

PRECISION AND RELIABILITY OF NANOPATE DIGITAL PCR SYSTEM FOR PORK DNA IDENTIFICATION AND QUANTIFICATION

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ABSTRACT

The adulteration and mislabeling of food ingredients, particularly meat products, pose a significant concern for consumers. In order to address this issue, it is crucial to use precise and sensitive methods for species identification, mainly when dealing with samples that contain a limited number of copies of DNA. The primary objective of this study is to design a digital nanoplate polymerase chain reaction (PCR) technique for the purpose of quantifying species identification. The primer used in this study was *cytochrome b gene*. The results showed that target DNA can be amplified using the digital PCR nanoplate until the lowest sample concentration of 0.0013 ng/μL. This study can be applied to enhance species identification processes in food samples by utilizing digital PCR nanoplates.



Keywords: Species Identification, Nanoplate Digital PCR, DNA Quantification

INTRODUCTION

Adulterating meat and meat products is a global problem when it comes to food safety. Consumers are frequently deceived by unclear labels about food product contents or by mixing low-quality meat. In addition, there are numerous deliberate instances of meat product adulteration and mixing, which violate the rights and confidence of consumers and are a significant issue for religious communities (e.g., halal for Muslims and other religious groups), as well as economic and health factors (e.g., the incidence of allergies) (Cao *et al.*, 2020; Khatun *et al.*, 2021). Identifying species in meat products with high accuracy and sensitivity is critical for detecting adulteration and meat mixing in food goods. There are numerous methods for detecting the presence of meat species in food products, such as meat's physical, chemical, and biochemical composition. Unfortunately, this method depends on the age, sex, and quality of the meat, making it highly challenging to identify the species reliably (Bhat *et al.*, 2015).

Using DNA-based techniques, such as DNA barcoding and real-time polymerase chain reaction (RT-PCR), has made substantial advances in animal species identification. Nevertheless, this technique remains semi-quantitative, meaning it can only be employed to identify the species present in a product and cannot be utilized to calculate or measure DNA concentration with absolute quantification. In addition, it should be noted that the accuracy of copy number calculations in quantitative polymerase chain reaction (qPCR) assays can be influenced by factors such as amplification efficiency, standard curve, and DNA purity. Consequently, it is imperative to explore alternative methodologies that offer enhanced sensitivity and enable absolute quantification of DNA content (Karpinen *et al.*, 2022; Ren *et al.*, 2017).

Protein-based techniques (ELISA, liquid chromatography (LC), high-performance LC, and ultra-performance LC) and DNA-based techniques (hybridization, PCR, single-strand conformation analysis, conformation-sensitive gel electrophoresis, and RLEP) have all been used to identify specific species. DNA from mitochondria has more variety than nuclear DNA, making it useful for identifying species (Ballin *et al.*, 2009).

The latest generation of PCR, known as digital PCR (dPCR), is an approach that does not require a standard curve to calculate the exact number of target DNA copies. In the dPCR technique, target DNA is multiplied by partitioning—splitting a single, large reaction into smaller, individual reactions. Because of this, the dPCR method is now more sensitive, tolerant of inhibitors, and able to detect target DNA even in very low quantities (Hindson *et al.*, 2011). Two types of digital PCR

systems are available: droplet-based and chamber-based dPCR (chip). These two dPCR techniques have been extensively used in various domains, including diagnostics, microbiology, diversity analysis, and species identification in food products. The study conducted by Basanisi *et al.* (2020) demonstrates that digital polymerase chain reaction (dPCR) has a high level of accuracy in species identification and possesses a remarkable degree of sensitivity. This is corroborated by a study Shehata *et al.* (2017) utilizing the dPCR method to compute the DNA content of target species even in food mixes. As a result, this technology has the potential to support food authenticity efforts.

In conjunction with technological advancements, a novel chamber-based digital polymerase chain reaction (dPCR) technique known as nanoplate dPCR has been devised. Using nanoplates in this dPCR technique enhances its sensitivity and accuracy, enabling precise determination of absolute quantities of target DNA. This study involved the development of a nanoplate digital polymerase chain reaction technology to detect and quantify the copy number of DNA from target species. In addition, we assessed the reliability of determining DNA copy counts, thereby suggesting the potential application of this approach as a supportive tool for species identification, particularly in the context of food goods

MATERIAL AND METHOD

Sample and Extraction DNA

The meat samples were procured from commercial establishments located in Jakarta. Samples of meat from pig species were used. The extraction process was conducted with the Dneasy Mericon Food kit, manufactured by QIAGEN. The meat sample was dissected into smaller fragments, weighing 200 mg and then transferred into a 2 ml tube. The DNA extraction procedure was conducted following the protocol provided by the DNeasy Mericon food kit. The concentration and purity of the DNA extraction outcomes were assessed using a UV/Vis nano spectrophotometer (Implen N80). After extraction, the DNA was kept at -20 °C before being used for PCR.

Amplifikasi Nanoplate Digital PCR (dPCR)

The detection of pork was accomplished through the utilization of Nanoplate Digital PCR (QIAcuity One 5plex, QIAGEN, Germany). The meat samples were procured from commercial establishments located in Jakarta and will be used for

the whole experiment. The extracted DNA was diluted from 10^{-1} to 10^{-5} and quantified using implan instrumen. However, due to the lack of sensitivity of the instrument, we are unable to quantify the template below 10^{-2} . Thus, we challenge the capability of dPCR to quantify the dilution of 10^{-3} on wards to see the linearity . The primers utilized to amplify pork DNA were taken from Tanabe et al. (2007) (Table 1).

Table 1 The primer used in this study

Forward	5'-CTTGCAAATCCTAACAGGCCTG-3'
Reverse	5' CGTTTGCATGTAGATAGCGAATAAC-3'
Probe	5'-FAM ACAGCTTTCTCATCAGTTAC-MGB-3'

The QIAcuity probe PCR Kit (manufactured by QIAGEN, Germany) was used to do digital polymerase chain reaction (dPCR) amplification,. The reaction mixture consisted of 40 μ L, with 10 μ L of QIAcuity probe PCR master mix, 4 μ L of primer-probe mix (with concentrations of 0.8 μ M and 0.4 μ M), 21 μ L of RNase Free Water, and 5 μ L of DNA template. The mastermix solution was introduced onto the QIAcuity nanoplate 26K 24 well-plate. The amplification process was conducted by initially activating PCR with a heat protocol at 95 °C for 2 minutes. This was followed by a denaturation cycle at 95 °C for 15 seconds and an annealing/extension phase at 60 °C for 30 seconds. This entire process was repeated for a total of 40 cycles. The imaging step is used gain six and exposure duration 500 ms. Collecting data in green channel. The amplification procedure was carried out in three repetitions. Once PCR is finished, QIAcuity Software Suite version 2.2.0 (QIAGEN, Germany) will automatically analyze the copy number.

RESULTS AND DISCUSSION

DNA Concentration and Purity

The primary step in genomic research is isolating genomic DNA, which can be used for species identification, gene polymorphism, DNA fingerprinting, or gene sequencing. In addition, measuring the outcomes of genomic DNA extraction is crucial. One method that can measure the concentration and purity of genomic DNA extraction results is a nano spectrophotometer with an absorbance length ratio of A260/A280, which indicates DNA purity. Meanwhile, DNA concentration can be quantified using the Lambert-Beer law from A260 wavelength analysis (Lewis et al. 2010).

According to the readings from the nano spectrometer, the DNA extraction concentration value obtained was 130 ng/L. The absorbance A260/A280 ratios were determined to range from 1.8 to 1.9. These results indicate that the purity of the DNA concentration obtained is good. Lucena-Aguilar et al. (2016) state that an absorbance value of 1.8 to 2.0 indicates good DNA purity. When the ratio value is less than 1.6, it suggests that contamination originates from protein, phenol, or other substances detectable at the A280 wavelength.

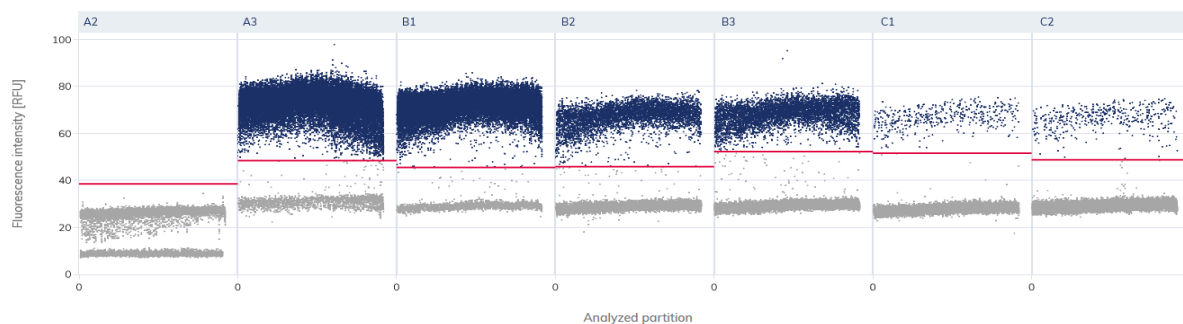
DNA Target Detection and Quantification by Nanoplate Digital PCR

The utilization of mitochondrial genes has been widely used to simplify the process of species identification. Even in the genomic era of today, species and species complexes are identified using a single locus of mitochondrial genes. A segment of the mitochondrial gene that possesses potential for species identification is *cytochrome b (cytb)*. The *cytb* gene is a mitochondrial gene that has been remarkably conserved throughout evolution. This particular gene is also present in all mammals, indicating a significant number of copies, and can be utilized for qualitative species identification (Floren et al., 2015).

The results of this study demonstrated that digital PCR can qualitatively identify species. The assay of nanoplate digital PCR obtained a range from 3051.8 copies/ μ l at the lowest dilution (10^{-3}) to 22.85 copies/ μ l at the highest dilution (10^{-5}) (Table 2). The success of amplification in digital PCR can be observed through a 1D scatterplot that demonstrates the presence of two fractions, with the upper fraction indicating the positive fraction (successful amplification of the target DNA) and the lower fraction indicating the negative fraction (absence of the target DNA) (Figure 1). Based on (Table 2), it can be observed that the results of the copy values from the three repetitions exhibit consistent values. This proves that the results of target DNA amplification can be consistently quantified using digital PCR. The research conducted by Sanders et al. (2011) demonstrates that digital PCR amplification yields high sensitivity and consistency for the same target DNA. Furthermore, a study conducted by Cao et al. (2020) aimed to compare the sensitivity of digital PCR and qPCR in detecting *Laptiplantibacillus plantarum*. The results showed that digital PCR was significantly more sensitive, as it was able to detect concentrations as low as 10^{-2} CFU/mL, whereas qPCR failed to detect at that concentration. According to Umi et al. (2023), digital PCR can do the quantification of porcine DNA down to ~1 copies/reaction where this result is undetected in qPCR

Table 2 DNA sample amplification quantification results

Dilution Factor	Quantification results (copies/ μ l)					
	Repetition 1	Repetition 2	Repetition 3	Mean	Standard Deviation	CV
10-3	3051.8	2842	3053.4	2982,1	121,5	4,1
10-4	294.3	277	297.6	289,6	11,06	3,8
10-5	22.85	21.65	32,24	25,58	5,7	22,6



Picture 1 1D scatterplot of the results of amplifying samples on digital PCR nanoplates. (A2) NTC; (A3-B1) sample with dilution 10^{-3} ; (B2-B3) sample with dilution 10^{-4} ; (C1-C2) sample with dilution 10^{-5}

PCR has been widely employed in a variety of genomic applications, including species identification. The use of microfluidic technologies led to the creation of digital PCR. Droplet-based technology digital PCR, often known as droplet digital PCR (ddPCR), is the most extensively used type of dPCR today. The target is disseminated into homogenous picoliter or nanoliter droplets that are largely independent using ddPCR. Droplet digital polymerase chain reaction (ddPCR) examination of samples is highly susceptible to bubbles and residual microbubbles, interfering with correct interpretation of sample results (Schuler et al., 2016). Various solutions have been developed to address this issue, including the use of a vacuum-Nakayama et al., (2010).

The chamber-based dPCR (cdPCR) was developed as a solution to address the bubble issue encountered in previous generations of dPCR. cdPCR uses a pressure mechanism to distribute targets, allowing them to enter and disperse uniformly within the chamber. This technique eliminates the possibility of bubbles in the sample. Duanmu et al. (2023), applying pressure on the microdroplet PCR

instruments has been found to mitigate the occurrence of microbubbles during the distribution of targets, enhancing the accuracy of the acquired results.

Nanoplate digital PCR is a type of chamber-based dPCR technique. Using the dPCR technology to check food product adulteration, in particular, has yet to be thoroughly investigated to improve species identification. Currently many meat adulteration is performed in qPCR platform as it provide fast and sensitive detection. However, according to He et al. (2022), it is difficult to decide if the adulteration was intentionally added or due to contamination. Hence, quantification is required to see the percentage of adulteration and dPCR can provide the absolute quantification. According to Umi et al. (2023), the dPCR has lower limit of detection compared to qPCR as well as PCR efficiency by looking at R2 values. It is mentioned that the PCR efficiency of dPCR and qPCR are 0.9998 and 0.9971 respectively. The findings of the aforementioned study support the reality of this claim. The dPCR nanoplate can recognize and measure target DNA that has been diluted up to 10^{-5} from its original concentration of 0.0013 ng/L. This work illustrates the feasibility of establishing a nanoplate digital PCR

technique to aid in the identification of species in samples, particularly those containing DNA with low copy number.

CONCLUSION

This research could help to improve analytical techniques used in quality control and food safety, notably in the detection and identification of species present in limited quantities. The use of the dPCR nanoplate technology has been demonstrated to improve sensitivity, efficiency, and consistency in DNA analysis. This technology is extremely advantageous since it includes a partition mechanism that effectively distributes DNA molecules into distinct nano partitions. Aside from that, the findings of this study support the use of the mitochondrial gene, *cyt b*, as a tool for identifying animal species.

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