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Lysis Buffers for Detecting Pork Content in Food Products

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Abstract: *The increasing number of counterfeit processed food products with non-halal ingredients, such as pork, has caused public concern about the halalness of Indonesian food products, especially in the Muslim community. This issue has prompted the development of halal authentication techniques, including deoxyribonucleic acid (DNA) isolation followed by polymerase chain reaction (PCR) amplification to detect pork DNA. The main purpose of this research was to modify the lysis buffer using tris-ethylenediaminetetraacetic acid, cetyl trimethylammonium bromide, salt, alkaline, and urea. Two DNA extraction methods were evaluated using kit-based and conventional methods with modified lysis buffer. Using a fluorometer, it was proven that the conventional method with modified urea lysis buffer produced quite competitive DNA concentrations (119 ng for pork meat and 100.05 ng for pork sausage), as confirmed by real-time PCR analysis. As a result, the modified urea lysis buffer can be further developed because it is more affordable.*

Keywords: *DNA extraction; halal foods; halal testing; lysis buffer; real-time PCR*

INTRODUCTION

Indonesia is recognized globally as the nation with the highest number of Muslims. In total, there are 276,534,400 people, and 87.4% of them are Muslims [1]. Muslims have provisions that must be followed, one of which is to comply with Islamic law regarding the halal and haram of foods consumed. The rise of the manufacture of processed food from non-halal ingredients is a particular concern for the Muslim community in Indonesia. In this case, the government plays an active role in increasing awareness of the importance of safe and halal food consumption in accordance with halal standards and regulations regulated in law [2].

The detection of food adulteration has become an emerging issue for authorities and industries [3]. In Islam, adulteration of any pig-derived ingredients—such as meat, skin, lard, enzymes, or other derivatives—is strictly prohibited, and their presence, regardless of the amount, renders the food product haram [4]. It has been reported that beef or mutton-based products often contain additional animal meats, particularly poultry and other types of meat such as pork. Some producers have fraudulently mixed different kinds of meat to reduce production costs and increase profits [5]. Furthermore, many scientific studies [6-9] have detected the presence of poultry and pork in raw, cooked, and processed meat products. Research on food adulteration have also been conducted by Karabasanavar [10], Song et al. [11], Chang et al. [12], and Hossain et al. [13]. Singh Yadav et al. [14] successfully extracted DNA from salted fish using the Dneasy Mericon Food Kit (50) from Qiagen, achieving a concentration range of 24,600–27,150 ng/μL.

Halal detection technology for a product that is currently widely used and offers fast results continues to be developed, one of which is a deoxyribonucleic acid (DNA)-based method that is then analyzed using real-time PCR. DNA is genetic information owned by every living thing in the form of a long double-helix molecule. It has a polymer chain that is paired and unbranched and is formed from the same four types of monomers. DNA monomers are called nucleotides. DNA is a double-stranded polynucleotide with characteristic constituent components, including deoxyribose sugar, phosphate groups, and nitrogenous bases (adenine (A), guanine (G), thymine (T), and cytosine (S)) [15].

The process of separating DNA from other cellular constituents, such as proteins, carbohydrates, lipids, and other impurities, is known as DNA extraction [16]. DNA extraction consists of three main stages: cell wall destruction (lysis), DNA separation from contaminants, and DNA purification. The lysis stage is the critical initial step in the DNA extraction process. During lysis, the cell membrane is disrupted, allowing the release of cellular contents, including DNA, into the extraction medium. This membrane breakdown can be achieved through various methods, including physical disruption, chemical treatment, or enzymatic digestion. This stage is crucial because if the cell membrane remains intact, the DNA will remain trapped within the cell, making it inaccessible for further processing.

DNA extraction methods are divided into 2 types: conventional and kit-based. Conventional methods require several stages and the addition of chemicals such as phenols and chloroforms to separate DNA from other cellular components. This method requires the preparation of relatively complicated tools and materials. Meanwhile, DNA isolation using a kit is more practical because one package of the kit contains a ready-to-use isolation solution that saves time but is relatively more expensive [17]. This commercial kit uses a filtration column or magnetic technology to separate DNA from the sample, allowing for a faster extraction process.

DNA extraction is a critical stage that plays an important role in determining the success of subsequent molecular analysis [18]. PCR is an *in vitro* DNA amplification technique that involves a repetitive cycle of three main stages [19]. The denaturation stage is performed at high temperatures to separate the double strands of DNA into single strands. Furthermore, during the annealing stage, the primer specifically attaches to the target region on the DNA of the template that has been separated. The final stage is elongation, where the Taq polymerase enzyme synthesizes a new DNA strand by adding complementary nucleotides to the primer. The main advantage of PCR is its high sensitivity, therefore, it has good potential for replicating DNA [20].

The PCR requires several critical components so that the process is effective. Among these, the DNA template serves as the starting material, providing the specific sequence to be amplified. The amplification process is guided by a pair of forward and reverse primers, which are short oligonucleotides designed to complement the target sequence within the DNA template. These primers are essential for initiating DNA synthesis during PCR. Additionally, deoxynucleotide triphosphates (dNTPs) are necessary substrates that provide the building blocks for the new DNA strands. The reaction also requires a PCR buffer that maintains the optimal pH and ionic strength, which are crucial for enzyme activity and stability. Magnesium chloride ($MgCl_2$) is another key component, as it acts as a cofactor for the enzyme Taq DNA polymerase, enhancing its activity and ensuring the accuracy of DNA synthesis. Thermus aquaticus is a thermophilic bacterium that produces the thermostable enzyme Taq DNA polymerase. This enzyme is responsible for the elongation of DNA strands by the addition of dNTPs to the DNA chain. Each of these components plays an indispensable role in the successful execution of the PCR process, contributing to the high specificity and efficiency of DNA amplification [19].

The detection of pig DNA in food has been widely performed in previous studies, but no studies have specifically compared the modifications of various lysis buffers and kits for real-time PCR analysis. Many studies have utilized conventional DNA extraction methods and applied variations at each process stage. However, this study made no modifications during the DNA separation from contaminants and precipitation stages. Instead, the focus was placed exclusively on the lysis stage, where different lysis buffers were used to assess their impact on producing crude DNA. Therefore, the objective of this work was to compare kit-based DNA isolation methods and conventional methods modified with lysis buffer in terms of the production of DNA concentrations from food samples and the effect of DNA concentration on cycle quantification (C_q) values in PCR analysis.

■ EXPERIMENTAL SECTION

Materials

Fresh pork meat and Sulawesi pork sausage were purchased from a supermarket in the Surabaya area of East Java. The chemicals used include aquifers, phenols:chloroform:isoamyl alcohol (24:24:1) (Himedia), Tris-HCl ($C_4H_{11}NO_3$, Promega), cetyltrimethylammonium bromide buffer (CTAB) ($C_{19}H_{42}BrN$, Promega), sodium chloride (NaCl, Sigma Aldrich), ethylenediaminetetraacetic acid (EDTA, $C_{10}H_{16}N_2O_8$, Promega), urea (CH_4N_2O, Sigma Aldrich), sodium hydroxide (NaOH, Sigma Aldrich), sodium dodecyl sulfate (SDS, $C_{12}H_{25}NaO_4S$, Sigma Aldrich), proteinase-K (Promega), isopropanol (C_3H_8O, Sigma Aldrich), ethanol (C_2H_6O), DNA isolation kit (Maxwell® RSC Pure Food GMO and authentication kit), Tris-EDTA buffer (Promega), and real-time PCR kit (Pork) Food Safety Halal Food Cytochrome (Lifif).

Instrumentation

The DNA extraction process was carried out using several instruments, including an analytical balance (RADWAG, AS220R2), a digital heat block (Benchmark, BSH1001), a vortex mixer (Bench Mixer), a microcentrifuge (Benchmark, MC-12), and an automated DNA extraction system (Promega, Maxwell® RSC). Meanwhile, the instruments used for analysis included a Quantus™ Fluorometer (Promega), a spindown (Benchmark, MyFuge™ Mini), and a real-time PCR system (Bio-Rad, CFX Connect™).

Procedure

DNA extraction

The DNA extraction process was carried out through 3 main stages: cell lysis, separation, and purification. The pre-treatment process was initiated by cleaning the fat remaining attached to the pork. The pork meat and sausage were cut into small pieces and mashed before being weighed. In this study, five modifications of the lysis buffer Tris-EDTA, CTAB, salt, alkaline, and urea) were carried out. The Tris-EDTA lysis solution was composed of 10 mM Tris-HCl, 20 mM EDTA, and 2% w/v SDS at pH 8.0. The CTAB lysis solution consisted of 10 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, and CTAB solution. The salt lysis solution was made of 10 mM Tris-HCl, 20 mM EDTA, 2% w/v SDS at pH 8.0 and NaCl 0.4 M. The alkaline lysis solution was made of 0.5 M NaOH, 20 mM EDTA, and 2% w/v SDS pH 8.0. The urea lysis solution was made of 10 mM Tris-HCl, 20 mM EDTA, 2% w/v SDS, and 6 M urea at pH 8.0.

Lysis buffer modification. This method was modified from Yalçınkaya et al. [21]. As much as 250 mg of the crushed sample was dissolved in 560 µL of a pre-prepared Tris-EDTA lysis solution. The mixture was vortexed for 3 s and then incubated for 15 min at room temperature of 37 °C. The pre-processed sample was added 30 µL of Proteinase-K and then mixed with the vortex. Then, it was incubated for 1 h at 60 °C. After incubation, the mixture was centrifuged at 16000 ×g for 5 min. The upper phase of the centrifugation result was transferred to a new tube. Next, 300 µL of phenol-chloroform-isoamyl alcohol solution with a ratio of (25:24:1) was added to the supernatant and homogenized using the pipetting technique. After the mixture was evenly distributed, it was centrifuged at 16000 ×g for 20 min. The upper phase of the centrifugation result was transferred to a new tube, and 300 µL of 10 M ammonium acetate was added. Then, the mixture was centrifuged at 16000 ×g for 10 min. The resulting supernatant was washed with 600 µL of isopropanol and centrifuged at 16000 ×g for 10 min. The resulting supernatant was discarded while the sediment (pellets) was washed with 300 µL of 70% ethanol. After the pellet dried, it was dissolved in 25 µL TE to quantify its DNA. The same process was performed for CTAB lysis solutions, salts, alkaline, and urea.

Pure food isolation kit. The process of DNA isolation using a commercial kit of pork begins by weighing

200 mg of the crushed sample and inserting it into a 2 mL microtube. The sample was added 600 µL CTAB buffer. Then, 2 µL of RNase A and 30 µL of Proteinase-K were added to each microtube. The liquid and sample were mixed using a vortex and stirred until well combined. After being evenly distributed, the samples were incubated in a heat block at 60 °C for 30 min. After incubation, the samples were homogenized using a vortex until smooth and then centrifuged at room temperature for 10 min at 1600 ×g. In the maxwell cartridge (#1) 300 µL of lysate sample was added, and it was ensured that only the liquid was transferred. Then, 300 µL of the lysis buffer was added to the maxwell cartridge in #1. The plunger was installed at position #8, and a 0.5 mL elution tube containing 100 µL of elution buffer was installed at the end of the tool. Samples were extracted using the maxwell semi-automatic extraction tool. The extraction process was completed in approximately 40 min. Successfully extracted DNA was placed in an elution tube [22].

Real-time PCR

In the second stage, real-time PCR was performed after obtaining the DNA solution. A real-time PCR kit (pork) Food Safety Halal Food Cytochrome was used to determine DNA amplification in the samples. There are 3 types of samples that need to be prepared, namely test DNA samples, positive controls, and negative controls. Table 1 shows the components of the samples used in the PCR analyses. This process consists of several stages, namely the target DNA amplification cycle, which consists of initial denaturation at 95 °C for 10 min, followed by 35 cycles with denaturation conditions at 95 °C for 10 s, annealing at 64 °C for 20 s, and elongation at 64 °C for 20 s. The detection of PCR products was performed continuously using fluorescent probes based on the increase in the fluorescent signal emitted by the specific probe, which allowed real-time quantification of amplicons.

DNA quantification

The concentration of isolated DNA from both methods was determined using fluorometric quantification with the Quantus™ Fluorometer. Before measurement, a standard sample was prepared by mixing 1 µL of standard Quantifluor Lambda DNA and 200 µL of Quantifluor ONE dsDNA Dye in a 0.5 mL microtube. Blank samples were prepared by adding 200 µL of Quantifluor ONE dsDNA Dye to a 0.5 mL microtube. The last stage was preparing samples to be tested. As much as 200 µL of Quantifluore One Dye was inserted into a new 0.5 mL microtube. In the same tube, 10 µL of sample was added and vortexed until evenly distributed. Next, the sample was incubated at room temperature for 5 min and make sure that the sample was protected from light. Quantus™ Fluorometer was calibrated with blank and standard sample readings. Once the calibration was complete, the tested sample can be inserted into the instrument and read for its DNA concentration.

Statistical analysis

Analysis of variance (ANOVA) is a statistical technique for comparing the means of two or more group [23]. ANOVA makes it possible to evaluate the influence of one or more independent variables on dependent variables, as well as identify possible interactions between variables. There are several types of ANOVAs, including one-way ANOVAs that analyze the influence of one factor on dependent variables and two-way ANOVAs that consider two factors at once and the interaction between the two. In this study, a full factorial design was used, in which a two-way ANOVA was applied to test the combined influence of two independent variables and their interactions on the observed results. This factorial design allows for a more in-depth and comprehensive analysis of the relationships between the variables tested.

Table 1. Components of the PCR test samples

Component	Sample tested	Positive control	Negative control
Extracted DNA	5 µL	-	-
Dissolved positive control	-	5 µL	-
Nuclease free water	15 µL	15 µL	20 µL

■ RESULTS AND DISCUSSION

DNA Extraction

DNA extraction is a fundamental process in molecular biology, crucial for isolating high-quality DNA for further analysis. To achieve optimal results, the extraction procedure must ensure that the DNA is intact and free of contaminants. This process involves carefully breaking down cellular barriers to access and purify DNA without compromising its structural integrity. In general, the three primary steps of DNA extraction are cell wall or membrane destruction (lysis), separation from impurities, and DNA purification [16]. In this study, several modifications were made to the lysis buffer. These modifications included the addition of EDTA, CTAB, salt, NaOH, and urea. The modification of the lysis buffer with the reagents aims to maintain the structure of DNA during the destruction and purification process, making it easier to remove proteins and prevent the activity of DNA-degrading enzymes and changes in DNA molecules. The pH of the modified lysis buffer was maintained at pH 8.

The cell is encased by an outer boundary known as the cell membrane, which encapsulates all its internal components. The membrane functions as a selective barrier, managing the movement of materials in and out of the cell, thus maintaining its internal environment. However, it is necessary to disrupt or break down the cell membrane to access the DNA located within the cell for purposes such as molecular diagnostics. Proper disruption techniques are essential in molecular biology to ensure that the DNA remains intact and uncontaminated for accurate testing [24]. Cell lysis is performed mechanically using a mortar and pestle to destroy the cell wall and chemically using detergents and enzymes. SDS as a detergent aims to dissolve lipids in the cell membrane so that cell membrane destabilization occurs [25]. Switzer stated that SDS plays a role in reducing the activity of nuclease enzymes, which are DNA-degrading enzymes [26]. Another detergent used in this study was CTAB, which can precipitate nucleic acids and acid polysaccharides from solutions with low ionic strength while proteins and neutral polysaccharides remain in solution [27]. In addition to detergents, the enzyme Proteinase-K was added to destroy proteins by breaking down protein peptide bonds during the extraction process. Furthermore, incorporating proteinase-K into the DNA extraction protocol has been reported to enhance both the concentration and purity of the extracted DNA [28].

The main component used to lyse the cell membrane in alkaline lysis is hydroxide (OH^-) ions. The OH^- ion breaks the fatty acid-glycerol ester bonds in the cell membrane, making the membrane permeable. Then, SDS dissolves the proteins and the membrane [24]. In addition to ionic and non-ionic detergents, chaotropic agents such as urea and EDTA can be used for cell lysis. These agents disrupt the structure of water, reducing its hydrophilicity and consequently weakening hydrophobic interactions. The addition of urea can be used as a protein regulator at a concentration of 6–8 M [29-30]. EDTA deactivates nuclease enzymes by binding magnesium and calcium ions, which are required as cofactors for DNase activity [16]. Another reagent of lysis buffer was Tris-HCl, which is used as a buffer to prevent DNA damage by maintaining a normal pH of pH 8.

In this study, the procedures and types of reagents used in the separation and purification stages were applied consistently to all samples, with no variations in treatment. This uniformity aims to minimize result variability that might arise from differences in procedures or reagents, allowing the research to focus more precisely on the effects of lysis buffer modifications on the resulting crude DNA. The separation stage was carried out by the addition of phenol:chloroform:isoamyl alcohol to bind proteins, lipids, carbohydrates, and other contaminants [31]. After centrifugation, two separate phases were formed, namely the organic phase in the lower layer and the aqueous (water) phase in the upper layer, while DNA and RNA are in the aqueous phase after centrifugation, while the denatured proteins are in the interphase and lipids are in the organic phase.

The purification stage was carried out by the addition of isopropanol followed by ethanol. The DNA obtained was still dissolved in water, so isopropanol was added for precipitation. Water molecules that previously formed a hydration layer around the DNA are attracted to the isopropanol molecule. This phenomenon causes the environment around DNA to become less polar, resulting in DNA becoming less soluble and precipitating. The ethanol washing stage was performed by centrifugation to purify DNA. Ethanol was evaporated in a vacuum chamber until a pure DNA pellet precipitate was obtained. Crude DNA was dissolved in TE buffer for the quantification.

The results of DNA extraction using conventional and kit-based methods can be seen in Table 2. Based on the results of measurements using a Quantus™ fluorometer on both samples, the DNA extraction results were obtained in the range of 6.55–16.50 ng/μL. DNA concentration is one of the important parameters that indicate the effectiveness of the extraction process. A high concentration indicates high efficiency of the production process. Table 2 shows that commercial kits produce higher DNA concentrations than conventional methods. This can happen because commercial kits have been optimized. In the conventional method, the highest concentration was obtained by modifying the lysis buffer with the addition of urea. Meanwhile, the modification with the addition of salt occupies the second position in producing the highest DNA concentration. This result is in accordance with a previous study [21], in which the addition of urea and NaCl to the lysis buffer resulted in a higher concentration of DNA. Yalçınkaya et al. [21] stated that the DNA concentration obtained from beef using the urea method was 41.3 ng/μL, while the DNA concentration obtained using the salt method was higher (49.6 ng/μL).

In the DNA extraction process, protein denaturation is an important step in separating DNA from protein contaminants. The denaturation mechanism by urea involves a combination of direct interactions with proteins and indirect effects through disruption of the solvent environment. Directly, urea binds to the peptide backbone and protein residues, especially after the protein's secondary structure is disrupted, replacing water in the formation of hydrogen bonds. Indirectly, urea disrupts the structure of water by weakening interactions between water molecules and reducing hydrophobic effects, thereby facilitating the dissolution of nonpolar residues. Bennion stated that at high concentrations, urea can accelerate the dissolution of hydrophobic groups by altering the orientation of surrounding water molecules and creating a more favorable solvent environment to expose nonpolar residues. The combination of these two effects accelerates protein unfolding and shifts the equilibrium towards a non-native (denatured) state. Similarly, urea may bind to water around the DNA, providing an opportunity for the DNA to detach from the protein complex [32].

The addition of salt to buffer lysis can damage cells. This happens when the salt concentration around the cell suddenly changes, creating a difference between the internal and external environments, allowing the cell membrane to be permeable to water due to osmosis [24]. When the salt concentration in the surrounding solution drops, water enters the cell, causing it to swell and eventually burst. In addition, high salt concentrations can cause the salting-out process, where proteins become less soluble and settle so that they separate from

Table 2. Crude DNA concentration measurements

Methods	Concentration (ng/μL)		Pellet DNA (ng)	
	Pork meat	Pork sausage	Pork meat	Pork sausage
Conventional –Tris EDTA lysis buffer	8.58 ± 0.78	9.80 ± 0.28	88.50 ± 7.78	98.00 ± 2.83
Conventional – Urea lysis buffer	11.90 ± 0.14	10.01 ± 0.01	119.00 ± 1.41	100.05 ± 0.07
Conventional – Salt lysis buffer	10.20 ± 1.13	9.55 ± 0.64	102.00 ± 11.31	95.50 ± 6.36
Conventional – Alkaline lysis buffer	8.10 ± 0.42	6.55 ± 0.64	81.00 ± 4.24	65.50 ± 6.36
Conventional – CTAB lysis buffer	7.05 ± 0.07	7.00 ± 0.57	70.50 ± 0.71	65.50 ± 6.36
Commercial kits	16.50 ± 0.71	16.00 ± 1.41	165.00 ± 7,07	160.00 ± 14.14

the DNA that remains soluble in the solution. Besides that, detergents can be used to disintegrate the cell membrane, which is composed of a bilayer of lipids containing both hydrophobic and hydrophilic molecules. Detergents can interfere with the interactions between lipid–lipid, lipid-protein, and protein-protein. Combining salt with detergents like SDS allows the release of DNA from the protein-DNA complex. This occurs because SDS has amphipathic properties, meaning it has hydrophilic and hydrophobic parts. SDS interacts with the hydrophobic region of the protein membrane to disrupt the hydrophobic interactions that stabilize the protein structure and cause denature. Meanwhile, salt, in this case, plays a role in helping to release proteins from the membrane so that it can increase the efficiency of denaturation by SDS.

Real-Time PCR

The results of real-time PCR amplification are presented in Table 3, in which the sample was analyzed with repeats accompanied by positive and negative controls. The analysis was conducted using CFX Maestro software to process and interpret the amplification data. The results showed that the samples that tested positive contained pig DNA. The samples that were indicated to contain the least pig DNA were pork meat samples treated with modified Tris-EDTA buffer lysis (13.05) and pork sausage samples treated with modified CTAB buffer lysis, which is 15.02. In general, the value of C_q is inversely proportional to the number of target nucleic acids in the sample. This is in line with research conducted by Whale et al. [33], a low C_q value indicates a high concentration of DNA, while a high C_q value indicates a low concentration of DNA. However, there were conditions where samples with high DNA concentrations exhibited high C_q values. This can be caused by several factors, such as the presence of PCR inhibitors in the sample that interfere with the efficiency

Table 3. Real-time PCR test result

Sample	Fluor	DNA Target	C_q value
Tris-EDTA lysis buffer – Pork meat	FAM	Pig	13.05 ± 1.07
	VIC	Vertebrate	24.11 ± 1.08
Tris-EDTA lysis buffer – Pork sausage	FAM	Pig	12.33 ± 0.28
	VIC	Vertebrate	N/A
Urea lysis buffer – Pork meat	FAM	Pig	11.85 ± 0.37
	VIC	Vertebrate	33.24 ± 1.17
Urea lysis buffer – Pork sausage	FAM	Pig	10.31 ± 1.39
	VIC	Vertebrate	27.47 ± 3.55
Salt lysis buffer – Pork meat	FAM	Pig	10.47 ± 0,49
	VIC	Vertebrate	30.33 ± 0.98
Salt lysis buffer – Pork sausage	FAM	Pig	10.72 ± 0.66
	VIC	Vertebrate	N/A
Alkaline lysis buffer – Pork meat	FAM	Pig	10.89 ± 0.47
	VIC	Vertebrate	25.53 ± 2.63
Alkaline lysis buffer – Pork sausage	FAM	Pig	13.78 ± 0.95
	VIC	Vertebrate	23.18 ± 2.67
CTAB lysis buffer – Pork meat	FAM	Pig	12.93 ± 0.56
	VIC	Vertebrate	N/A
CTAB lysis buffer – Pork sausage	FAM	Pig	15.02 ± 0.29
	VIC	Vertebrate	N/A
Commercial Kits – Pork meat	FAM	Pig	12.78 ± 0.19
	VIC	Vertebrate	26.42 ± 1.22
Commercial Kits – Pork sausage	FAM	Pig	12.06 ± 0.37
	VIC	Vertebrate	N/A

of amplification, resulting in the reaction running more slowly despite the abundant amount of DNA available. In addition, DNA degradation can occur, where the quality of DNA decreases despite high quantities, thereby reducing amplification efficiency.

Samples detected to contain pig DNA have different levels, so the amount of pig DNA after amplification can be estimated using the standard curve equation as follows Eq. (1);

$$y = -0.126x + 16.433 \quad (1)$$

where y is the positive C_q value and x is the estimated concentration of pig DNA in the sample after PCR.

Table 4 shows that the use of real-time PCR resulted in exponential amplification of the target DNA amplicon during repeated cycles. These data show that the number of copies of DNA amplicons from the target DNA that was originally small can increase significantly as the number of cycles increases. A typical real-time PCR chromatogram is presented in Fig. 1. The threshold was set above the detection limit but was still well below the plateau phase, where DNA amplification slowed. A horizontal blue line indicates the threshold points in Fig. 1. In the initial PCR cycle, all DNA in the sample was detected. For example, a pork meat sample was detected at 160 RFU in the 0th cycle.

PCR-based species detection and differentiation methods are commonly applied due to their high specificity, sensitivity, and speed [34]. In this study, the focus of amplification is solely aimed at pig DNA. Therefore, during the denaturation stage, DNA from other species are destroyed, or they do not participate in the amplification process. Only pig DNA is amplified and detected in the final result. This is in line with the research conducted by Che Man [35], where all DNA extraction results can be read via electrophoresis, as shown by the presence of different base pair values. However, after the amplification process using 12S rRNA primers, only samples containing pig DNA remain

Table 4. Estimation of pig DNA concentration after PCR

Lysis methods	Concentration (ng/μL)	
	Pork meat	Pork sausage
Conventional –Tris EDTA lysis buffer	26.84 ± 8.53	32.60 ± 2.19
Conventional – Urea lysis buffer	36.38 ± 0.18	48.85 ± 11.04
Conventional – Salt lysis buffer	47.32 ± 3.93	45.33 ± 5.28
Conventional – Alkaline lysis buffer	43.95 ± 3.75	21.08 ± 7.56
Conventional – CTAB lysis buffer	27.79 ± 4.45	11.19 ± 2.33
Commercial kits	29.18 ± 1.73	34.70 ± 2.92

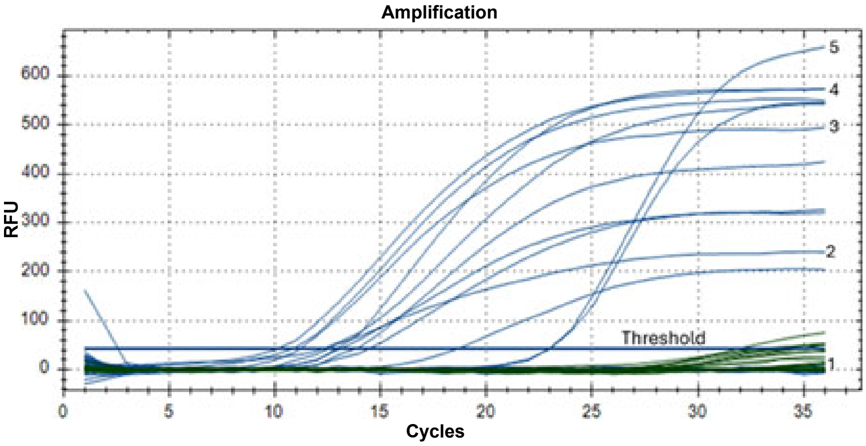

Fig 1. Typical RT-PCR amplification curve. (1) Negative control; (2) pork meat sample with CTAB lysis buffer modified; (3) pork sausage sample with salt lysis buffer modified; (4) pork meat sample with commercial kit; and (5) positive control

readable, and base pair values are generated during the electrolysis process at 387 bp. This suggests that only pig DNA was successfully amplified, whereas DNA from other components was not detected after amplification, confirming the selectivity of amplification against specific DNA targets [35]. Similarly, research conducted by Mohd Hafidz et al. [36] reported that during the initial screening for the presence of pork, the agarose gel electrophoresis analysis of PCR products revealed that 8 of the processed meat samples produced a specific single band for pork DNA, consistently readable at 387 bp.

Overall, Fig. 1 presents the various amplification curves in which most samples eventually crossed the threshold, indicating the success of DNA amplification. However, some curves may remain close to the baseline, indicating that the DNA in the sample was below the limit of detection and may not provide an adequate amplification signal.

Statistical Analysis Using Design Expert

In statistical analysis, the F-value and *p*-value are crucial indicators of a model's effectiveness and the significance of its terms. The F-value assesses how well the model fits the data compared to the variation caused by random error, while the *p*-value helps determine the importance of individual model terms. Together, these values provide insight into whether the relationships observed within the model are statistically meaningful or if they may have arisen by chance. The results of the experiments presented in Table 2 and Table 3 were then analyzed using a factorial design that implemented two-way ANOVA to analyze the combined influence of two independent variables, namely the lysis method and sample type, on the observed results (DNA pellets and C_q value).

The ANOVA results presented in Table 5 indicate that the high Model F-value of 41.07 suggests that the model is significant, with only a 0.01% probability that such a large F-value would occur due to random variation. Similarly, Table 6 shows that the models F-value of 7.97 suggests that the model is significant, with only a 0.06% probability that such a large F-value would occur due to random variation. In this case, the lysis method significantly influenced the DNA pellets and C_q values, which are characterized by *p*-values in the linear model of < 0.05. Meanwhile, the analysis of the sample type variable showed a p-value more than 0.05, which means that there was no significant difference between the sample types, which means that the variation in the sample type did not directly affect the observed results. The 2-way interaction between the lysis method and sample type significantly influenced the DNA pellet and C_q value. This condition was marked by a *p*-value of < 0.05. From the statistical analysis, the R-squared (R^2)

Table 5. The ANOVA results of pellet DNA

Source	Sum of squares	df	Mean square	F-value	*p*-value	
Model	23358.1200	11	2123.4700	41.0700	< 0.0001	significant
A-lysis method	22407.8700	5	4481.5700	86.6700	< 0.0001	
B-sample type	287.0400	1	287.0400	5.5500	0.0363	
AB	663.2100	5	132.6400	2.5700	0.0841	
Pure error	620.5000	12	51.7100			
Cor total	23978.6300	23				

Table 6. The ANOVA result of C_q value

Source	Sum of squares	df	Mean square	F-value	*p*-value	
Model	56.1900	11	5.1100	7.9700	0.0006	significant
A-lysis method	29.9400	5	5.9900	9.3400	0.0008	
B-sample type	2.2700	1	2.2700	3.5400	0.0843	
AB	23.9800	5	4.8000	7.4800	0.0021	
Pure error	7.6900	12	0.6409			
Cor total	63.8800	23				

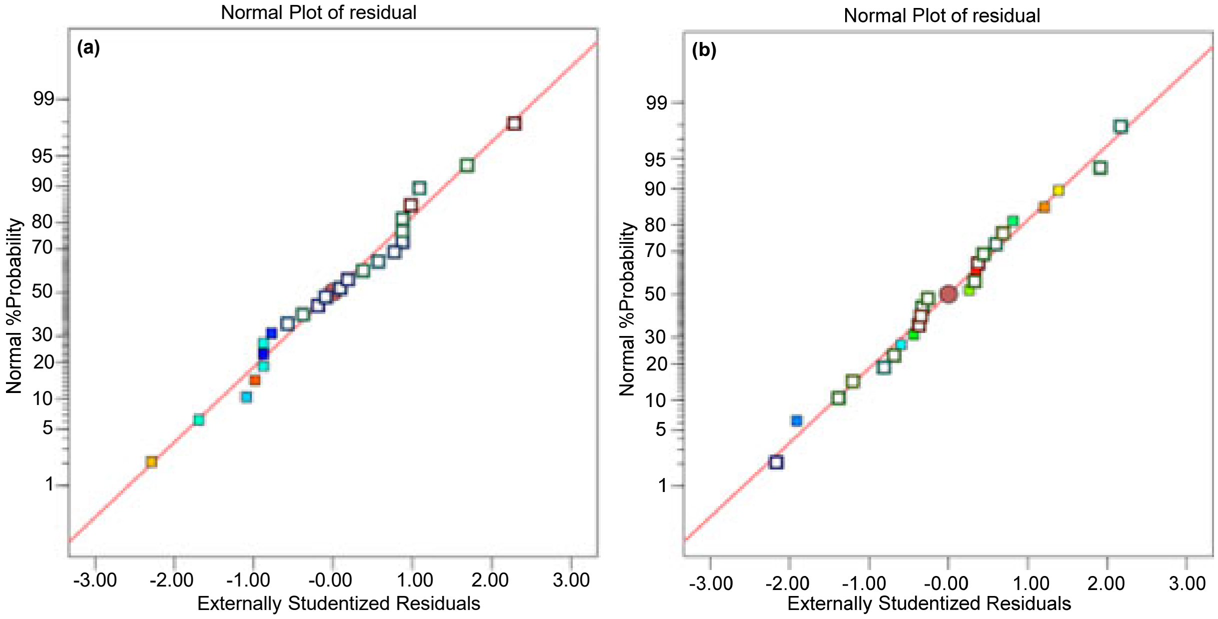

Fig 2. Normal probability plot: (a) response is pellet DNA and (b) response is C_q value

Table 7. Statistical analysis of solutions for combinations of categoric factor levels

Lysis method	Sample type	Pellet DNA	C_q value	Desirability
Commercial kits	Pork sausage	12.060	16.000	0.965
Commercial kits	Pork meat	12.755	16.500	0.955
Urea	Pork meat	11.845	11.900	0.942
Salt	Pork meat	10.470	10.200	0.942
Urea	Pork sausage	10.315	10.005	0.941
Salt	Pork sausage	10.710	9.550	0.932
EDTA	Pork sausage	12.325	9.800	0.914
Alkali	Pork meat	10.895	8.100	0.905

value is 0.9741. The values are close to 1, indicating that the model has a good fit with the data. A high R^2 value reflects that most of the variation in the response variable can be explained by the model.

The normal probability plot graph shows the distribution of pellet DNA data (in nanograms) that tends to follow the normal distribution (Fig. 2(a) and (b)). The dots on the graph show a standardized residual distribution, whereas the red straight line is a reference line that represents the ideal normal distribution. Overall, the data points generally follow a straight line, which indicates that the assumption of data normality is met. This shows that the model is quite valid in terms of the assumptions of residual normality.

The solutions for combinations of categoric factor levels show that commercial kits occupy the top position in DNA extraction effectiveness, indicated by the highest composite desirability values, which are 0.965 for pork sausage and 0.955 for pork (Table 7). This indicates that this method results in a high concentration of DNA and an optimal C_q value. However, conventional methods using urea also show good potential with a composite desirability value of 0.942 in pork and 0.941 in pork sausage, making it a viable alternative to consider in DNA extraction testing. These results emphasize that although commercial kits provide the best results, the urea method remains a competitive choice, especially in cost efficiency and tool availability.

■ CONCLUSION

Using real-time PCR, it was demonstrated that the modified lysis buffer effectively detected pig DNA in all samples, as evidenced by the cycle quantification (C_q) values. The lower the C_q value indicates a higher concentration of pig DNA in the sample. The kit-based DNA isolation method was proven to produce higher DNA concentrations than conventional methods for pork and pork sausage samples (165 and 160 ng, respectively). However, the modification of the conventional method by adding urea to the lysis buffer shows the potential that can be developed in DNA extraction. The DNA pellets of pork meat and pork sausage were 119 and 110.50 ng, respectively. As a result, the modified urea lysis buffer can be further developed because it is more affordable.

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■ CONFLICT OF INTEREST

The authors declare no conflict of interest.

■ AUTHOR CONTRIBUTIONS

Conceptualization, methodology, investigation, data curation, writing—original draft preparation, Ulfah Lailatul Khoiriah; software, Fadlilatul Taufany; validation, resources, Nasori; formal analysis, project administration, Pramudja Baydillah; writing—review and editing, visualization, Raden Darmawan; supervision, Setiyo Gunawan and Fadlilatul Taufany; funding acquisition, Setiyo Gunawan All authors have read and agreed to the published version of the manuscript.

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