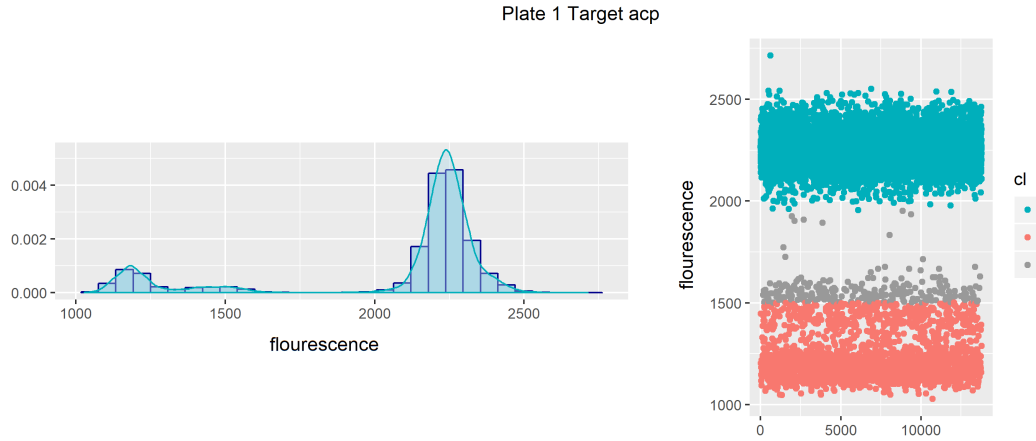


Figure 2.2: One replicate of the DNA target acp Plate 1 from Lievens et al. (2016) dataset. Left panel shows the fluorescence densities. Right panel is the result of droplet categorization using cloudy

It is noted that the context of the threshold setting here is based on in-house data for studying dPCR experiment optimization, and not necessarily as a classifier for external data. The constant threshold criterium enables the control for other PCR experimental factors, such as sonication, PCR enhancers, annealing conditions, and number of cycles, to achieve optimization or diminishing rain droplets for dPCR experiments.

2.1.4 K-Nearest-Neighbors (KNN)

Based on the research papers curated by Peterson (2009), K-nearest-neighbor (KNN) is a unsupervised clustering approach that should be among the first methods considered for data with little to no information about its distribution. This clustering method operates on the chosen distance measure — commonly the Euclidean distance — between the observations. Due to its simplicity, data from various fields have applied KNN such as in a movie recommendation system (Ahuja, Solanki, & Nayyar, 2019), climate classification (Shi & Yang, 2020), breast cancer diagnostics (Mittal, Aggarwal, & Mahajan, 2019), among others.

An open-source tool developed by Jones et al. (2014a), called *definetherain*, utilizes the KNN algorithm in identifying rain droplets. According to their research, they claim that *definetherain* is accurate in estimating assays with low template numbers, which is particularly applicable in research fields such as the HIV-1 cure research. *definetherain* follows these steps for classification:

1. Setup a positive control sample of known input copy numbers.
2. Cluster the droplets using kNN with $k = 2$. The cluster on the left is the negative cluster, and on the right is the positive cluster.
3. Observations between the range of the negative cluster’s mean + 3 standard deviations and the positive cluster’s mean - 3 standard deviations are classified as rain.
4. Rain droplets are not included in the final calculation of the concentration estimate.

Unlike the other methods discussed here, this tool produces two cutoff values — one for each cluster. The droplets falling between these two values are classified as rain. These cutoff values are solely dependent on the control sample. The disadvantage of this is that the control has to be representative of the NA target; otherwise, concentration estimates would be biased.

2.1.5 Model-Based Clustering w/ Non-parametric Density Estimation

As opposed to the distance-based clustering in Section 2.1.4, a probabilistic approach is achieved with model-based clustering. According to McNicholas (2016),

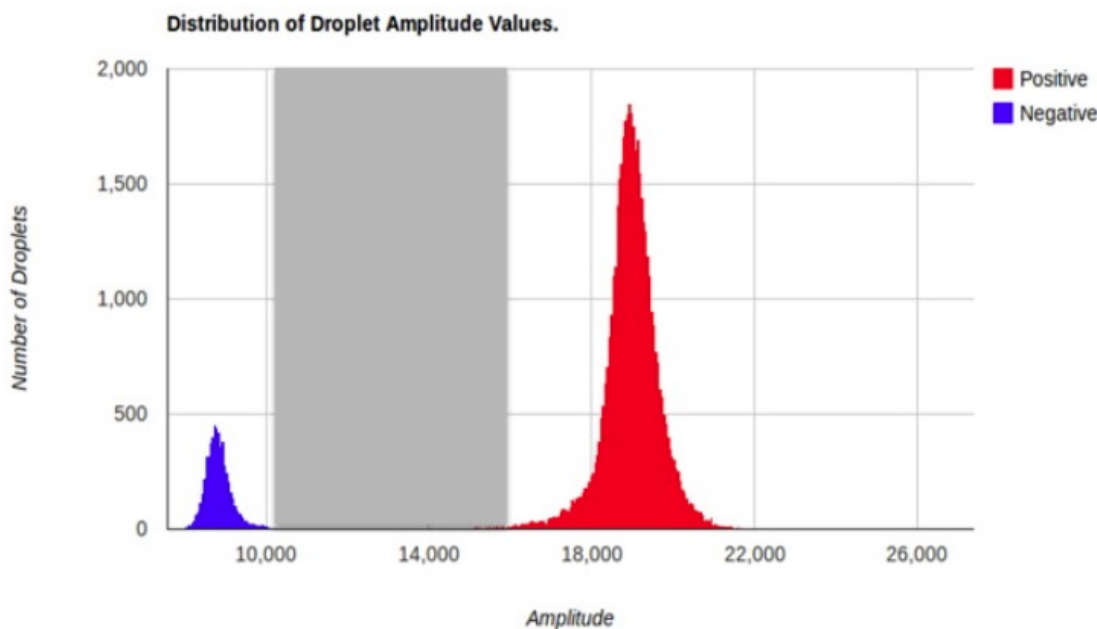


Figure 2.3: Determined thresholds calculated by definetherain, reprinted from (Jones et al., 2014b)

a finite mixture model is a sum of weighted density components. This mixture model has to be appropriate such that its parameters are flexible for fitting the characteristics of the data. In this approach, each unimodal density component are defined as a cluster; and each observation has a calculated probability of it belonging to a cluster.

In an electricity usage profiling study, Li et al. (2018) noticed several elongated ellipses in the scatterplot of electricity usage data, urging them to use Gaussian components for their mixture model. In another example, (Choy, Lam, Yu, Lee, & Leung, 2017) performed image segmentation using the generalized Gaussian density model, where each cluster formed is interpreted as an object. Their algorithm was able to segment objects such as a starfish, cat, or tree in photographs. Additionally, using genetic data, Li et al. (2018) discovered a potential differentially-variable microRNA (miRNA) not yet reported in literature, upon fitting a three-component multivariate normal distribution to miRNA expression levels. The assumption of the Gaussian/normal component distributions are common in model-based clustering; however, in the succeeding texts, dPCR droplets is claimed to violate this assumption.

Jacobs et al. (2017) developed Umbrella, a model-based clustering for dPCR droplets using nonparametric density estimation (<https://github.com/statOmics/umbrella>). Umbrella requires a representative NTC sample(s), the procedure then follows a

series of estimation and assumptions in deriving the final estimated concentration. An oversimplification of the Umbrella procedure, that skips the detailed steps for handling specific cases, are as follows:

1. The NTC distribution $f_0(x)$ is assumed to follow a unimodal distribution. The location and variation is estimated by the mode and mean of absolute deviation (MAD), respectively.
2. The fluorescence intensities x observed in partitions of a target partition set A is assumed to have a mixture density

$$f_A(x) = p_{0,A}f_{0,A}(x) + (1 - p_{0,A})f_{1,A}(x)$$

- $p_{0,A}$ = proportion of negative partitions
 - $f_{0,A}(x)$ = densities of the partitions without target copy (null component of the target partition set A)
 - $(1 - p_{0,A})$ = proportion of positive partitions
 - $f_{1,A}(x)$ = densities of the partitions with target copy
3. Begin estimating the parameters by first aligning the modes of the null component of $f_A(x)$ and the NTC reference $f_0(x)$.
 4. Discretize the aligned distributions by generating a histogram with the same bins.
 5. The bin counts of the aligned distribution are modeled using a Poisson regression model, resulting in estimates for $\hat{p}_{0,A}$, $\hat{f}_0(x)$, and $\hat{f}_A(x)$.
 6. The posterior probability that partition i is void of the NA target with fluorescence intensity x_i of partition set A , $\hat{p}_{0,A}$, can be defined from the estimated $\hat{p}_{0,A}$ from the previous step as

$$\hat{p}_{i,0,A} = \hat{p}_{0,A} \left(\frac{\hat{f}_{0,A}(x_i)}{\hat{f}_A(x_i)} \right)$$

7. The Umbrella threshold estimator is then determined by the estimated $\hat{p}_{i,0,A}$. For intensity value i , the interpretation of the posterior probabilities are
 - $\hat{p}_{i,0,A} > 80\%$ are considered negative partitions with a probability of $\leq 20\%$ to be false negatives
 - $\hat{p}_{i,0,A} < 5\%$ are considered positive partitions with a probability of $\leq 5\%$ to be false positives
 - $5\% \leq \hat{p}_{i,0,A} \leq 80\%$ are considered as rain

The mode and MAD, as the location and spread estimators for the null NTC distribution, $f_0(x)$, are chosen due to its robustness and insensitivity to skewed tails. Only observations within 10 deviations of the mode are included for the null model.

Following the assumption of a mixture density in step 2, unlike most model-based clustering algorithms, Umbrella does not assume normal densities for $f_{1,A}(x)$ and $f_{0,A}(x)$, the partitions with and without the NA target, respectively. This is due to the exhibition of dPCR fluorescence intensities to be non-normal, as clusters tend to have heavy tails to the left or to the right. The solution for this is the use of non-parametric density estimation in step 3.

After estimating all the components in the mixture model from steps 3 - 5, the component of interest $p_{0,A}$ is then used to determine $\hat{p}_{i,0,A}$ in step 6. Finally, this is used as the basis for Umbrella threshold estimator in step 7. It is cautioned that Umbrella may not be precise in detection experiments for low copy samples, as classifying individual samples is not the strength of this method.

2.2 Expectation-Maximization (EM) Clustering

Recall from Section 2.1.5, model-based clustering refers to fitting a finite mixture model given the data set X ; then the cluster membership of observation x_i is determined by the highest probability of it belonging to a density component. Building the mixture model $f(x|\Theta)$ requires the determination of 1) G — the number of mixture components (clusters), and 2) $f_g(x|\theta_g)$ — the distribution assumed to be followed by the mixture component g .

A common method for determining G is by selecting the model with the lowest Bayesian Information Criterion (BIC) amongst the proposed G -component mixture models. As mentioned in the model-based clustering examples in Section 2.1.5, the mixture components are frequently assumed to follow a Gaussian distribution, and is also known as Gaussian Mixture Model (GMM). Although popular, GMMs poorly fit the data that exhibit skewness and different levels of kurtosis, consequently leading to overestimation on the number of clusters (Dang, Gallagher, Browne, & McNicholas, 2019). Alternatively, the following distributions can better generalize these kinds of data: multivariate t , skew- t , multivariate power exponential, variance-gamma, generalized hyperbolic, etc. Unlike Gaussian, these component models are flexible in data of varying tail weight, peakedness, and skewness.

After determining the distribution of G mixture components, the next prob-

lem is on how to estimate its corresponding parameter set Θ^* . Expectation-Maximization (EM), a well-known parameter estimation algorithm, is an iterative procedure that maximizes the likelihood of the parameters given the observed data (Garriga, Palmer, Oltra, & Bartumeus, 2016). In the EM procedure, the parameter set is initially guessed and is re-estimated in every iteration of the E-step and M-step, until the parameter set reaches convergence. E-step computes the likelihood weight, or the posterior probability, of each data point x_i belonging to a component g . M-step re-estimates new parameters that maximize the likelihood of these weights for each component. The result of EM guarantees to reach a local maximum for parametric distributions. The direct application of using the final EM posterior probabilities in assigning data points to groups is called EM Clustering (EMC) (Garriga et al., 2016).

For dPCR droplets classification, since the groups of interest are the positive and negative droplets, a two-component mixture model suffices. However, there is room for research in identifying the fluorescence intensity distribution that will fit the characteristics of the positive/negative groups. Since heavy tails are observed in fluorescence densities in Figure 2.2, its distribution appears to be nonnormal, as also stated by Jacobs et al. (2017).

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