International Journal of Forensic Medicine

E-ISSN: 2707-4455
P-ISSN: 2707-4447
IJFM 2024; 6(2): 30-34
www.forensicpaper.com
Received: 03-07-2024
Accepted: 07-08-2024

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Why optimizing thresholds in forensic genetics matters

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DOI: https://doi.org/10.33545/27074447.2024.v6.i2a.92

Abstract
In forensic genetics, the accurate analysis and interpretation of DNA trace is paramount. To ensure reliability, scientists employ cut-off and threshold values at various stages of DNA analysis, including quantitative real-time PCR (qPCR) and capillary electrophoresis of short tandem repeat (STR) amplicons. These thresholds help distinguish true genetic signals from background noise, ensuring the integrity of forensic trace evidence.

Keywords: Analytical thresholds, DNA, forensic genetics, quantification cut-off values, stochastic threshold

Introduction

DNA profiling methodologies are used due to their high discrimination power and sensitivity, allowing profiles to be generated from small quantities of DNA trace. (Gill, Haned,; Bleka, Hansson, Dørum, Egeland, 2015; Børsting, C.; Morling, 2015; Butler 2012 Butler, J.M., 2015) [1-4]. Enhancing methods to generate high-quality DNA profiles from samples with low DNA concentration is an important objective for forensic DNA laboratories. Processing DNA evidence samples typically involves extraction, quantification, and short tandem repeat (STR) amplification.

DNA extraction can result in the loss of a portion of the original DNA trace and may increase the risk of introducing external DNA contaminants, which is particularly problematic when dealing with forensic evidence containing low levels of DNA· ((Alketbi, S.K., 2024, Cavanaugh, S.E. and Bathrick, A.S., 2018) [5, 6]. This may result in non-existent or incomplete profiles with a significant prevalence of stochastic effects, hence complicating interpretation. DNA extraction is a crucial step that purifies nucleic acids by removing cellular debris, endogenous proteins, and external inhibitors. This process is vital as it ensures the success of PCR amplification of STR loci, a key technique in molecular biology. However, depending on the extraction method and its efficiency, this process can lead to a loss of approximately 70% to 85% of the initial DNA template. (R.A.H. van Oorschot, D.G. Phelan, 2003; R. Ottens, J. Templeton, V. Paradiso, *et al*., 2013) [7, 8].

Factors influencing DNA extraction efficiency include the number of tube transfers, washing steps, and the propensity of DNA to adhere irreversibly to plastic consumables and extraction matrices (C. Gaillard, F. Strauss, 1998;. Schiffner, E.J. Bajda, M. Prinz, 2005) [9, 10]. In silica-based extraction protocols, for instance, silica matrices possess a fraction of irreversible binding sites that can permanently sequester nucleic acids (K.J. Shaw, L. Thain, P.T. Docker, *et al*., 2009; Thather) [11, 12]. Centrifugal filter devices are commonly employed to concentrate DNA samples; however, they can lead to significant DNA loss, ranging from 33% to 67%. (A.M. Garvin, A. Fritsch, 2013; Doran, D.R. Foran, 2014) [13, 14]. This loss is primarily due to DNA becoming trapped within the device. However, the inclusion of carrier RNA in the extraction process has proven to be helpful, significantly enhancing DNA recovery by increasing the overall nucleic acid concentration and improving the binding efficiency to the silica matrix. (R. Kishore, W. Reef Hardy, V.J. Anderson, *et al*., (2006), Pearma [15, 16]; Implementing strategies to minimize sample loss during extraction is not just a recommendation, but a necessity, especially when working with limited or degraded DNA trace. Optimizing extraction protocols by reducing tube changes and washing steps and incorporating carrier molecules like RNA is crucial.

These strategies can significantly improve DNA yield and the success of subsequent analyses, making them indispensable in the laboratory.

In standard DNA processing workflows, quantifying the extracted DNA is essential to determining its concentration, selecting the optimal amount for amplification, and choosing the most appropriate downstream genotyping analysis methods. Ricci, U., Ciappi, D., Carboni, I., Centrone, C., Giotti, I., Petti, M., Alice, B. and Pelo, E., 2024) [17]. In capillary electrophoresis (CE) analysis of short tandem repeats (STRs), a stochastic threshold is employed to account for the potential challenges of allele drop-out or drop-in events.

This threshold, typically set around 150–200 relative fluorescence units (RFUs), is a crucial tool in addressing these challenges, ensuring the reliability of the data. (Stephens, K., Snedecor, J. and Budowle, B., 2022) [18]. Stochastic effects in PCR amplification manifest when DNA inputs are minimal or the DNA quality is subpar. Stochastic thresholds are established for DNA analysis in human identification to signify the likelihood of absent data in compromised samples. The stochastic thresholds for analysing STRs by CE are defined as the peak height at which there is a significant likelihood of an absent allele.

The setting of thresholds at Capillary Electrophoresis and STR Analysis is to empower laboratories to reliably provide precise and reproducible allelic designations while detecting possible allelic drop-out, underpinned by internal validation data and established laboratory processes. Analytical thresholds are necessary when a laboratory's data processing entails binary determinations on the detection or non-detection of peaks in casework. Likewise, stochastic thresholds are essential if the laboratory assesses the likelihood of allele drop-out in its analyses. (Gill, P., Haned, H., Bleka, O., Hansson, O., Dørum, G. and Egeland, T., 2015) [19]

The implementation of thresholds inevitably poses the danger of classification inaccuracies. Analytical thresholds can lead to the misclassification of non-reproducible noise peaks as alleles if they above the threshold, or the omission of real alleles if their peaks are below the threshold. Stochastic thresholds have inherent dangers, including the misclassification of heterozygous genotypes as homozygous when one allelic peak is absent, or the erroneous identification of homozygous genotypes as potentially heterozygous if the solitary peak falls below the threshold (Gill, P., Haned, H., Bleka, O., Hansson, O., Dørum, G. and Egeland, T., 2015) [19].

Defining thresholds by statistical analysis of empirical data enables laboratories to assess the relative risk of mistakes associated with every specific threshold. Employing a statistically robust methodology, laboratories can determine acceptable error rates for forensic casework analysis, so guaranteeing a balance between dependability and practicality in their analyses.

Discussion

Quantitative real-time PCR is a technique used to quantify the amount of DNA in a sample. In forensic genetics, qPCR helps determine whether a sample contains sufficient DNA for further analysis. Thresholds in qPCR are used to:

- **Set Detection Limits:** Establishing a baseline fluorescence level above which DNA is considered detectable.
- **Assess Sample Quality:** Determine if the DNA quantity meets the minimum requirements for subsequent procedures, such as STR analysis and an indication of degradation.

Accurate threshold settings in quantification of extracted DNA trace (i.e. qPCR) are vital to avoid misinterpretation of DNA quantities, which can lead to either unnecessary consumption of resources on samples with insufficient DNA or the overlooking of samples that are actually suitable for analysis. DNA quantification is essential to adjust the DNA template amount to an optimal range for STR amplification reactions. This quantification ensures that STR profiles fall within the linear detection range of the capillary electrophoresis instrument, minimizing artefacts and stochastic effects. For example, Ricci *et al*., [17] use the DNA quantification results below 0.003 ng/µL in the minimum extraction volume of 40 µL to decide against further analysis of the DNA extract. This decision is based on performing validation in the laboratory by evaluating their method's limitations and a strong focus on cost-benefit analysis. By ensuring the financial prudence of their choice, they use the minimal amount of DNA necessary to create robust genetic profiles suitable for inclusion in the Italian DNA Forensic Database. Ricci, U., Ciappi, D., Carboni, I., Centrone, C., Giotti, I., Petti, M., Alice, B. and Pelo, E., 2024.) [17].

Forensic DNA profiling use the established technique of PCR. High quantities of DNA typically yield clear profiles; nevertheless, stochastic effects can significantly influence results with low copy number DNA. (Weusten, J. and Herbergs, J., 2012) [20]. Thus, the outcomes become less definitive when mixtures are examined, particularly when the DNA quantity is quite low. It is crucial to comprehend the stochastic effects that can occur during PCR amplification.

When DNA inputs are low or of poor quality, stochastic effects during PCR amplification become significant, as extensively documented in the literature. Each DNA strand has a limited probability of successful amplification, meaning it may not always produce a detectable signal. With abundant DNA, the likelihood that none of the strands amplify is negligible; however, with only a few DNA strands, this probability increases. Consequently, stutter peaks can reach intensities comparable to allelic peaks, some alleles may not amplify to detectable levels (allele drop-out), and replicate analyses of the same DNA sample can yield varying results. (Weusten, J. and Herbergs, J., 2012; Whitaker, Cotton, Gill, 2001; Gill, Curran, Elliot, 2005; Gill, Brenner, Buckleton, Carracedo, Krawczak, Mayr, Morling, Prinz, Schneider, Weir, 2006; Michel, Bast, Vandenbroere, Froment, 2009; Cowell, 2009; Cowell, Lauritzen, Mortera, 2010; Petricevic, Whitaker, Buckleton, Vintiner, Patel, Simon, Ferraby, Hermiz, 2010) [20-27]. This interpretation threshold is referred to sometimes as the stochastic threshold.

These effects, which manifest when DNA inputs are scarce or of inferior quality, can lead to phenomena such as allele drop-out, allele drop-in, sister allele imbalance, and increased stutter. Understanding these effects is vital for accurate forensic genetic analysis.

Capillary electrophoresis is employed to separate and detect STR amplicons based on size. The resulting data are visualized as electropherograms, where peaks represent

alleles. In capillary electrophoresis (CE) for short tandem repeat (STR) analysis, stochastic thresholds (the point at which the likelihood of data omission in compromised samples becomes significant) signify the likelihood of data omission in compromised samples. These thresholds denote the maximum height at which there is a fair to high likelihood of an absent allele (Stephens, K., Snedecor, J. and Budowle, B., 2022.) [18] The thresholds in forensic DNA analysis serve as benchmarks to differentiate between genuine genetic data and artefacts or noise. They are essential for [18]:

- **Ensuring Analytical Accuracy**: By setting appropriate thresholds, laboratories can minimize the risk of false positives (detecting a signal when none exists) and false negatives (failing to detect a true signal).
- **Standardizing Interpretations**: Thresholds provide a consistent framework for interpreting DNA profiles, which is crucial for reproducibility and comparison across different cases and laboratories.
- **Maintaining Legal Integrity**: In the judicial system, the admissibility and credibility of DNA evidence depend on standardized and validated analytical methods, including the use of thresholds.

Thresholds allow you to assess data quality. For example, on an Applied Biosystems 3500 series capillary electrophoresis instrument, peaks heights correlate to signal intensity or relative fluorescent units (or RFU's). Laboratories have confidence in peaks that reach or exceed your threshold. In forensic DNA analysis, two principal thresholds are utilised in CE: analytical and stochastic:

Analytical Threshold

Analytical threshold represents the minimum relative fluorescence unit (RFU) value that differentiates authentic DNA signals from background noise. Signals above the threshold may be DNA peaks, while signals below are usually disregarded.

Stochastic Threshold (ST)

A higher threshold indicating the peak height above which it is unlikely that allelic dropout has occurred, ensuring that detected alleles are not artifacts. Stochastic thresholds help scientists evaluate when alleles of a heterozygote pair may not be detected. Signals beyond this threshold may indicate either DNA peaks or artefacts, whilst those falling below are generally ignored. Determining this threshold requires assessing the baseline noise of both the chemical processes and the instrumentation. Historically, differing noise levels, particularly in the presence of DNA, required distinct analysis thresholds for each colour. Contemporary amplification kits, including the Applied Biosystems GlobalFiler™ kit, demonstrate negligible noise across all dye channels, even when amplifying negative controls or samples containing 1 ng of DNA. Therefore, a singular analytical threshold may be adequate for these kits.

Upper thresholds are important when reviewing data from samples with high quantities of amplified DNA. Thresholds help assess data quality. Factors that can influence a laboratory's threshold include:

- **Instrument sensitivity**: Different types of instruments, such as capillary electrophoresis (CE) and slab-gel instruments, have different sensitivities.
- **Noise**: The background noise of the chemistry and instrument can vary

Setting thresholds appropriately through internal validation is crucial to accurately interpret DNA profiles, especially in complex cases involving low-template DNA or mixed samples. (National Institute of Standards and Technology. (2022) [28]. Incorrect threshold settings can lead to misidentification of alleles, potentially implicating or exonerating individuals erroneously.

Case Study: Queensland, Australia

The forensic DNA laboratory in Queensland faced significant scrutiny due to its handling of DNA trace, particularly concerning threshold settings. An inquiry revealed that changes in DNA testing thresholds led to the misclassification of samples, adversely affecting the investigation of serious crimes, including rapes and murders [29, 30]. In 2018, the laboratory implemented higher thresholds for DNA detection, resulting in numerous samples being reported as having "no DNA detected" or "DNA insufficient for further processing," even when they contained viable genetic material (reference) This practice potentially compromised thousands of criminal cases, as critical DNA trace may have been overlooked, hindering the resolution of serious offenses [29, 30]

Experts argued that the thresholds were not scientifically validated and were arbitrarily high, excluding potentially probative evidence [29, 30]. Recommendations included lowering thresholds to scientifically justified levels and reanalyzing samples using updated methods. The Commission of Inquiry into Forensic DNA Testing in Queensland concluded that the laboratory's threshold policies were scientifically unsound and recommended a comprehensive review of affected cases [29, 30]. The Queensland experience highlights several key lessons for forensic laboratories worldwide [29, 30].

- **Scientific Validation:** Thresholds must be established based on rigorous scientific validation to ensure they accurately reflect the capabilities and limitations of the analytical methods employed.
- **Regular Review:** Threshold settings should be periodically reviewed and adjusted in light of technological advancements and emerging scientific knowledge.
- **Transparency and Oversight:** Clear documentation and external oversight are essential to maintain the integrity of forensic analyses and uphold public confidence in the justice system.

Case Study: Amanda Knox and Raffaele Sollecito (Meredith Kercher Murder Case, Italy)

In the high-profile case of Meredith Kercher's murder in 2007, DNA evidence played a significant role in the trials of Amanda Knox and Raffaele Sollecito [31]. The interpretation of low-template DNA results and the use of thresholds in analyzing the DNA evidence were heavily scrutinized [31] DNA from a knife handle was reported to match Amanda Knox's DNA, and a trace amount of Meredith Kercher's DNA was found on the blade. Critics argued that the low peak heights and noise levels questioned the validity of the allelic assignments [31]. The stochastic threshold used was deemed inappropriate for low-template DNA, increasing the likelihood of allelic drop-in or drop-out.

Independent experts appointed by the court concluded that the evidence was unreliable due to the absence of rigorous validation for the thresholds used [31] The DNA evidence was ultimately excluded, contributing to Knox and Sollecito's eventual acquittals.

Case Study: The Omagh Bombing Case (United Kingdom)

In the aftermath of the 1998 Omagh bombing, forensic DNA evidence faced scrutiny due to the interpretation of partial DNA profiles. Partial profiles were obtained from LTDNA samples, but the application of thresholds led to exclusions of certain alleles [32]. Defense experts argued that allelic drop-out and stochastic effects were not adequately accounted for in the laboratory's threshold settings. The court found that the DNA evidence lacked robustness due to poor threshold validation and the inherent limitations of LTDNA profiling. This case highlighted the need for well-defined thresholds in cases involving LTDNA to avoid miscarriages of justice [32].

Case Study: The Krystal Beslanowitch Murder Case (United States)

In this cold case from 1995, advancements in DNA technology eventually solved the murder, but the initial investigations faced criticism for their threshold application [33]. Early testing methods excluded potential suspects due to high thresholds for detecting DNA. Later, with the introduction of more sensitive techniques (e.g., Touch DNA), evidence initially deemed insufficient yielded profiles leading to the conviction of the perpetrator. The initial thresholds failed to account for low-template and degraded DNA, delaying justice for over two decades [33].

Lessons from Criticized Threshold Applications

Thresholds must be rigorously validated to reflect the sensitivity and specificity of the technology used. Clear records of how thresholds are determined and applied are essential for transparency and reproducibility. In cases of outdated or criticized threshold applications, a systematic review and reanalysis using updated methods are crucial. Ensuring that forensic analysts and legal professionals understand the implications of thresholds can prevent misinterpretation in court. These cases underscore the critical role of scientifically sound thresholds in ensuring the reliability and admissibility of forensic DNA evidence.[34]

Conclusion

Cut-off and threshold values are fundamental components of forensic genetic analysis, ensuring that interpretations of genetic data are accurate and reliable. The issues encountered in various countries, including Queensland serve as a cautionary tale of the potential consequences when these thresholds are improperly set or applied. Forensic laboratories must prioritize the scientific basis of their methodologies, maintain transparency, and commit to continuous improvement to support the fair administration of justice.

Author Contribution

Kate Simon: Conceptualization, Writing-original draft. Joe H Smith: Supervision, Writing- original draft, review & editing.

Acknowledgements

Declared none.

Conflict of Interest

The authors declare no conflict of interest financial or otherwise.

Financial Support

Not available

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How to Cite This Article
Simon K, Smith JH. Why optimizing thresholds in forensic genetics matters. International Journal of Forensic Medicine. 2024;6(2):30-34.