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Validation of porcine DNA analysis method for food products using selected primer and exogenous internal positive control in real-time PCR

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A B S T R A C T

The method for porcine DNA analysis using real-time PCR is widely applied in the halal certification process and post-market monitoring. Therefore, this research aimed to validate porcine DNA analysis method using selected primer and exogenous internal positive control (IPC) as an alternative. The experiment was conducted in various stages, namely primer selection, sample extraction, efficiency testing, and method validation. The results of efficiency tests showed that using IPC at half concentration (Exo IPC Mix 5X and Exo IPC DNA 25X) provided reliable amplification with a Ct value of 27.57 ± 0.28 and RFU of 205.5 ± 14.85. The maximum DNA concentration that could be amplified without inhibition was 100 ng/µl. Validation tests showed specificity, sensitivity, linearity, PCR efficiency, and robustness. Among 23 positive and 23 negative samples, two positive samples (porcine collagen and collagen peptide) produced false negatives, while three negative samples had false positives after Ct 42.26. The method achieved a limit of detection (LOD) of 0.01 ng/l at Ct 33.29 ± 0.92, with linearity (r^2 = 0.996) and PCR efficiency (ϵ = 96.32). The results showed robustness to variations in master mix type, primer concentration, and annealing temperature, as well as resistance to inhibitors such as alginate, cellulose, EDTA, calcium ions, collagen peptide, and polysaccharides at 1 g/l. The performance of this method was also compared to the SNI ISO/TS 20224-3:2020 standard, showing potential as a viable alternative for porcine DNA testing. Moreover, further comparative research were recommended to fully establish the efficacy against national standards.

ARTICLE INFO

Keywords:
Internal positive control
Method validation
Porcine DNA
Primer
Real-time PCR

History:
Received 31-03-2024
Revised 12-06-2024
Accepted 15-06-2024

1 Introduction

The examination of food products for potential non-halal content is an essential component of ensuring compliance with halal certification. This process is particularly important in Indonesia, where halal certification is mandated by JPH Law No. 33 (2014) for food, beverages, and various other consumer goods. Therefore, the Ministry of Religion Regulation No. 26 (PP 2019) has set the timeline for mandatory halal certification, starting with food and beverage products between October 17, 2019, and October 17, 2024. To achieve the timeline, the contribution of laboratories is essential by performing various experiments to identify ingredients of questionable halal status. According to Government Regulation No. 31 (2019), several organizations also play significant roles such as Halal Product Assurance Organizing Agency (BPJPH), which is required to collaborate with Food and Drug Monitoring Agency (BPOM).

Regarding product authentication, there are several analytical methods that have been effective, particularly DNA-based. These include real-time PCR, PCR-RFLP, LAMP, and sequencing, as well as protein and lipid-based methods including ELISA, LC-MS/MS, and FTIR (Nakyinsige *et al.* 2012). Among these methods, real-time PCR has been extensively used due to specificity and sensitivity in detecting porcine DNA serving as a popular alternative for species identification and product authentication in the food supply chain (Lazaro & Hernandez 2013).

In Indonesia, porcine DNA testing using real-time PCR varies across laboratories. Some laboratories design apply primers and probes, while others use commercial kits, which offer validated results. Recent research by Cahyaningsari *et al.* (2019) has Qiagen Mericon Pig Kit to successfully detect farmed pork and wild boar DNA in beef-based food. In detecting bovine and porcine gelatin, Zilhadia *et al.* (2017) showed the superior performance of fluorescent probes over SYBR Green. Demirhan *et al.* (2012) also identified porcine DNA in processed food products such as marshmallows and gummy candies using the SureFood® Animal ID Pork Sens Kit, although limited transparency is associated with commercial kits which often conceal the sequences of primers, probes, and target DNA.

The ability of primers to accurately recognize and amplify the target DNA is an essential factor in DNA testing (Lazaro & Hernandez 2013). Regarding porcine DNA analysis, primers obtained from published research, ensure specificity, optimized performance, and validate efficiency through in silico as well as in vitro testing (Bustin & Hugget 2017). Previous research conducted by Brezna & Piknova (2013) has identified highly sensitive primers such as the Tanabe primer that targets the mitochondrial cytochrome b gene using a TaqMan MGB probe. Despite the significant contribution, the successful application of PCR can be compromised by inhibitors found in food matrices affecting DNA polymerase enzymes and nucleic acids. To address this problem, internal positive control (IPC) is often used to monitor and mitigate the effects caused by inhibitors (Schrader *et al.* 2012). Generally, IPC can be endogenous or exogenous which provides more accurate for detecting inhibitors (Ke *et al.* 2000).

There is currently no national or international standard method (SNI, ISO, or AOAC) for porcine DNA testing, showing the need for laboratories to validate their methods. Therefore, this research aimed to validate porcine DNA testing method using selected primers and exogenous IPC as an alternative at the LPPOM MUI Laboratory. The performance of the method was assessed using real-time PCR and results were compared with the SNI ISO/TS 20224-3:2020 standard to provide a reliable procedure for halal certification. The experiment was performed to address the absence of a standardized testing method and provide an alternative that could be widely adopted. This research also addressed challenges in PCR such as the presence of inhibitors in certain food matrices that could cause false-negative results.

2 Methodology

2.1 Materials and Chemicals

This research was carried out between September 2019 and April 2020 at the LPPOM MUI Laboratory, Global Halal Centre Building, Jl. Youth No. 5, Bogor City, 16161 West Java, Indonesia. The equipment comprised Bio-Rad CFX96 real-time PCR, centrifuge, spectrophotometer, analytical

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Halal Studies and Society
Journal homepage https://journal.ipb.ac.id/index.php/hass
https://doi.org/10.29244/hass.1.3.5-11

Balance, Spinner, Vortex Mixer, Reactor thermos-shaker, and micropipettes. Disposable materials were face masks, PCR tube strips, sterile microtubes, spin column tubes, sterile tip filters of various capacities, and powderless gloves.

The materials used for the experiment conducted in this research were divided into samples and reagents. The samples consisted of various meats and meat products, including pork, beef, goat, chicken, duck, salmon, gelatin, collagen, leather, and other derivatives. These samples were sourced from markets, commercial products, and the LPPOM MUI Laboratory collection. Meanwhile, the reagents used consisted of XYZ extraction kit, ABC Master Mix, specific primers and probes, nuclease-free water, and ProAnalyst ethanol.

2.2 Research Procedure

This research was conducted in two stages, the first included determining efficiency of IPC and sample DNA concentration, followed by method validation. The sample extraction validation used a solid-phase extraction method with a spin column binding DNA to the solid (silica) and washing with a wash buffer. The experiment started with a primary selection of published primers based on literature review, focusing on primer sensitivity, target gene, and probe. Based on the results, the selected primers from Tanabe *et al.* (2007) showed the highest sensitivity of 0.0001 ng/µl for pig DNA, targeting the mitochondrial Cyt b gene with a TaqMan MGB probe. Subsequently, the PCR Mix was prepared following the procedures of Tanabe *et al.* (2007) with modifications, including the addition of IPC and specific sample DNA quantities. The PCR conditions followed the ABC Master Mix User Guide with enzyme activation at 95℃ for 10 minutes, followed by 45 amplification cycles. Data analysis was carried out by evaluating IPC concentration efficiency, DNA sample concentration, specificity, sensitivity, linearity, amplification efficiency (ϵ), and robustness tests.

3 Result

3.1 Internal Positive Control Concentration Efficiency

The use of an internal positive control (IPC) is a specific method to ensure true negative results for the PCR inhibition process. The PCR reaction process using IPC is included in multiplex PCR, namely, a PCR amplification process including two or more target DNA. In this research, IPC used was Exogenous Internal Positive Control Reagents, which consisted of 50X Exo IPC DNA with DNA IPC template and 10X Exo IPC Mix containing IPC primers and probes. IPC template DNA is synthetic DNA that does not exist in nature or the tested samples (Schrader *et al.* 2012).

In the PCR reaction process, IPC DNA was detected using a probe labeled with fluorescent VIC, and pig DNA was detected through a probe labeled with fluorescent FAM (6-Carboxyfluorescein). True positive and true negative results were indicated by the amplification of IPC on VIC fluorescein. However, when IPC is not amplified, there is inhibitory activity in the PCR process. IPC performance can be evaluated through experiments with variations in IPC concentration as shown by the amplification results, namely the Ct value when crossing the threshold line and the retention percentage unit (RFU) value.

The experiment was conducted to determine the lowest concentration of IPC that could be used to reduce testing costs. IPC in Formula 2 (Exo IPC Mix 5X and Exo IPC DNA 25X), namely the concentration made half lower than the initial concentration (Formula 1), was still amplified at a Ct value of 27.57 ± 0.28 and RFU 205.5 ± 14.85, as shown in Table 1 and Figure 1a. Meanwhile, IPC in Formula 3 (Exo IPC Mix 2.5X and Exo IPC DNA 12.5X), a quarter of the initial concentration, was still amplified but at a greater Ct (29.74 ± 0.78) with a lower RFU value and close to the threshold line, namely 103 ± 8.49, as shown in Table 1 and Figure 1c. Lower concentrations of IPC DNA were amplified at greater cycles, while IPC Mix provided lower fluorescence values and faster plateau curve formation when compared with Formulas 1 and 2, as shown in Figures 1b and 1d. The concentration of primers, probes, master mix, and DNA templates was considered the limiting factor in PCR amplification (Jansson & Hedman 2019). In Formula 4, there was a minimum concentration limit that was unable to amplify the IPC DNA and was considered unsuitable for application. According to the results, to obtain good IPC amplification performance, efficiency could be achieved by using half the initial concentration.

3.2 Maximum DNA Sample Concentration Efficiency

The efficiency test was carried out by examining pork DNA at an initial concentration and diluting to produce 100% true positives in 3 repetitions, as shown in Figure 2. As shown in Table 2, pork DNA used had the highest concentration obtained from the specificity test, namely from the extraction results of pork 3 samples at 133.86 ng/µl with a purity of 1,948.

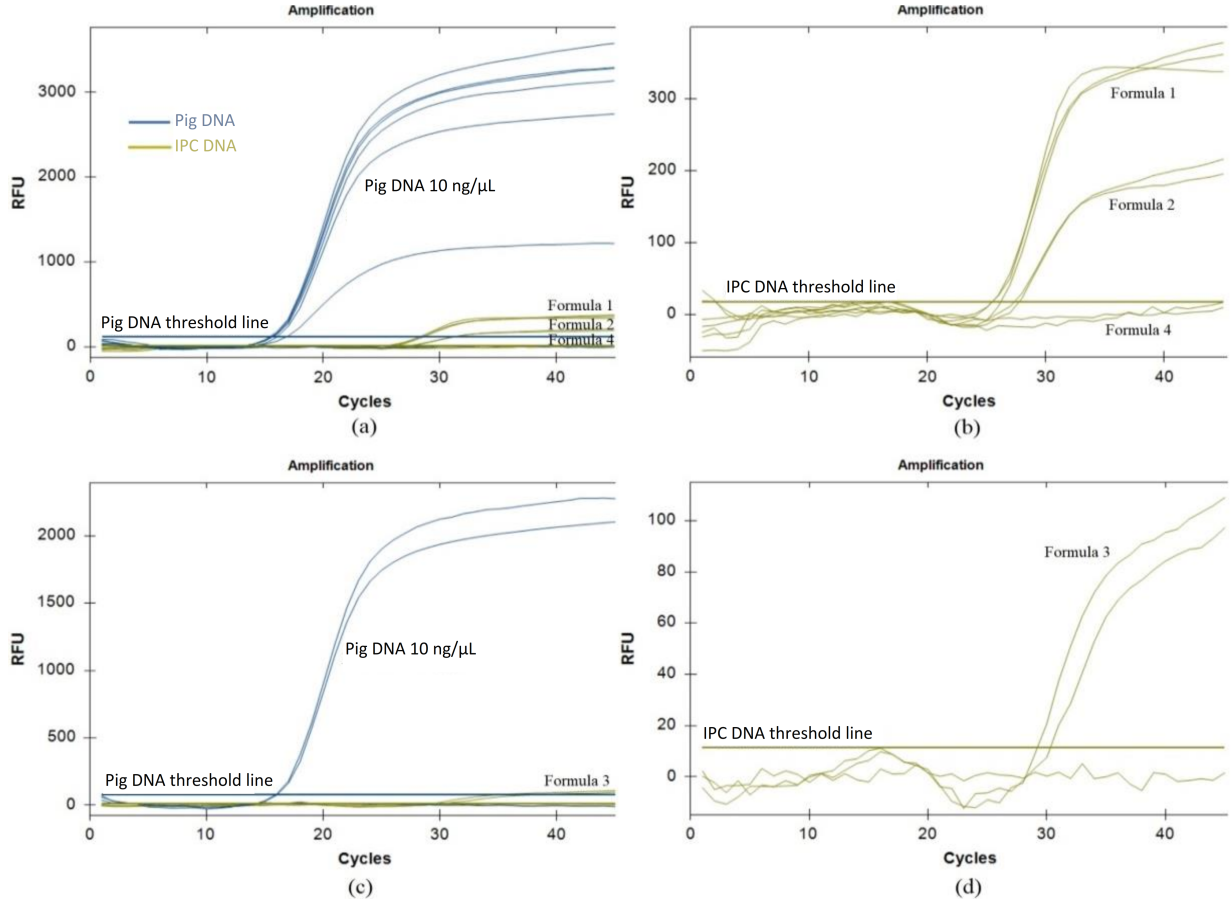

Figure 1: Amplification curve of internal positive control efficiency test results on 10 ng/µl pig DNA (a) and IPC (b) in Formula 1, 2, and 4, and 10 ng/µl pig DNA (c) and IPC (d) in Formula 3; retention percentage unit (RPU)

Table 1: Internal positive control (ICP) efficiency test results on 10 ng/µl pig DNA

Formula	Formula component	Pig Ct DNA (FAM)	Ct IPC (VIC)	RFU IPC average
1	Exo IPC Mix (10X) Exo IPC DNA (50X)	15.86 ± 0.28	25.87 ± 0.50	370.0 ± 11.31
2	Exo IPC Mix (5X) Exo IPC DNA (25X)	15.58 ± 0.02	27.57 ± 0.28	205.5 ± 14.85
3	Exo IPC Mix (2.5X) Exo IPC DNA (12.5X)	16.13 ± 1.03	29.74 ± 0.78	103.0 ± 8.49
4	Exo IPC Mix (1X) Exo IPC DNA (5X)	15.95 ± 0.11	Did not pass the threshold	

retention percentage unit (RPU)

Based on ISO 21571:2005 (ISO 2005), the DNA concentration range used for quantification tests was 2 ng/µL to 50 ng/µL. Therefore, concentrations above 50 ng/µL were selected in this research, namely 75 ng/µL, 100 ng/µL, and 150 ng/µL. The concentration of 150 ng/µL was adjusted to the highest concentration of pork DNA obtained at 133.86 ng/µl. The results showed that there was a false negative at an initial DNA isolate concentration of 133.86 ng/µl, which was indicated by the absence of IPC amplification. An excessively high amount of DNA can cause failure of primer attachment. Additionally, the concentration of contaminants or inhibitors remained high, showing the tendency for an inhibition process to occur during PCR.

Table 2: Results of the maximum concentration efficiency test for DNA isolates

No	DNA isolate (ng/µL)	Ct FAM	Ct FAM average	Ct VIC	Ct VIC average
1	Pig DNA 133.86	Negative	N/A	Negative	N/A
		19.59		26.26	
		19.29		26.24	
2	Pig DNA 100	19.77	19.65	25.60	26.08
		19.55		26.60	
		19.62		26.05	
3	Pig DNA 75	20.13	20.17	26.23	26.19
		20.05		26.10	
		20.33		26.23	

Pig Ct DNA (FAM), Ct internal positive control (VIC), not applicable (N/A)

Although contaminants can be observed in the purity of the DNA isolate, not all contaminants act as inhibitors. The purity of the DNA isolates was determined by the nucleic acid absorbance value at OD260 which was compared with the protein at OD280, with a purity range of 1.8 to 2.0, at OD260/280 (Lorenz 2012). Based on standard requirements, the purity value of 2.0 shows the presence of impurities from RNA. Other contaminants were shown by the OD260/230 ratio, such as guanidine thiocyanate (GTC), guanidine hydrochloride (GuHCl), EDTA, non-ionic detergents, proteins, phenols, and polysaccharides. BVL (2016) stated that the acceptance criteria for the purity of DNA isolates were OD260/230 > 2.0, while OD260/280 > 1.8.

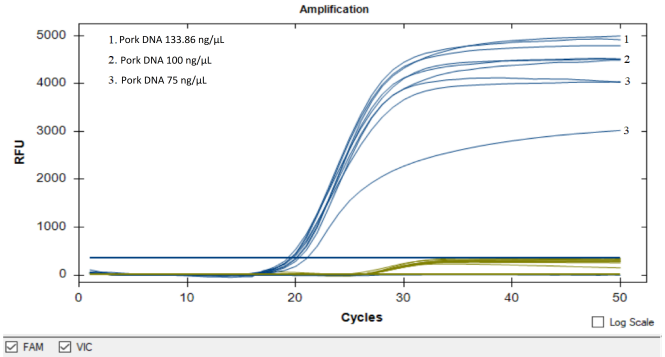

Figure 2: Amplification curve of maximum concentration efficiency test results for DNA isolates; Pig Ct DNA (FAM), Ct internal positive control (VIC)

3.3 Specificity Test

Based on in silico tests (theoretical tests) through BLAST, forward primers for pigs from Tanabe *et al.* (2007) are in line with the species Sus scrofa (wild boar) and Sus scrofa domesticus (cattle pig). However, the reverse primer for pigs from Tanabe *et al.* (2007), apart from having lineations with the species, also has lineations with *Phacochoerus africanus* (warthog pig), *Atherurus africanus* (African horsetail porcupine), *Thryonomys swinderianus* (cane rat), mammals and *Phlebotomus perniciosus* (sand fly). The specificity test was carried out by in vitro testing carried out on 23 samples containing pork and 23 samples that did not contain pork, as shown in Tables 3 and 4.

Table 3: Specificity test for positive samples[a]

No	Samples contain pork	DNA concentration (ng/µL)[b]	DNA $_{OD260/280}$ purification	Ct FAM
1	Pork 1	91.438	2.257	23.94 ± 0.71
2	Pork 2	62.792	1.869	20.66 ± 1.16
3	Pork 3	133.860	1.948	19.82 ± 0.33
4	Pig gelatin	11.664	1.297	24.55 ± 0.27
5	Pig marshmallow	5.742	2.784	30.74 ± 0.28
6	Raw lard	5.885	1.403	29.65 ± 0.69
7	Pig collagen	14.322	1.691	Negative[c]
8	Pig peptide collagen	6.780	1.408	Negative[c]
9	Pigskin crackers	71.194	1.864	34.75 ± 1.53
10	Pork salami	148.460	1.897	15.22 ± 1.40
11	Pork pastrami	48.002	1.898	15.56 ± 0.10
12	Pork jerky	94.822	1.883	11.73 ± 0.18
13	Pork floss	60.543	1.847	13.83 ± 0.80
14	Pork sausage	64.506	1.869	13.71 ± 0.01
15	Pork meatballs 1	65.725	2.057	19.94 ± 0.58
16	Pork meatballs 2	12.112	1.730	14.89 ± 0.18
17	Corned pork	13.656	1.397	19.83 ± 0.96
18	Bacon	3.775	1.519	19.10 ± 0.14
19	Pork patty	64.094	1.904	13.34 ± 0.11
20	Pig tanned leather	3.751	2.804	29.88 ± 0.26
21	Pig placenta	42.967	1.856	25.90 ± 0.01
22	Pork capsule shell	6.356	2.459	35.69 ± 0.95
23	Pig hair	6.452	2.038	34.16 ± 0.08

IPC (internal positive control) DNA was amplified in all samples, except pork collagen (a), the maximum concentration of DNA run was 100 ng/µL (b), false negative results due to collagen inhibitory activity and inappropriate extraction methods for collagen (c)

Table 4: Specificity test for negative samples[a]

No	Samples contain pork	DNA concentration (ng/µL)	DNA $_{OD260/280}$ purification	Ct FAM
1	Beef	156.880	1.949	Negative
2	Lamb	139.150	1.953	Negative
3	Chicken meat	97.378	1.997	Negative
4	Duck meat	85.257	2.037	Negative
5	Salmon	3.679	1.909	Negative
6	Cow gelatin	6.027	1.621	Negative
7	Cow marshmallow	2.662	2.939	Negative
8	Raw goat fat	7.524	1.833	Negative
9	Fish collagen	3.284	1.092	Negative
10	Beef skin crackers	15.628	1.791	Negative
11	Cow salami	70.065	1.918	Negative
12	Cow pastrami	41.423	1.887	44.00_b
13	Cow jerky	72.399	2.713	Negative
14	Cow floss	12.762	1.528	Negative
15	Beef sausage	85.712	1.989	Negative
16	Beef meatball	108.210	1.946	43.66 ± 1.97
17	Corned chicken	59.039	1.879	Negative
18	Smoked beef	72.459	1.874	Negative
19	Beef patty/burger	31.867	1.770	Negative
20	Cowhide leather	3.702	2.154	Negative
21	Flour	46.736	1.775	Negative
22	Cow capsule shell	4.089	2.538	Negative
23	Human hair	2.812	1.387	47.75_b

IPC (internal positive control) DNA was amplified in all samples (a) One of two samples replicate was negative (b)

There were three false-positive results at the rate of 13.04% in samples that did not contain pork, namely human hair, beef pastrami, and beef meatballs. The occurrence of false positives was attributed to sample contamination with positive material (cross-contamination) or contamination of reagents with amplification products (carry-over contamination) (Sefers *et al.* 2005). Contamination from the extraction process and environment (cross-contamination) in this research was minimized using extraction blanks (FDA 2019). Extraction blanks were carried out by rinsing the equipment that was used for preparation with distilled water that was extracted from the sample. When the PCR results showed amplification in the extracted NTC, contamination occurred during the extraction process (LPPOM MUI 2019). Furthermore, reagent contamination from carry-over amplification results was minimized by using ABC Master Mix containing heat-labile Uracil-N-glycosylase (UNG) which degraded dU in PCR carry-over products (Sefers *et al.* 2005; AppliedBiosystems 2016). Another possibility was the stability of PCR reagent components, such as the Taq polymerase enzyme, primers, and probes which decreased as the number of amplification cycles increased. Primer instability can cause errors in the hybridization of the primer and probe against non-target DNA (mishybridization). Martel *et al.* (2002) stated that increasing the number of cycles was essential for the occurrence of false positives.

The acceptability of the specificity test was significantly determined by the false positive and negative results plotted in the form of a receiver operating characteristic (ROC) curve, as shown in Figure 3a. This ROC

curve can be used to determine the cut-off Ct value based on estimates from the intersection of true positive and false positive from several samples tested (Tilaki 2013). Generally, this application is determined by testing in the medical field to ensure accuracy. The specificity of a primer and the stability performance of all PCR reagent components were determined in areas with no false positive or negative results. The highest Ct value of the 23 positive samples tested was 35.69 ± 0.95 which detected pork gelatin capsule shells. These results were considered to be the limit that did not yield false negative values.

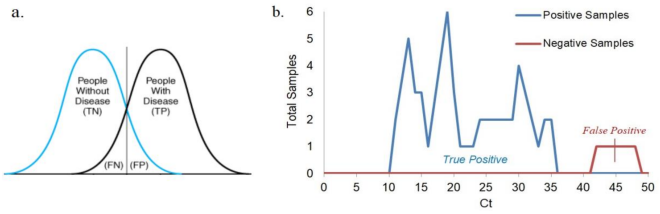

Figure 3: ROC (receiver operating characteristic) curve (a) (source: www.accessmedicine.com), and distribution curve of Ct values in the specificity test (b)

3.4 PCR Sensitivity, Linearity, and Efficiency Test

Determination of the limit of detection (LOD) value was obtained by testing the sensitivity, linearity, and PCR efficiency. A sensitivity test was performed using pork DNA from the results shown in Table 2. Pork DNA was diluted by spiking non-target DNA (background DNA), namely wheat DNA, at uniform concentrations of 100, 10, 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 ng/µL. Each concentration was repeated 14 times and the LOD was determined for the PCR reagent. Subsequently, testing laboratory, as the user of this method, validated the LOD for various food matrices (FDA 2019).

Broeders *et al.* (2014) stated that there were two methods for determining LOD, namely serially diluting CRM as positive material, or diluting background DNA (non-target DNA). In this research, the LOD was determined using background DNA to evaluate the interaction between the target and Non-target DNA. Furthermore, non-target DNA can be obtained from several sources, such as wheat flour (Tanabe *et al.* 2007), plant, and salmon sperm (BVL 2016). Wheat flour is known to be one of the food ingredients that is widely consumed in Indonesia in various food products, such as instant noodles, bread, bread flour, and others. It contains several compounds such as polysaccharides (Lin *et al.* 2019) and iron from fortifiers (MENKES 2003) which have inhibitory properties in the PCR reaction. Scrader *et al.* (2012) reported an inhibitory mechanism of polysaccharides that could be cross-linked to nucleic acids and metal ions capable of reducing primer specificity. The inhibitory properties were evaluated for their influence on the performance of PCR reagents using Taqman Exogenous

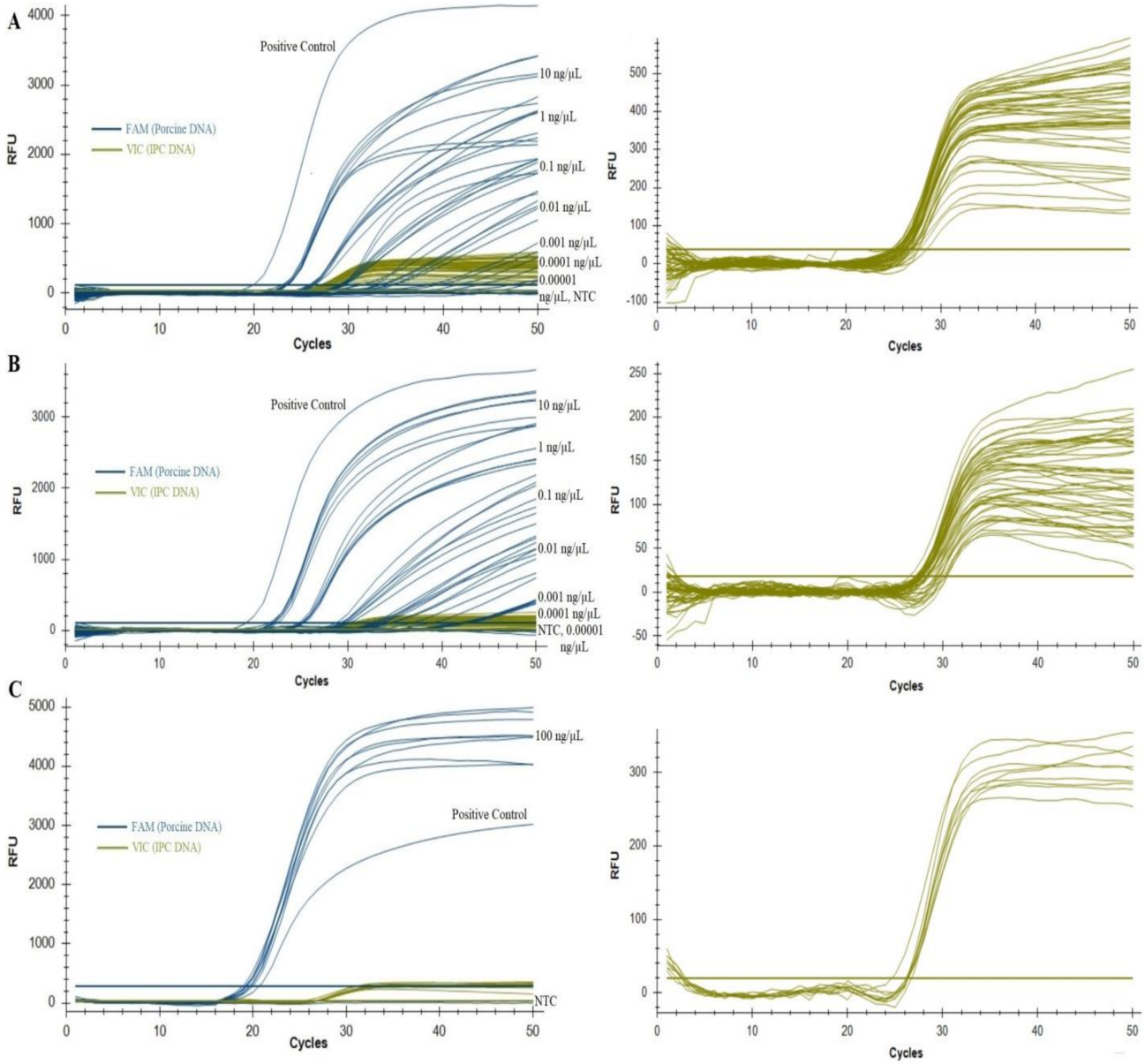

Figure 4: Amplification curve of pig DNA concentration 10 to 0.00001 ng/µl in repetitions 1-7 (a), in repetitions 8-14 (b), and pig DNA concentration of 100 ng/µl in repetitions 1-14 (c); retention percentage unit (RFU)

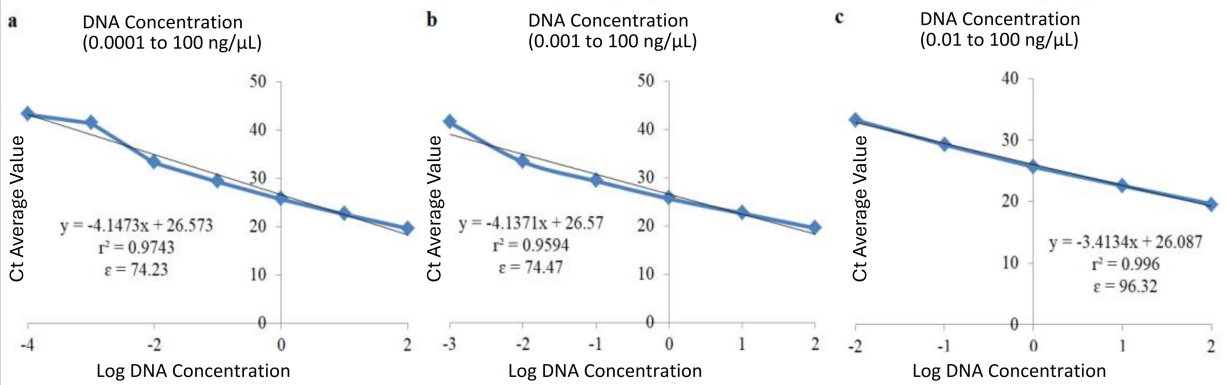

Figure 5: Linear regression and PCR (polymerase chain reaction) efficiency at pig DNA concentrations of 0.0001 - 100 ng/µl (a), 0.001 - 100 ng/µl (b), and 0.01 - 100 ng/µl (c)

IPC and ABC Master Mix. The results showed that there was no inhibition process from the interaction of target and background DNA at all concentrations, indicating the efficiency of IPC in amplification, as presented in Figure 4. The primer-probe combination used was considered robust against the two inhibitors found in wheat flour DNA. Based on Table 5, the LOD value was obtained at 0.01 ng/µl determined from the smallest target DNA concentration that still produced positive results in all replications (12 repetitions) (BVL, 2016). The LOD value was also determined by acceptable PCR linearity and efficiency (ϵ) values. Broeders *et al.* (2014) stated that the acceptability of the linearity value (r^2) for quantitative and qualitative tests with multiplex PCR $\geq$ 0.98, had slope values in the range -3.9 $\leq$ slope $\leq$ -2.9, and PCR efficiency values in the range 80–120%. The PCR linearity, slope, and efficiency values within the acceptance ranged from concentrations of 100 ng/µl to 0.01 ng/µl with linearity r^2 = 0.996, slope -3.4134, and PCR efficiency ϵ = 96.32, as shown in Figure 8. Moreover, a total of 100 DNA concentrations (0.01 ng/µl) were detected at 33.29 ± 0.92 cycles.

Table 5: FAM (Pig Ct DNA) Ct value in the pork DNA sensitivity test in wheat DNA

Repli-cates	DNA concentrations of pork in flour DNA (ng/µl)							
	100	**10**	**1**	**0.1**	**0.01**	**0.001**	**0.0001**	**0.00001**
1	19.77	23.43	26.12	29.36	34.26	39.19	-	-
2	19.55	23.32	26.26	29.23	33.17	43.56	-	-
3	19.62	23.44	26.34	29.36	33.72	41.30	-	-
4	19.72	22.85	25.90	28.88	31.19	-	-	-
5	19.45	23.21	26.95	29.16	32.62	38.25	-	-
6	19.47	23.35	25.67	29.46	33.33	46.88	48.63	-
7	19.98	23.08	25.97	29.07	34.63	-	-	-
8	19.48	22.39	25.04	30.07	34.34	36.56	-	-
9	19.31	22.07	26.03	29.59	32.80	41.35	-	-
10	19.36	22.04	25.09	29.11	32.40	42.05	42.39	-
11	19.59	21.71	24.99	29.76	33.29	42.02	-	-
12	19.34	21.48	25.23	29.15	32.82	41.75	-	-
13	19.35	22.11	24.95	29.39	34.17	42.42	38.62	-
14	19.85	23.23	24.62	28.61	33.38	-	-	-
Average	19.57	22.62	25.65	29.30	33.29	41.39	43.21	N/A
SD	0.21	0.69	0.67	0.36	0.92	2.74	5.06	N/A

standard deviation (SD), not applicable (N/A)

At a pig DNA concentration of 0.001 ng/µl, 11 of the 14 replicates were amplified (78.57%) in 41.39 ± 2.74 cycles with a linearity value of r^2 = 0.9594, slope -4.1371 and a PCR efficiency value of ϵ = 74.47%. Based on Broeders et. al. (2014) and BVL (2016), this concentration did not fall within the LOD acceptance range, as shown in Figure 5. The low value of PCR efficiency at a concentration of $\leq$ 0.001 ng/µL was related to the stability of PCR reagents over the number of PCR cycles, such as the DNA Taq polymerase enzyme, primers, and probes (Martel *et al.* 2002). The same results were obtained at a lower concentration of pig DNA (0.0001 ng/µL). Only 3 of 14 replicates (21.4%) were amplified at 43.21 ± 5.06 cycles with r^2 = 0.9743, slope -4.1473 and ϵ = 74.23%, which was not within the acceptable range (Figure 5).

Based on the results, the smallest LOD value or pig DNA concentration within the acceptance range was 0.01 ng/µl. This LOD value was greater compared to Tanabe *et al.* (2007), who obtained 100 fg/µL (0.0001 ng/µL) in cycle 40. However, a concentration of 0.001 ng/µL was detected in the same cycle at Ct 35 due to differences in the reagents and test treatments. Tanabe *et al.* (2007) used the singleplex PCR method with one primer. This research used a multiplex PCR method with two primers that amplify two target DNA in one PCR reaction. Additionally, the LOD determination in Tanabe *et al.* (2007) was carried out with 3 repetitions at each concentration, and there was no PCR efficiency test.

3.5 Robustness Test

A robustness test was conducted to assess the effects of small unintentional changes during the testing process. These small changes were determined using several parameters, as shown in Table 6. Differences in PCR machines were not the object of this research because of the availability of PCR machines in the laboratory. The sample tested was pig DNA spiked into wheat DNA at the LOD point (0.01 ng/µl). The results showed that all combinations still produced positive values, namely target DNA and IPC remained amplified in the Ct range at the LOD point (33.29 ± 0.92), as shown in Table 6. This suggested that the determination was robust to small changes in test conditions.

Despite the significant results, the Ct value of the LOD after the robustness test was not verified in this research. LOD verification was carried out by the user laboratory as a form of quality assurance program for test results within the scope of the validated matrix. This can be performed by spiking program for the target DNA at the LOD concentration of non-target DNA from each matrix area. The accuracy of the obtained Ct value was evaluated against the Ct value at the LOD point from the validation results. Based on data from the LPPOM MUI Laboratory using a different method with LOD value of 0.0005 ng/µl at Ct 32.37 ± 0.49, the Ct value obtained from spiking results was still within the range of 32.14 ± 0.65.

The robustness test of the method was carried out on several inhibitors which were often found in food product matrices, such as alginate, cellulose, EDTA, calcium ions, collagen peptides, and polysaccharides. This test method was found to be resistant to inhibitors at a concentration of 1 µg/µl which was equivalent to 9.99 mM $CaCO_3$ and 2.97 mM Na_2EDTA, as shown in Table 7. When present in DNA isolates, an inhibitor concentration of 1 µg/µL was classified as high, equivalent to 1000 ng/µL. This concentration was greater than the maximum limit for DNA isolate samples to be amplified, namely 100 ng/µL, as shown in Table 3. Additionally, the concentration of 1 µg/µL was higher compared to the results of McCord *et al.* (2015), who identified several similar inhibitors with 50% PCR inhibition activity. These included calcium at a concentration of 1 mM, EDTA at 1.2 mM, and collagen at 100 ng/µL. Therefore, the ABC Master Mix used in this research was considered to be more resistant to inhibitors. As shown in Figure 6, it was discovered that the presence of inhibitors in DNA isolates could be properly monitored using IPC.

Resistance to inhibitors has been reported due to the use of a polymerase chain reaction (PCR) buffer system as an amplification facilitator. Several commercial buffers have been used to minimize inhibition, such as Tth buffer (Roche, Germany) containing Tween 2 detergent, bovine serum albumin (BSA), and CertAmp buffer (Biotools, Spain) which has glycerol. Each amplification facilitator has a specific mechanism for reducing the inhibitory activity. BSA shows the potential to bind heme inhibitors, melanin, and organic molecules and act as a competitive target for protease enzymes. Tween 20 detergent can reduce the inhibition of samples from feces, polysaccharides, and phenolic components (Hedman & Radstrom 2013). Based on the results, this method was tested for resistance to inhibitors of alginate, cellulose, EDTA, calcium ions, collagen peptides, and polysaccharides. This test method used ABC Master Mix with components that were not kept secret by the kit manufacturer but were designed for multiplex PCR and were resistant to

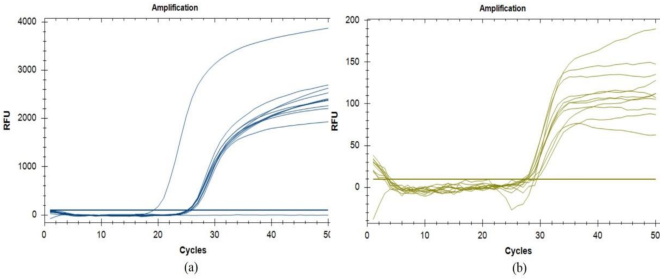

Figure 6: Amplification curves of target DNA (a) and IPC (internal positive control) DNA (b) resulting from spike inhibitors on pig DNA; retention percentage unit (RFU)

Table 6: Robustness test results

Factor	Combination 1	Combination 2	Combination 3	Combination 4
PCR machine	CFX96	CFX96	CFX96	CFX96
PCR kit (master mix)	Taqman	Taqman	Kogene	Kogene
Primer concentration	Constant	-30%	Constant	-30%
Probe concentration	Constant	-30%	-30%	Constant
Volume master mix	17.6 µL	17.6 µL	19.4 µL	19.4 µL
Annealing temperature	Plus 1 ℃	Minus 1 ℃	Plus 1 ℃	Minus 1 ℃
Ct FAM average (pig)	32.27 ± 0.16	33.20 ± 0.24	32.87 ± 0.53	32.54 ± 1.62
Ct VIC average (IPC)	25.78 ± 0.44	27.68 ± 0.15	27.76 ± 0.18	28.23 ± 0.20

polymerase chain reaction (PCR), Pig Ct DNA (FAM), Ct internal positive control (VIC), internal positive control (IPC)

Table 7: Amplification results of 1 µg/µl inhibitor in 10 ng/µl pig DNA

Inhibitor	Sample type	Inhibitor type	Ct FAM	Ct VIC
Alginate	Alginate	Taq polymerase	25.37 ± 0.13	27.34 ± 1.01
Cellulose	CMC	DNA	25.10 ± 0.01	27.29 ± 0.62
EDTA	Na_2EDTA	Taq polymerase	25.80 ± 0.28	31.42 ± 4.18
Potassium ion	$CaCO_3$	Taq polymerase	25.19 ± 0.04	28.40 ± 1.40
Collagen	Peptide collagen	DNA/taq polymerase	25.16 ± 0.06	27.49 ± 0.08
Polysaccharide	Xanthan gum	DNA	25.57 ± 0.06	28.14 ± 1.49

Pig Ct DNA (FAM), Ct internal positive control (VIC), Ethylenediaminetetraacetic acid (EDTA)

4 Discussion

The success of a PCR reaction is significantly determined by the concentration of the DNA isolate added as the template DNA. Lavanya *et al.* (2014) stated that the ideal amount of template DNA per PCR reaction is 60 ng. In this research, the amount of template DNA added per reaction was 6.5 µl with a DNA concentration ranging from 10 ng/µl. When the concentration of the obtained DNA isolate is greater, dilution with nuclease-free water is essential to obtain the ideal amount of template DNA (10 ng/µL). The dilution process is considered capable of diluting the concentration of contaminants or inhibitors contained in the sample DNA isolate and the target DNA, thereby producing false-negative results.

The process of diluting DNA isolates needs to be minimized by carrying out an efficiency test for the maximum concentration of samples that still show true positive results to increase sensitivity. Due to the unavailability of certified reference material (CRM) for pig DNA testing, this research used another reference material, namely DNA extracted from pork. The selection of pork was due to the availability as a single raw material with good traceability, integrity, and repeatability of the target DNA isolates. This was different when positive samples from processed pork products were used. The DNA isolate obtained was heterogeneous, consisting of a mixture of target and non-target DNA. Additionally, the integrity of DNA decreased with a high level of processing, which affected the repeatability of the results when used as a reference material.

DNA isolates with a concentration of $\leq$ 100 ng/µL produced 100% true positives, showing that the target DNA and IPC DNA were properly amplified. Based on these results, a concentration of 100 ng/µL pork DNA isolate was the maximum concentration that did not produce a false negative. The same results were shown for processed meat products (pork salami) in the specificity test, which had a false negative at an initial concentration of 148.46 ng/µL, with a purity of 1.897. Generally, concentrations above 100 ng/µL have been established to inhibit PCR. A true positive result was observed after the pork salami DNA was diluted to half its initial concentration.

The number of samples in this specificity test was determined based on the minimum of 20 for each positive and negative required by Broeders *et al.* (2014). Sample types were determined based on variations in product types, such as raw materials, meat products, and highly processed products originating from animal ingredients. Raw meat materials and products have a high DNA content, while highly processed products, such as gelatin, marshmallow, capsule shells, collagen, and tanned leather, contain low DNA residues. This was caused by processing processes including hydrolysis with acid and alkaline treatment, and enzymes, as well as a purification process using an ion-exchange resin. However, animal hair, which is commonly used as a tool for spreading margarine, contains a significantly lower amount of DNA than meat.

The type of sample was determined based on the representation of products containing animal ingredients except dairy products, eggs, and fish in BPOM Regulation No. 34 (BPOM 2019) concerning the Food Category. Based on these 16 food categories, the product represented in this specificity test belonged to categories 2.0 (animal fat), 5.0 (marshmallow and gelatin as raw materials), and 8.0 (meats and their products).

During pork determination, two samples produced false negatives at the rate of 8.7%, namely, collagen and collagen peptide samples. IPC was not amplified in the pig collagen sample but was amplified in the pig collagen peptide sample. This showed that there was a PCR inhibitory activity caused by hydrogen bonding to the DNA template which formed aggregates and could damage DNA stability (McCord *et al.* 2015), as well as inhibiting DNA Taq Polymerase (Kim *et al.* 2000). The amplification of IPC in pig collagen peptides was due to the incomplete collagen structure from the total hydrolysis process into a peptide structure, causing loss of the inhibitory activity of the DNA template. Since the extraction process had not been optimized for collagen or collagen peptide extraction, the target DNA was not amplified. Additionally, gelatin was amplified in both the target and IPC DNA in positive sample as the result of partial hydrolysis of collagen using chemicals, enzymes, or their combination (Nurilmala *et al.* 2017; Nurilmala *et al.* 2020). This research showed that the collagen hydrolysis process was able to eliminate inhibitory activity in the PCR amplification process.

False positive results from negative samples were detected at Ct = 42.26, in line with the research of qPCR on single copies that showed values at ~42 cycles. Tanabe *et al.* (2007) also carried out a correlation between the minimum concentrations of pig DNA that were still quantified at Ct < 40. These results were not in line with the trend of several pig DNA testing laboratories in Indonesia which extended the PCR cycle < 40 to achieve a higher level of sensitivity. In this research, an intersection between true and false positive results was not formed related to the number of samples used, as shown in Figure 6b. However, the maximum limit which produced true positive result was Ct 35.69 ± 0.95. The number of samples in the sensitivity test could be increased to improve the cut-off value on the ROC curve.

5 Conclusion

In conclusion, this research showed validation results of the pig DNA testing method using selected primers, exogenous IPC, and ABC Master Mixes, as a suitable alternative for detecting pig DNA through real-time PCR. The method showed strong sensitivity in detecting pig DNA at concentrations of 0.01 ng/µl with a cycle threshold (Ct) of 33.29 ± 0.92, r^2 = 0.996, and amplification efficiency (ϵ) of 96.32%. Pig DNA with highly processed products was also identified effectively such as gelatin, marshmallow, leather, hair, and capsule shells, with a maximum Ct of 35.69 ± 0.95. The method proved robust against minor procedural variations such as changes in master mix type and volume, primer, and probe concentration, including annealing temperature, which consistently amplified pig DNA within the Ct range of 33.29 ± 0.92 at LOD. Additionally, the method showed resilience to inhibitors such as alginate, cellulose, EDTA, calcium ions, collagen, and polysaccharides at concentrations up to 1 µg/µl. The validation success was further supported by the efficient use of IPC at half the initial concentration, with Exo IPC Mix 5X and Exo IPC DNA 25X still providing reliable amplification at Ct 27.57 ± 0.28 and RFU 205.5 ± 14.85. There was also successful amplification of pig DNA from DNA isolates to a concentration of 100 ng/µL without inhibition, eliminating the need for sample dilution before PCR.

For this method to meet ISO 17025 accreditation requirements in user laboratories, further validation should be carried out. This included validating the method with LOD parameters on non-target sample matrices intended for accreditation and refining extraction methods. Further research was recommended to optimize collagen sample extraction for true-positive results and DNA sequencing could be used to confirm the absence of false-positive outcomes.

Conflict of Interest

The authors declare no conflict of interest.

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