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ARTICLE

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Optimization of DNA Extraction Methods in Fresh Meat (Rat and Chicken Meat) based on Incubation Time

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Abstract: *DNA (deoxyribonucleic acid) extraction method is the process of separating DNA from the sample. In this process, the DNA obtained must be protected from contamination by RNA, carbohydrates, lipids, and proteins. Contamination of RNA, carbohydrates, lipids, and proteins can increase DNA purity. DNA purity was measured using a NanoDrop 2000 spectrophotometer measured by the absorbance ratio at 260 nm and 280 nm wavelengths. Good quality DNA will have an A_{260}/A_{280} ratio of 1.7–2.0 and a concentration > 0.03 pg. This study aimed to obtain the appropriate DNA extraction method for fresh meat samples (a mixture of rat and chicken meat). This research consisted of two stages: the DNA extraction stage using the Progenus EasyFast™ Extraction Kit for Meat Products and the amplification stage using the EASYFAST™ Rat Detection Kit. This study used 16 samples of a mixture of rat meat and chicken with concentrations of rat meat: 5, 10, 15, and 20%. At the extraction stage, the incubation time was optimized for 15, 30, 45 minutes, and 1 hour. The results showed that the one hour incubation had a lowest CT value in the results of PCR amplification.*

Keywords: *DNA extraction, incubation time, real-time PCR*

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1. Introduction

Food, a fundamental human necessity, demands meticulous and accurate processing to yield health benefits. Food diversity is expanding rapidly with the emergence of novel culinary preparations, such as meatballs, sausages, nuggets, and corned beef. Among these, processed meat stands out as a widely favored meat-based delicacy among Indonesians, particularly children; however, there is a discernible rise in instances where food safety is no longer guaranteed (Aminah et al., 2019; Newsome et al., 2014). Processed meat is sometimes mixed with non-permissible meat types for Muslims, often driven by the intent to reduce production costs (Ali et al., 2012; Lever & Fischer, 2018; Thomas et al., 2017). Several cases have surfaced across various regions, revealing instances of adulterating beef meatballs with rat meat, eliciting public alarm and disquiet (Choudhary et al., 202[28] Lestari et al., 2022).

In 2015, Rizki Widiyanti conducted a study to detect rat meat in meatballs from food stalls utilizing polymerase chain reaction (PCR) techniques, revealing that one out of five samples analyzed contained rat meat. In 2019, Tri Susilowati organized a comparable study focusing on identifying pork DNA contamination through real-time PCR (RT-PCR) analysis of meat milling samples sourced from Pasar Surya, Surabaya City. Pork DNA was detected in five out of the 30 samples analyzed. Sunaryo et al. (2022) conducted a study employing the RT-PCR method to detect rat DNA contamination in 30 sausage samples, revealing one positive sample. Notably, a corresponding inquiry was undertaken by Widiyanti (2015), focusing on detecting rat DNA contamination in meatballs using the PCR method, revealing one out of the five samples tested positive for rat DNA contamination.

Mitigating production fraud in processed meat necessitates identification of extraneous material contamination—a pivotal factor in ensuring product safety adherence to halal standards. DNA amplification, through RT-PCR, is a precise method for detecting such contamination. This approach can concurrently monitor the progression of PCR reactions and quantify the expression of PCR products (Adawiyah et al., 2023; Rachmawati et al., 2017), rendering it a productive tool in this context.

The RT-PCR method comprises two phases: DNA extraction and the RT-PCR amplification stages. The DNA extraction method entails segregating DNA from the sample, commencing with disruption of the cell wall followed by isolation of the DNA from other constituents (e.g., fat, protein, carbohydrate, and RNA), culminating in DNA purification (Corkill & Rapley, 2008). During the extraction phase, DNA is acquired, and its subsequent quantificat[11]n for concentration and purity involves use of the NanoDrop 2000 Spectrophotometer with readings at wavelengths of 260 nm and 280 nm. Desirable DNA has a purity ranging from 1.7 to 2.0 (Adriany et al., 2020) and a concentration above 0.03 p[7] López-Andreo et al., 2005).

Two DNA extraction and purification procedures for food analysis were compared, Wizard Magnetic DNA Purification for Food (P[7]mega Italia S.r.l., Milan, Italy) and DNeasy Tissue Kit (QIAGEN, Hilden, Germany), in term[4] of extraction efficiency, DNA purity, and DNA suitability for amplification (Di Pinto et al., 2007). The quality and quantity of DNA extracted from food products tend t[4]decrease with the extent to which the food is processed. Additionally, exposure to heat can lead to the fragmentation of high molecular weight DNA, physical and chemical treatments can cause random breaks in DNA strands and may reduce the size of DNA fragments. The type of sample used for DNA extraction can also affect the quality and qu[18]ity of DNA (Demeke & Jenkins, 2010). There are ten methods for DNA extraction such as: the Tris-EDTA, modified cetyltrimethylammonium bromide (CTAB), alkaline, urea, salt, guanidinium isothiocyanate (GuSCN), wi[14]d, QIAGEN, zymogen, and gene Spin (Paireder et al., 2013; Rohland & Hofreiter, 2007; Schiebelhut et al., 20[10]; Yahya et al., 2017; Yalçınkaya et al., 2017). Determination and quantification of species using RT-PCR are influenced by temperature, duration of the heat treatment, and size of the DNA fragment to be amplified (Şakalar et al., 20[3]).

Traditional digital PCR (ddPCR) is a method of absolute nucleic acid quantification based on the partitioning of individual analyte molecules into many replicate reactions at limiting dilution, with most [3]actions resulting in one or zero molecules. Recently, advanced technology has emerged that allows reactions to be divided into nanoliter-sized droplets in oil rather than multiwell plates. Rapid microfluidic anal[15]s of thousands of droplets per sample makes ddPCR practical for routine use. The combination of nanoliter-sized droplet technology with ddPCR holds promise for highly precise, absolute nucleic acid quantification (Hindson et al., 2013). Molecular technologies like PCR can be used as an accurate alternative solution to authenticate/ensure that a ground food sample[37] a local market does not contain contamination from meat prohibit[12]n Islamic Sharia law (Baihaqi et al., 2019).

RT-PCR with SYBR Green I dye is used as a simple, fast, sensitive, and reliable method for the detection and quantification of pork meat. This method was validated using blind mixtures and

subsequently employed to measure the presence of pork meat in commercially available processed poultry meat products (Soares et al., 2013). Detection of pork DNA in binary meat mixtures and various commercial food products can be performed using conventional PCR and RT-PCR (Al-Kahtani et al., 2017). Various analytical methods rely on protein or DNA measurements to identify meat species. Most of these methods have been replaced by more accurate and sensitive detection methods, such as DNA-based techniques. Emerging technologies like DNA barcoding and mass spectrometry are still in the early stages of use in meat detection. Gold nanobiosensors have shown some promise, but their applicability in small-scale industries is still distant (Zia et al., 2020). Materials used are literature sources such as research journals, research data, and books. Research method employed is the descriptive method, discussion, and study (Kulsum et al., 2019). One way to reduce production costs is by mixing beef with meat from other animals in the manufacturing process, resulting in a similar product but at a much lower cost. One common case that occurs is the mixing of beef with rat meat (Septiani & Pendrianto, 2018). one method for analyzing rat meat fat in beef meatballs is the Soxhlet method, which uses only one solvent, resulting in less waste and simplifying the process (Rosyidi & Khamidinal, 2019). Multiplex PCR is reliable for identifying chicken, beef, pork, and goat species in meat products. However, multiplex PCR may not be sensitive enough for testing processed meat products (Zhang, 2013).

Authenticity and traceability of meat are highly important issues because there have been numerous recent incidents of meat product fraud (Sentandreu & Sentandreu, 2014). Identification of meat species and authentication of animals in meat products can be done through analysis based on proteins or DNA. However, the latest and most accurate technology is DNA-based analysis (Alikord et al., 2018). Authenticity of meat products includes concerns such as pork substitution, undisclosed use of blood plasma, use of prohibited ingredients, use of pork intestine casings, and non-halal slaughter methods. Analytical methods used for halal authentication of meat and meat products include PCR, enzyme-linked immunosorbent assays, mass spectrometry, chromatography, electronic nose, and spectroscopy (Nakyinsige et al., 2012). Currently many efforts have been made to develop more effective halal authentication detection systems. Conventional techniques, such as dielectric and electrophoresis, were employed to detect halal components before the invention of PCR in 1984. PCR has been commonly used since its introduction. Additionally, spectroscopy techniques have been used for decades and recently gained popularity when combined with chemometrics for data processing and treatment (Ng et al., 2021). DNA detection using powerful RT-PCR technique has been proven to be a highly specific and sensitive authentication tool. Furthermore, RT-PCR also efficiently extracts DNA in terms of both quality and quantity (Khairil Mokhtar et al., 2020). RT-PCR is a fast and reliable method for detecting species in meat products (Dalsecco et al., 2018). Critical points in food can be identified based on the processes, storage, additives, and the use of raw materials (Adawiyah & Kulsum, 2019; Cankar et al., 2006).

This present study employed the *Progenus EasyFast™ Extraction Kit for Meat Products*, which has previously been used by multiple researchers. Sunaryo et al. (2022) used this kit on sausage samples, showcasing DNA purity spanning from 1.47 to 2.57, accompanied by an average DNA concentration ranging between 1248.4 ng and 3142 ng. Similarly, in 2021, Choirunisah employed the same kit on nugget samples, yielding DNA purity values within the range of 1.12 to 1.87 and an average DNA concentration spanning from 718.8 ng to 4703.4 ng. In the context of the present study, the DNA extraction method was optimized on fresh meat samples consisting of a combination of rat and chicken meat based on varying incubation lengths.

2. Materials and Methods

The tools and instruments used in this study were micropipette (Bio-Rad™), heat block (My block, Benchmark), mini centrifuge (My Fuge, Benchmark), RT-PCR (CFX96 Deep Well, Bio-Rad™), NanoDrop 2000 spectrophotometer (Thermo Scientific™), analytical balance, mortar and pestle and scalpel.

The samples and chemicals used in this study were a DNA extraction kit consisting of solutions A and B (*Progenus EasyFast™ Extraction Kit for Meat Products*), an RT-PCR amplification kit consisting of MIX and EPC (*External Positive Control*) reagents (*Progenus EASYFAST™ Rat detection kit*), nuclease free water (Promega), rat meat, chicken meat, 1.5 mL microtube, and PCR tube.

2.1. Sample Preparation

Samples of mixed rat and chicken meat (rat meat concentration 5%, 10%, 15%, and 20%) were carefully weighed as much as 250 mg using an analytical balance. Then the sample was mashed using a mortar

and pestle. The mashed sample was put into a 1.5 mL microtube and labelled with the code ZA1 (5% concentration), ZA2 (10% concentration), ZA3 (15% concentration), ZA4 (20% concentration). The sample then continued to the DNA extraction step.

2.2. Extraction of DNA

This research used a DNA extraction kit (*Progenus EasyFast™ Extraction Kit for Meat Products*). Extraction was started by adding 500 μL of Solution A reagent to a 1.5 mL microtube containing the sample, then homogenized using a vortex for 10 seconds. The vortexed sample was heated at a temperature of 95°C for 1 hour for the tube coded "ZAX1", 45 minutes for the tube coded "ZAX2", 30 minutes for the tube coded "ZAX3", 15 minutes for the tube coded "ZAX4" using heating-blocks. The tube containing the sample and Solution A which had been heated was allowed to reach room temperature. Then 500 μL of Solution B was added to the tube, then homogenized using a vortex for 15 seconds. The sample was allowed to settle for 15 minutes. The supernatant was transferred into a sterile microtube then diluted 10x using nuclease free water.

2.3. Measurement of DNA Concentration

DNA purity was measured using a NanoDrop 2000 spectrophotometer (Thermo scientific™). The first step was selecting the nucleic acid application from the main menu. Select the type of nucleic acid for measuring DNA purity, then a blank was inserted after which the sample ID was entered according to the number printed on the tube, then the instrument arm was lifted and 1 μL of the sample was dripped onto the optical device. After the measurement was complete the instrument arm was lifted, and the optical instrument was cleaned using a tissue. The measurement results were entered automatically on the connected PC.

2.4. PCR Amplification

The amplification process began by preparing 18 PCR tubes labeled on the edge of the tube cap (N= Negative Control, P= Positive Control, S = Sample). After that, 18 μL of the MIX reagent (green cap) was added to each PCR tube, then 2 μL of eluent (DNA) to the tube code "S", 2 µL positive control (EPC) on tube code "P" and, 2 µL *nuclease free water* on the tube code "N". After that, it was homogenized in an *up and down* method, then the PCR tube was closed. Then the PCR was turned on by setting the pre-denaturation cycle at a temperature 95°C for 3 minutes for 1 cycle, and 40 cycles at the denaturation and anneling-extension stages. Denaturation at 95°C for 15 seconds, and anneling – extension at 60°C for 60 seconds. *Channel fluorescent* used was FAM (494/520 nm) with DNA target of *Rattus* species and VIC (538/554 nm) with target of vertebrate (internal control). Then the PCR tube was inserted, and the PCR device was started to run.

3. Results and Discussion

3.1. Optimization of Incubation Time at the DNA Extraction Stage

Optimization of the incubation time was carried out on mixed samples of rat and chicken meat at a concentration of 5%, the incubation times used were 15, 30, 45 minutes, and 1 hour. The kit used in the extraction stage is a commercial kit *Progenus EasyFast™ Extraction Kit for Meat Products*. This kit has the same basic principle of extraction as other kits, there are several stages of extraction, namely the process of destroying cells, separating contaminants, and purifying DNA (Adriany et al., 2020).

The DNA extraction process begins with sample preparation, then the DNA extraction stage. The DNA results obtained continued with the measurement of DNA concentration and purity using the NanoDrop 2000 Spectrophotometer with a ratio of A_{260}/A_{280}. The results of the measurement of DNA concentration and purity can be seen in Table 1. A good DNA result has a purity of 1.7–2.0 (Adriany et al., 2020) and has a concentration > 0.03 pg (López-Andreo et al., 2005). Table 1 shows the results of DNA concentration values obtained between 164.0–452.6 ng/μL and DNA purity obtained from 1.40–1.52. Purity values below 1.5 can occur due to contamination from protein and if the purity value is above 2.0 there is RNA contamination (Teare et al., 1997). Protein contamination can occur because this extraction kit does not have a precipitation step so that there is still protein remaining. Kusumadewi et al (2012) said the value of DNA purity results ranging from1.076–1.988 can still be used in PCR amplification.

Table 1. Results of DNA Concentration and Purity

Incubation time	Concentration (ng/μL)	Purity (A_{260}/A_{280})
1 hour	164.0	1.52
45 minutes	452.6	1.44
30 minutes	244	1.46
15 minutes	246.8	1.40

3.2. Real-Time PCR Amplification

The RT-PCR amplification stage begins with entering the Master Mix *Progenus EasyFast™ Rat Detection kit* in each PCR tube, then adding eluent (DNA) on the sample tube, EPC on the positive control tube, and nuclease-free water on the negative control tube, then the amplification process was carried out using the CFX96 Touch™ tool. *The Progenus EasyFast™ Rat Detection kit* is a commercial RT-PCR amplification kit that specifically identifies rats, consisting of two r(FAM) and vertebrate (VIC) target genes. The CT (cycle threshold) values of RT-PCR amplification can be seen in Table 2.

The amplification results in the table show that rat DNA was detected all samples and there was no contamination because the negative controls did not have CT on FAM (rats) and VIC (vertebrata). FAM (rat) C values ranged from 19.11–23.01 and VIC (vertebrata) CT values ranged from 16.51–29.58. The results of the amplification curve comparison can be seen in Figures 1 to 5.

Figure 1 describes the amplification curve of a mixed sample of rat and chicken meat, which was incubated for one hour at the extraction stage. The ZA1 code shows a sigmoid increase in the curve in FAM (rat DNA detection) with a CT value of 21.28 and VIC (vertebrate DNA detection) with a CT value of 17.11. The ZA2 code shows a sigmoid increase in the curve in FAM with a CT value of 21.34 and VIC with a CT value of 16.65. Code ZA3 shows a sigmoid increase in the curve in FAM with a CT value of 19.45 and VIC with a CT value of 17.16, while the ZA4 code shows a sigmoid curve increase in FAM with a CT value of 19.11 and VIC with a CT value of 17.11.

Table 2. Results of RT-PCR Amplification CT (Cycle Threshold) Values

Sample Code	Incubation Time	CT Value	
		FAM	VIC
ZA1[1]	1 hour	21.28	17.11
ZA2[1]		20.34	16.51
ZA3[1]		19.45	17.16
ZA4[1]		19.11	17.11
ZA1[2]	45 minutes	22.68	17.70
ZA2[2]		22.01	18.17
ZA3[2]		22.35	18.12
ZA4[2]		20.35	17.47
ZA1[3]	30 minutes	28.22	18.02
ZA2[3]		22.03	17.01
ZA3[3]		20.72	17.78
ZA4[3]		23.01	17.52
ZA1[4]	15 minutes	22.80	17.25
ZA2[4]		23.28	17.95
ZA3[4]		20.48	17.25
ZA4[4]		19.58	17.82
Negative Control		N/A	N/A
Positive Control		28.40	29.58

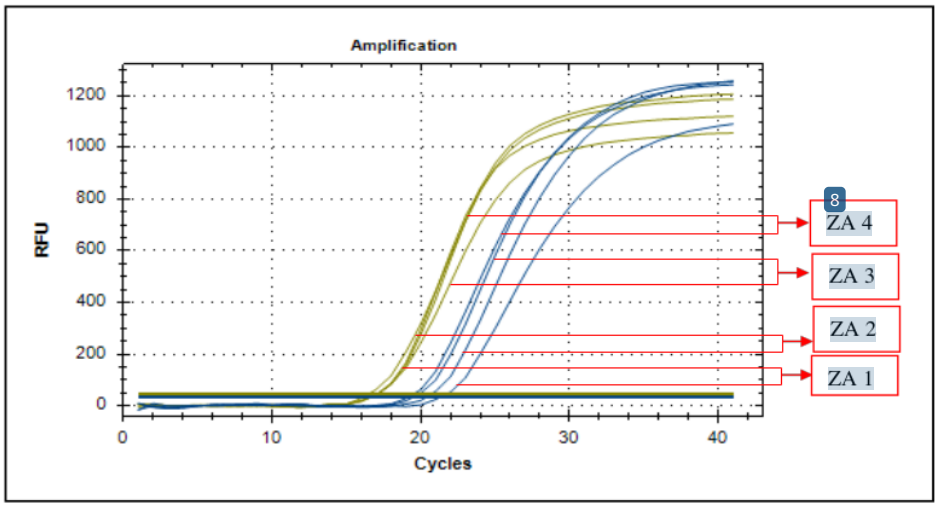

Figure 1. Amplification curve at one hour incubation (green: VIC; blue: FAM; concentration: ZA1 (5%), ZA2 (10%), ZA3 (15%) ZA4 (20%); relative fluorescence unit (RFU)).

Figure 2 describes the amplification curve of a mixture of rat and chicken meat samples, which were incubated for 45 minutes at the extraction stage. The ZA1 code showed a sigmoid increase in the curve in FAM (mouse DNA detection) with a CT value of 22.68 and VIC (vertebrate DNA detection) with a CT value of 17.70. The ZA2 code shows a sigmoid increase in the curve in FAM with a CT value of 22.01 and VIC with a CT value of 18.17. The ZA3 code shows a sigmoid increase in the curve in FAM with a CT value of 22.35 and VIC with a CT value of 18.12 while the ZA4 code shows a sigmoid increase in the curve in FAM with a CT value of 20.35 and VIC with a CT value of 17. 47. Figure 3 describes the amplification curve of a mixed sample of rat and chicken meat, which was incubated for 30 minutes at the extraction stage. The ZA1 code shows a sigmoid increase in the curve in FAM (rat DNA detection) with a CT value of 28.22 and VIC (vertebrate DNA detection) with a CT value of 18.02. The ZA2 code shows a sigmoid curve increase in FAM with a CT value of 22.03 and VIC with a CT value of 17.01. The ZA3 code shows a sigmoid increase in the curve in FAM with a CT value of 20.71 and VIC with a CT value of 17.78, while the ZA4 code shows a sigmoid curve increase in FAM with a CT value of 23.01 and VIC with a CT value of 17.52.

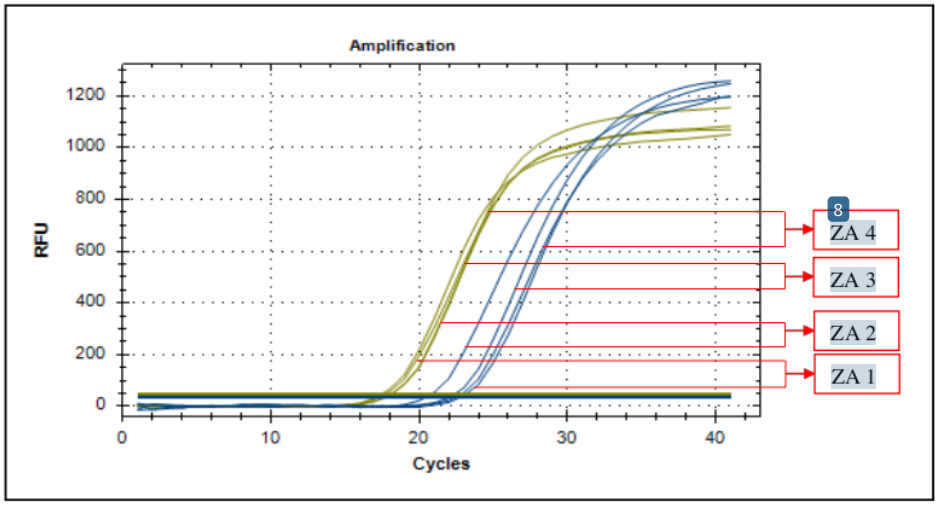

Figure 2. Amplification curve at 45 minutes incubation (green: VIC; blue: FAM; concentration: ZA1 (5%), ZA2 (10%), ZA3 (15%) ZA4 (20%); relative fluorescence unit (RFU)).

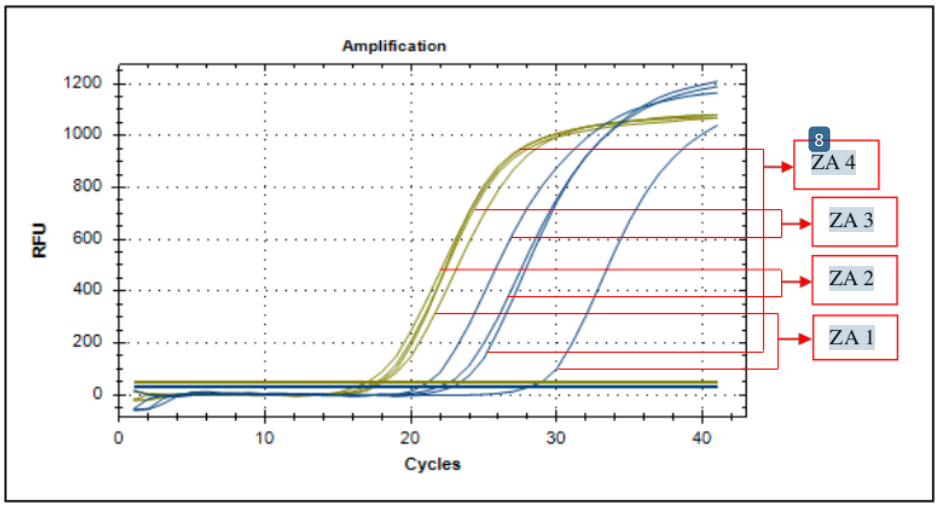

Figure 3. Amplification curve at 30 minutes incubation (green: VIC; blue: FAM; concentration: ZA1 (5%), ZA2 (10%), ZA3 (15%) ZA4 (20%); relative fluorescence unit (RFU)).

Figure 4 describes the amplification curve of the rat and chicken sample which was incubated for 15 minutes at the extraction stage. The ZA1 code shows a sigmoid increase in the curve in FAM (rat DNA detection) with a CT value of 22.80 and VIC (vertebrate DNA detection) with a CT value of 17.25 then in the ZA2 code it shows a sigmoid increase in the curve in FAM with a CT value 23.28 and VIC with a CT value of 17.95. The ZA3 code showed a sigmoid increase in the curve in FAM (rat DNA detection) with a CT value of 20.48 and VIC (vertebrate DNA detection) with a CT value of 17.25. ZA4 code sample showed a sigmoid increase in the curve in FAM (rat DNA detection) with a CT value of 19.85 and VIC (vertebrate DNA detection) with a CT value of 17.82.

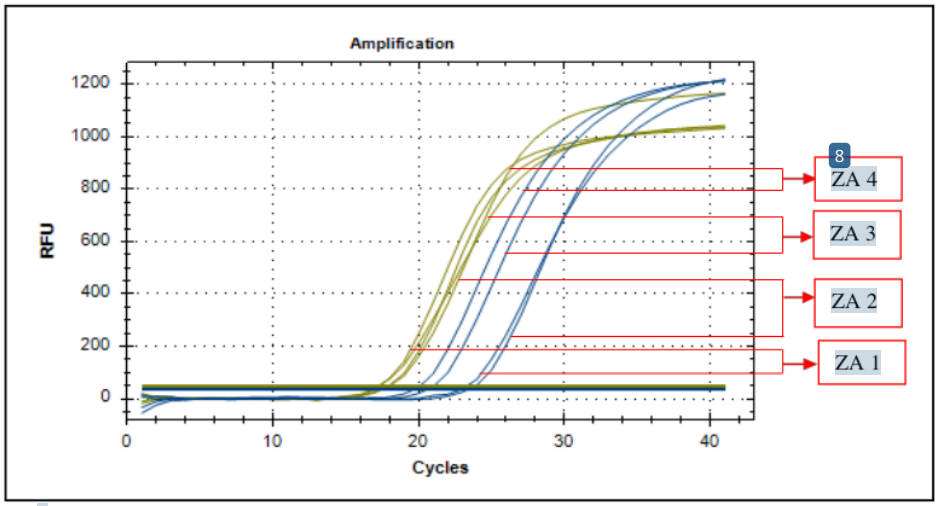

Figure 4. Amplification curve at 15 min incubation (green: VIC; blue: FAM; concentration: ZA1 (5%), ZA2 (10%), ZA3 (15%) ZA4 (20%); relative fluorescence unit (RFU)).

Figure 5 describes the comparison of the positive control curve and the negative control curve. There is a sigmoid amplification curve in FAM with a CT value of 28.40 and VIC with a CT value of 29.58 for positive controls and no increase in curves in FAM and VIC for negative controls (NTC). Positive and negative controls showed good results in accordance with the validation results recommended by the kit, positive control results CT FAM and VIC <30, while for negative controls CT FAM and VIC > 38, so it can be said the PCR amplification process showed valid results following the *Progenus EasyFast™ Rat Detection Kit* manual. The sensitive and specific amplification results are shown in Figure 1, namely the amplification results of a mixture of chicken and rat samples which were

incubated for one hour during the extraction stage.

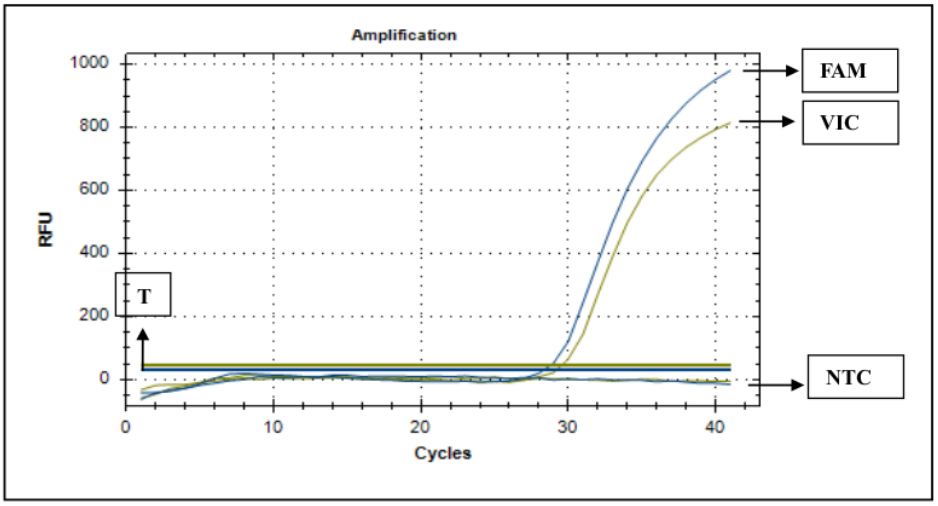

Figure 5. Comparison of positive control (green: VIC; blue: FAM; negative control: no template control (NTC); threeshold (T); relative fluorescence unit (RFU)).

4. Conclusion

There were differences between the RT-PCR amplification results for samples incubated for 15 minutes, 30 minutes, 45 minutes and 1 hour. The highest CT value was found in samples incubated for 30 minutes, while the lowest CT value was in samples incubated for 1 hour. The best incubation time for the extraction kit (*Progenus EasyFastTM Extraction Kit for Meat Products*) for fresh meat-based chicken samples is one hour. For future research, if used different sample types, optimization must be carried out first.

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