Model-Based Clustering of Digital PCR Droplets using Expectation Maximization

A Thesis Proposal
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of the Requirements for the Degree of
Master of Science in Statistics

by

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Abstract

From 150 to 200 words of short, direct and complete sentences, the abstract should be informative enough to serve as a substitute for reading the thesis document itself. It states the rationale and the objectives of the research.

In the final thesis document (i.e., the document you'll submit for your final thesis defense), the abstract should also contain a description of your research results, findings, and contribution(s).

Keywords can be found at `http://www.acm.org/about/class/class/2012?pageIndex=0`. Click the link "HTML" in the paragraph that starts with "The **full CCS classification tree**...".

Keywords: Keyword 1, keyword 2, keyword 3, keyword 4, etc.

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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 (?, ?).

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 (?, ?). 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" (?, ?).

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.

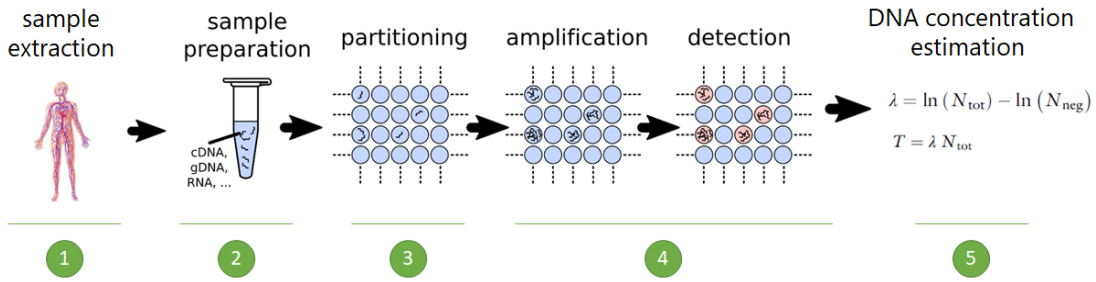

Figure 1.1: The dPCR workflow

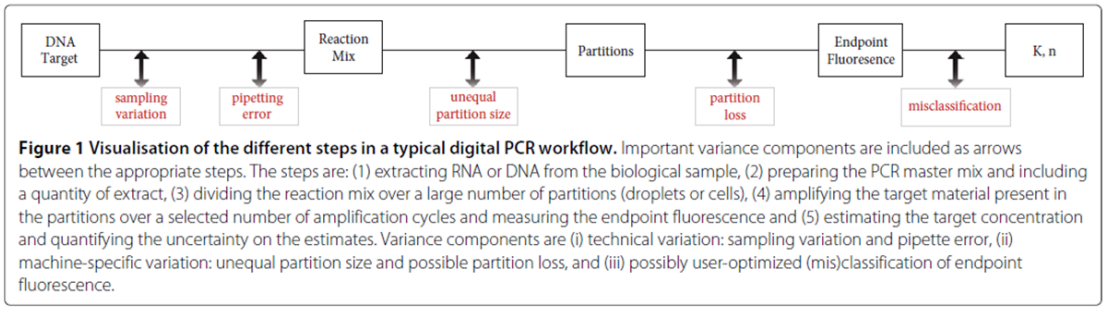

Figure 1 Visualisation of the different steps in a typical digital PCR workflow. Important variance components are included as arrows between the appropriate steps. The steps are: (1) extracting RNA or DNA from the biological sample, (2) preparing the PCR master mix and including a quantity of extract, (3) dividing the reaction mix over a large number of partitions (droplets or cells), (4) amplifying the target material present in the partitions over a selected number of amplification cycles and measuring the endpoint fluorescence and (5) estimating the target concentration and quantifying the uncertainty on the estimates. Variance components are (i) technical variation: sampling variation and pipette error, (ii) machine-specific variation: unequal partition size and possible partition loss, and (iii) possibly user-optimized (mis)classification of endpoint fluorescence.

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 (n.d.) 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 (n.d.) 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 repititions of DNA target cru

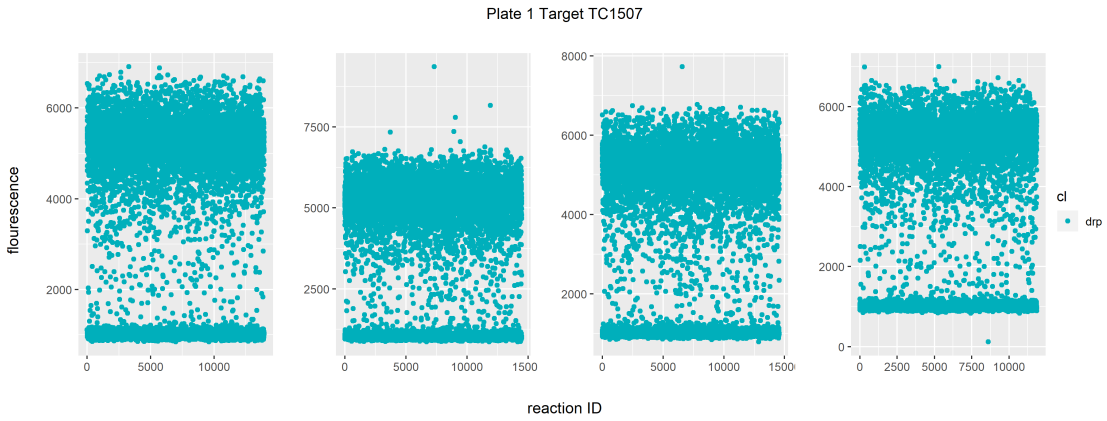

Figure 1.4: Fluorescence readings of 4 repititions of DNA target TC1507

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, n.d.). 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 dPCR Droplet Classification Methods

2.1.1 Threshold

The most common method in classifying positive and negative droplets are by enforcing a hard threshold. All droplets with a fluorescence amplitude greater than this threshold is then classified as positive, and negative otherwise. The following sections explore how a threshold can be determined with statistical methods.

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 digital PCR System package. It allows for the setting up of sample and experiments, running and controlling the instrument, and 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 over 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 droplet dPCR 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, 2017; 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 features of the QuantaSoft software, the focus of this section is on how the software sets the threshold. 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.

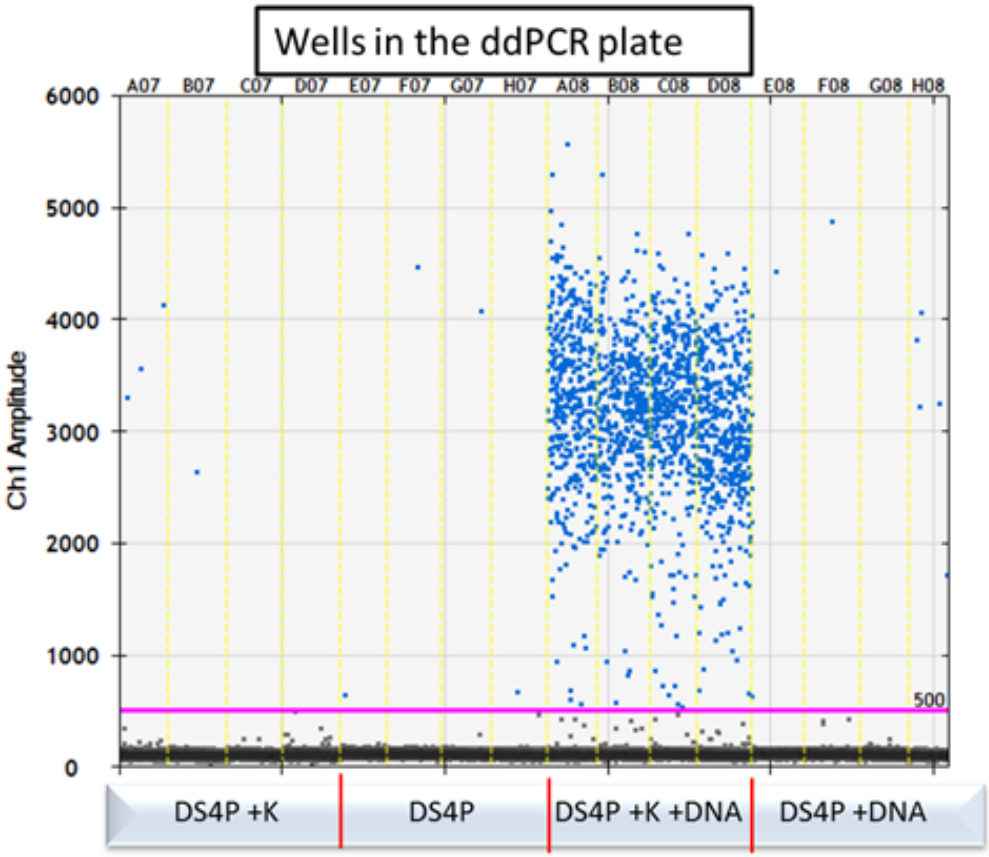

Figure 2.1: QuantaSoft threshold in pink line from (Hussain & Bowers, 2017)

TODO - look for Quantasoft experiments

[1] For samples exhibiting a substantial amount of intermediate fluorescence, the QuantaSoft software fails to determine a threshold, resulting in a "No call" output. This was the case when a study of two bacteria (Dreo et al., 2014) was ran on very high concentration. In the case of low bacteria concentrations, droplets near the negative droplets were classified as positives.

TODO - look for 2 more other Quantasoft experiments with bad results

These studies 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 Population Detection of Gaussian Kernel Densities

Although not named in the paper of Lievens et al. (2016), cloudy is the name of the function in their source code provided in their supplementary file. Cloudy implements the following steps for threshold calculation, which is also documented in their paper:

1. Estimate the density function of the fluorescence using a Guassian kernel density with a minimum bandwith 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).

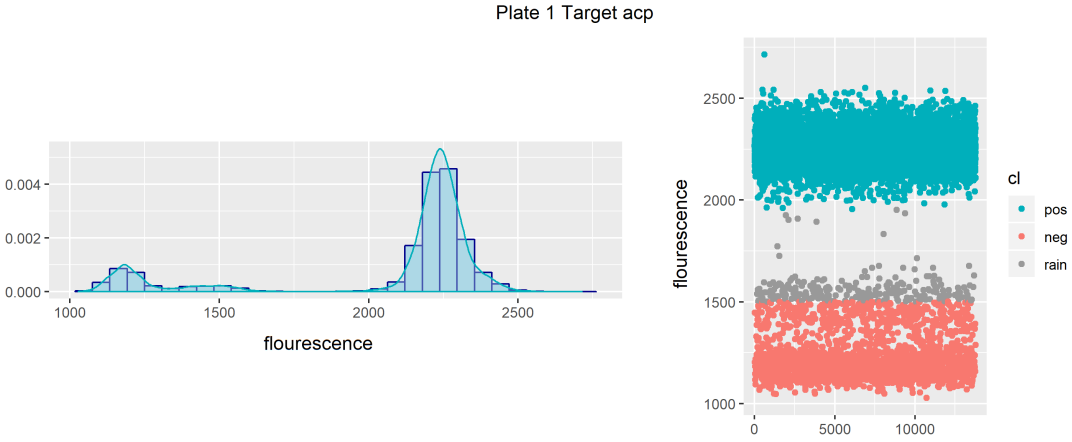

Figure 2.2: One replicate of the DNA target acp Plate 1 from Lievens et al. (2016) dataset. Left panel shows the fluorescence densities. Right panel is the result of droplet categorization using cloudy

TODO - add their results from this algorithm

2.1.3 K Nearest Neighbors

definetherain follows these steps for classification:

- Setup a control well of known input copy numbers.
- Cluster using k-nearest neighbor with $k = 2$.

- Using cluster means and standard deviation, rain in subsequent test samples are classified if it is not within the range of mean ± 3 standard deviations of the negative cluster and mean ± 3 standard deviations of the positive cluster.

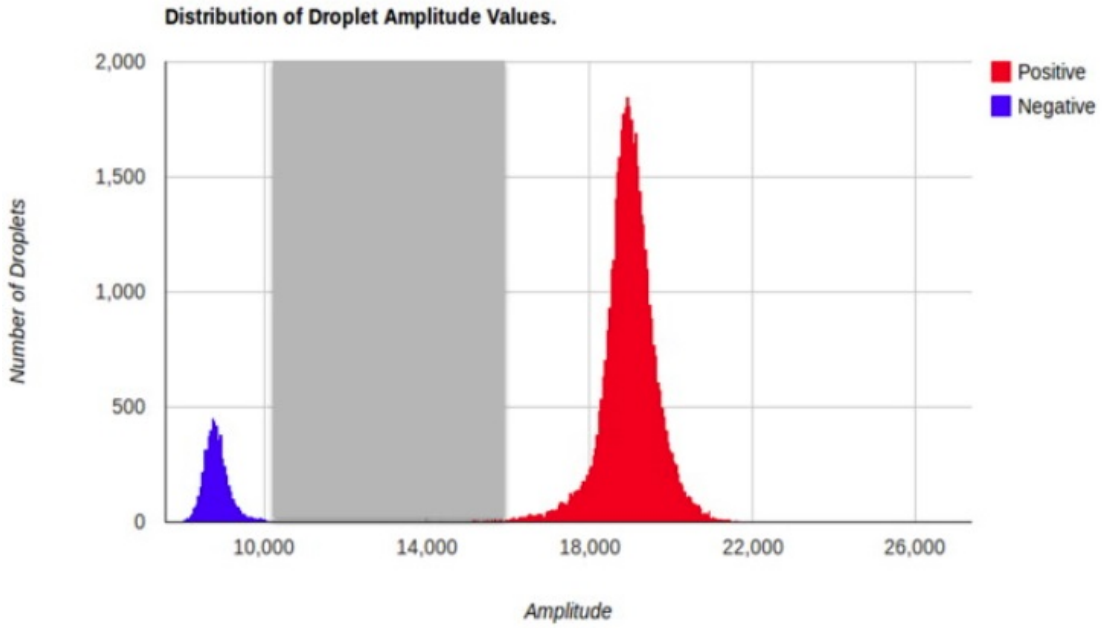

Figure 2.3: Determined thresholds calculated by definetherain, reprinted from (Jones et al., 2014b)

2.1.4 Non-parametric Mixture Models

2.2 Model-Based Clustering

Chapter 3

Theoretical Framework

3.1 G-component Finite Mixture Density

3.2 Expectation Maximization Clustering

Chapter 4

Methodology

4.1 Data

4.1.1 Rain Experiment Dataset

Plate 2 - Primer and Probe Concentration Gradient

Plate 4 - PCR Enhancers Experiment

Plate 5 - Cycle Gradient

Plate 6 - Sonication Gradient

Plate 7 - Annealing Temperature Gradient

4.1.2 DNA Quantification Dataset

Plate 3 - Rain Dilution Series

Albumin

4.2 Model Fitting and Classification

4.3 Performance Evaluation

Appendix A

Diagrams and Other Documentation Tools

This appendix may consist of proposed architectural design, algorithms, scientific formula for MSCS and Data Flow Diagrams, Fishbone for MSIT.

Appendix B

Theoretical and/or Conceptual Framework

Discusses the basic framework/foundation the thesis is based on. This section is normally referred to when discussing Scope and Limitations, and Research Methodology

Appendix C

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