

**DETECTION OF PORCINE DERIVED IN SWEET JELLY
PRODUCT THROUGH PCR AMPLIFICATION OF THE
MITOCHONDRIAL DNA ND5 GENE**

PUTRI JUNAIDAH BINTI JAELAN

UNIVERSITI SAINS MALAYSIA

2026

**DETECTION OF PORCINE DERIVED IN SWEET JELLY
PRODUCT THROUGH PCR AMPLIFICATION OF THE
MITOCHONDRIAL DNA ND5 GENE**

By

PUTRI JUNAIDAH BINTI JAELAN

Dissertation submitted in partial fulfillment of the requirement

for the

Degree of Bachelor of Science (Honours) (Forensic Science)

January 2026

CERTIFICATE

This is to certify that this dissertation title “**Detection of Porcine Derived in Sweet Jelly Product Through PCR Amplification of The Mitochondrial DNA Gene**” is bona fide record of final year project done by Ms Putri Junaidah Binti Jaelan during the period of October 2025 to February 2026 under my supervision. I have read this dissertation and that in my point of view it conforms to acceptable standards of scholarly presentations and is fully adequate, in scope and quality, as dissertation to submitted in partial fulfilment for the Bachelor of the Degree of Bachelor of Science (Honours) (Forensic Science).

Main Supervisor,

Dr. Nur Haslindawaty Binti Abdul Rashid

Lecturer

School of Health Science

Universiti Sains Malaysia

Health Campus

16150 Kubang Kerian

Kelantan, Malaysia

Date: _____

DECLARATION

I hereby declare that this dissertation is the result of my own investigation, except where otherwise cited and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any degrees at Universiti Sains Malaysia or any other institution. I grant Universiti Sains Malaysia the right to use to use the dissertation for teaching, research and promotional purposes.

(PUTRI JUNAIDAH BINTI JAELAN)

Date:

ACKNOWLEDGEMENT

I would like to express my sincere appreciation and deepest gratitude to my supervisor, Dr Nur Haslindawaty binti Abd Rashid for her invaluable guidance and support throughout my Final Year Project. I am truly grateful for her time and dedication that greatly contributed to the successful completion of this project.

I would also like to express like to sincerely thank all laboratory assistants for their kind guidance and helpful support in using laboratory instruments and handling chemicals throughout this study I am sincerely thankful my friends who have helped me directly and indirectly throughout this journey, especially Nurina, who has always been there to listen and comfort me whenever I needed it.

Lastly, I am deeply grateful and forever indebted to my beloved parents and siblings for their constant prayers, unwavering moral support, patience, and encouragement throughout my journey, which enabled me to remain motivated and complete this project successfully. They have always been my strongest supporters.

TABLE OF CONTENTS

CERTIFICATE.....	III
DECLARATION.....	IV
ACKNOWLEDGEMENT.....	V
TABLE OF CONTENTS	VI
LIST OF TABLES	X
LIST OF FIGURES	XI
LIST OF APPENDICES	XIII
LIST OF ABBREVIATIONS	XIV
LIST OF SYMBOLS	XVI
ABSTRAK	XVII
ABSTRACT.....	XVIII
CHAPTER 1 INTRODUCTION	1
1.1 Research Background	1
1.2 Problem Statement	4
1.3 Significance of Research.....	4
1.4 Objectives	5
1.4.1 Main Objectives	5
1.4.2 Specific Objectives	5
CHAPTER 2 LITERATURE REVIEW	6

2.1	Sweet Jellies and Gelatine-Based Product	6
2.2	Mitochondrial DNA (mtDNA)	8
2.2.1	mtDNA Gene Regions Used in Species Authentication.....	9
2.3	Polymerase Chain Reaction (PCR).....	11
CHAPTER 3 METHODOLOGY		14
3.1	Materials	14
3.1.1	Chemicals and Reagents	14
3.1.2	Equipment and Instruments	16
3.1.3	Buffer and Other Solution Preparation	16
3.2	Sample Collection and Preparation.....	19
3.3	DNA Extraction	21
3.3.1	DNA Extraction of Fresh Raw Meats	21
3.3.2	DNA Extraction of Candy Samples Product	22
3.4	Agarose Gel Electrophoresis of Genomic DNA	24
3.5	DNA Quantification.....	25
3.6	Oligonucleotide Primers	25
3.7	In-silico Primer Analysis	26
3.8	Polymerase Chain Reaction (PCR) Optimisation	27
3.8.1	Annealing Temperature Optimisation.....	28
3.8.2	Specificity Optimisation	28
3.8.3	Sensitivity Optimisation.....	29

3.9	Agarose Gel Electrophoresis of Amplified PCR Product.....	29
3.10	Polymerase Chain Reaction (PCR) Amplification of Sweet Jelly Samples	30
CHAPTER 4 RESULT		31
4.1	Extracted Genomic DNA of Fresh Muscle Tissues and Sweet Jelly Samples	31
4.2	Spectrophotometry DNA Quantification	34
4.3	<i>In silico</i> Primer Validation.....	36
4.4	PCR Optimisation	38
4.4.1	Annealing Temperature Optimisation.....	38
4.4.2	Specificity Test	41
4.4.3	Sensitivity Test.....	44
4.5	PCR Amplification of Sweet Jelly Samples	47
CHAPTER 5 DISCUSSION		56
5.1	Extracted Genomic DNA of Fresh Muscle Tissues and Sweet Jelly Samples	56
5.2	Spectrophotometry DNA Quantification	57
5.3	<i>In silico</i> Primer Validation.....	58
5.4	PCR Optimisation	59
5.4.1	Annealing Temperature Optimisation.....	59
5.4.2	Specificity Test	60
5.4.3	Sensitivity Test.....	60
5.5	PCR Amplification of Sweet Jelly Samples	60
CHAPTER 6 CONCLUSION.....		63

6.1	Conclusion	63
6.2	Limitation and Future Recommendations.....	64
REFERENCES.....		65
APPENDICES.....		70
Appendix A: Sweet Jelly Products		70

LIST OF TABLES

Table 3.1: List of chemicals and reagents and consumables.....	14
Table 3.2: List of instruments and equipment.....	16
Table 3.3: Product information of candy samples used in this study.....	20
Table 3.4: Gelatine content and halal status of candy products.	21
Table 3.5: Primer sequences used in this study.....	26
Table 3.6: Cycle parameters for gradient PCR	28
Table 3.7: Cycle parameters for specificity and sensitivity optimisation.	28
Table 4.1: DNA quantification of raw meat samples using a DeNovix DS-11 NanoDrop Microvolume Spectrophotometer.....	34
Table 4.2: DNA quantification of sweet jelly products using a DeNovix DS-11 NanoDrop Microvolume Spectrophotometer.....	35
Table 4.3: Taxonomy report from NCBI BLAST analysis	36

LIST OF FIGURES

Figure 4.1: Agarose gel electrophoresis of high molecular weight (HMW) DNA extracted from raw fresh meat samples.	32
Figure 4.2: Agarose gel electrophoresis of HMW DNA extracted from sweet jelly samples (S1-S20).	33
Figure 4.3: In silico alignment of the forward mtDNA ND5 primers with the <i>Sus scrofa</i> sequence and non-target species sequences using BioEdit software.	36
Figure 4.4: In silico alignment of the reverse mtDNA ND5 primers with the <i>Sus scrofa</i> sequence and non-target species sequences using BioEdit software.	37
Figure 4.5: Agarose gel electrophoresis of gradient PCR products amplified using the eukaryotic universal primer.	39
Figure 4.6: Agarose gel electrophoresis of gradient PCR products amplified using mtDNA ND5 primer.	40
Figure 4.7: Specificity test of PCR amplification using eukaryotic universal primer against target species and non-target species.	42
Figure 4.8: Specificity test of PCR amplification using mtDNA ND5 primer against the target species and non-target species.	43
Figure 4.9: Sensitivity test of PCR amplification using eukaryotic universal primer.	45
Figure 4.10: Sensitivity test of PCR amplification using mtDNA ND5 primer.	46
Figure 4.11a: PCR amplification of eukaryotic universal primer for sample S1 to S5.	48
Figure 4.11b: PCR amplification of eukaryotic universal primer for sample S6 to S10.	49
Figure 4.11c: PCR amplification of eukaryotic universal primer for sample S11 to S15.	50
Figure 4.11d: PCR amplification of eukaryotic universal primer for sample S16 to S20.	51
Figure 4.12a: PCR amplification of the mtDNA ND5 primer for sample S1 to S5.	52

Figure 4.12b: PCR amplification of the mtDNA ND5 primer for sample S6 to S10.	53
Figure 4.12c: PCR amplification of the mtDNA ND5 primer for sample S11 to S15.....	54
Figure 4.12d: PCR amplification of the mtDNA ND5 primer for sample S16 to S20.	55

LIST OF APPENDICES

Appendix A: Sweet Jelly Products.....	70
---------------------------------------	----

LIST OF ABBREVIATIONS

ATR-FTIR	Attenuated Total Reflection Fourier Transform Infrared Spectroscopy
Avg.	Average
BLAST	Basic Local Alignment Search Tool
bp	Base pair
COI	Cytochrome oxidase I
COII	Cytochrome oxidase II
CTAB	Cetyltrimethylammonium Bromide
cytb	Cytochrome b
ddH ₂ O	Double distilled water
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide Triphosphates
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
HCl	Hydrochloric acid
HMW	High molecular weight
JAKIM	Department of Islamic Development Malaysia
Kbp	Kilo base pair
LC-MS/MS	Liquid Chromatography–Tandem Mass Spectrometry
LOD	Limit of detection
Mg ²⁺	Magnesium ion

MgCl ₂	Magnesium chloride
mtDNA	Mitochondrial DNA
NaCl	Sodium chloride
NADH	Nicotinamide Adenine Dinucleotide Hydride
NaOH	Sodium hydroxide
ND1	NADH Dehydrogenase 1
ND2	NADH Dehydrogenase 2
ND5	NADH Dehydrogenase 5
nDNA	Nuclear DNA
NIR	Near-Infrared Spectroscopy
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
qPCR	Real-time PCR
RNA	Ribonucleic Acid
rRNA	Ribosomal ribonucleic acid
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
ssDNA	Single-stranded DNA
T _a	Annealing temperature
TBE	Tris Borate EDTA
TE	Tris EDTA
tRNA	Transfer ribonucleic acid
UV	Ultraviolet

LIST OF SYMBOLS

%	Percent
°C	Degree Celsius
g	Gram
M	Molar
mg	Miligram
min	Minute
mL	Mililiter
mM	Milimolar
ng	Nanogram
pg	Picogram
rpm	Revolutions Per Minute
s	Second
V	Volt
w/w	Weight by weight
μL	Microliter

PENGESANAN KANDUNGAN KHNINZIR DALAM PRODUK GULA-GULA JELI MELALUI AMPLIFIKASI PCR GEN DNA MITOKONDRIA ND5

ABSTRAK

Produk gula-gula jeli yang terdapat di pasaran kebanyakannya dihasilkan menggunakan gelatin yang berasal daripada khinzir atau lembu. Risiko pemalsuan dan salah label sumber gelatin telah menjadi kebimbangan dalam kalangan pengguna disebabkan oleh kepercayaan agama, pemakanan dan isu kesihatan. Oleh itu, kajian ini bertujuan untuk mengesan bahan terbitan khinzir dalam gula-gula jeli melalui amplifikasi PCR menggunakan primer khusus spesies gen ND5 mtDNA. Sebanyak 20 sampel produk gula-gula jeli yang diperolehi daripada pelbagai sumber telah diekstrak menggunakan kaedah CTAB-fenol-kloroform dan dianalisis menggunakan PCR konvensional yang menyasarkan gen ND5 mtDNA khinzir. Dalam kajian ini, DNA yang diekstrak daripada daging khinzir mentah yang segar berperanan sebagai kawalan positif untuk DNA khinzir. Hasil kajian menunjukkan primer ND5 mtDNA adalah sangat khusus terhadap spesies sasaran khinzir dan mempunyai had pengesanan sehingga 1 pg DNA. Amplifikasi PCR menggunakan primer khusus spesies ND5 mtDNA terhadap semua produk gula-gula jeli menunjukkan bahawa hanya satu sampel S2, didapati positif DNA khinzir. Menariknya, satu sampel S12, yang jelas diisytiharkan mengandungi gelatin khinzir dalam produknya, menunjukkan keputusan negatif dalam amplifikasi PCR menggunakan primer ND5 mtDNA. Keadaan ini berkemungkinan disebabkan oleh pendedahan terhadap suhu tinggi semasa proses pembuatan serta proses enzimatik, menyebabkan degradasi DNA yang ekstrem. Kesimpulannya, primer gen ND5 mtDNA yang direka bentuk secara dalaman telah dibuktikan bersifat khusus kepada spesies khinzir dan boleh digunakan untuk pengesanan bahan terbitan khinzir dalam produk gula-gula jeli.

DETECTION OF PORCINE-DERIVED IN SWEET JELLY PRODUCT THROUGH PCR AMPLIFICATION OF THE MITOCHONDRIAL DNA ND5 GENE

ABSTRACT

Sweet jelly products that are available in the market are predominantly made from either porcine or bovine gelatine derived. The risk of adulteration and mislabeling of gelatine origin has become a concern among consumers due to religious beliefs, dietary and health issues. Therefore, this study aims to detect the porcine derived ingredients in sweet jelly products through PCR amplification of the mtDNA ND5 gene species-specific primer. A total of 20 samples of sweet jelly products obtained from various sources were extracted using the CTAB-phenol-chloroform method and analysed using conventional PCR targeting the mtDNA ND5 gene of porcine mtDNA. In this study, the DNA extracted from fresh pork raw meat was used as a positive control for porcine DNA. The result shows the mtDNA ND5 primer was highly specific to the porcine target species and has a limit of detection of 1 pg of DNA. PCR amplification of the mtDNA ND5 species-specific primer against all sweet jelly products showed that only one sample S2, was positive for porcine DNA. Interestingly, one sample S12, clearly declared to contain pig gelatine in its product, was negative for PCR amplification using the mtDNA ND5 primer. This could be caused by exposure to high temperature during the manufacturing and the enzymatic processes, resulting in extremely degraded DNA. In conclusion, the in-house designed primer mtDNA ND5 gene was proven as a porcine species-specific primer and can be used for the detection of porcine-derived from sweet jelly products.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Sweet jellies are generally classified as a chewy confectionery. These sweets are well-known among people of all ages due to their sweets taste and special texture (Park *et al.*, 2021). Sweet jellies undergo extensive thermal and chemical processing in their production with a gelling agent as key ingredients (Agung Cendekiawan *et al.*, 2025; Wang and Hartel, 2022). The gelling agent usually from hydrocolloids such as agar, pectin, starch, carrageenan and gelatine (Agung Cendekiawan *et al.*, 2025).

Gelatine is widely used for gummy production such as gummy candies and vitamin gummies. In 2023, gelatine led the market with a 67.8% revenue share worldwide. Gelatine is favour in manufacture because of its stability and compatible with variety of other ingredients such as flavours, minerals and vitamins (Grand View Research, 2025). Additionally, gelatine is frequently utilised as thickening, stabilizing, and texturizing properties in food, pharmaceutical, and cosmetic industries (Kang *et al.*, 2018; Rather *et al.*, 2022; Zhu *et al.*, 2023). Commercial gelatine is primarily derived from mammalian, poultry and fish (Rather *et al.*, 2022). This gelatine is extracted from animal parts such as bones, skin, and connective tissue collagen (Alipal *et al.*, 2019; Rather *et al.*, 2022). Approximately 46% gelatine sources come from porcine skin, 23.1% and 29.4% from bovine bones and hide, respectively and 1.5% from fish (Alipal *et al.*, 2019; Rather *et al.*, 2022; Sultana *et al.*, 2018).

The porcine-derived ingredients in sweet jellies have raised concern among consumers in Islamic countries, for instance Malaysia, Indonesia and Turkey (Kang *et al.*, 2018). Porcine-

derived ingredients are substances originating from pigs (*Sus scrofa*) or their by-products, such as gelatine, enzymes, lard, collagen and porcine blood plasma (Kim *et al.*, 2023; Omar *et al.*, 2018). This ingredient is strictly forbidden (Haram) in Islamic religious laws alongside alcohol and harmful materials (Agung Cendekiawan *et al.*, 2025; Kang *et al.*, 2018; Kim *et al.*, 2023). Therefore, those ingredients must not include in halal-certified products (Kim *et al.*, 2023).

Halal certification or halal logo has become a symbol to inform consumers that a product is compliance with Islamic law (Agung Cendekiawan *et al.*, 2025). The certificate is given by specific national or institutional bodies such as Department of Islamic Development Malaysia (JAKIM) in Malaysia (Fazmin, 2023). To receive the halal accreditation, manufacturers must go through a series of process, which can be time-consuming (Fazmin, 2023; Kim *et al.*, 2023). However, some irresponsible producers and product owners take the easy route, illegally use the halal logo to gain access to the Islamic market (Sultana *et al.*, 2018; Zhu *et al.*, 2023). Therefore, an analysis to verify the authentic and source origin of gelatine in processed food is required.

Several analytical approaches have been employed to authenticate and determine the source origin of porcine-derived gelatine. These techniques are generally classified into DNA-based and protein-based methods. Protein-based analytical approaches employed for species identification include Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR) (Zhu *et al.*, 2023), Enzyme-Linked Immunosorbent Assay (ELISA) (Salamah *et al.*, 2021; Zhu *et al.*, 2023), Near-Infrared (NIR) Spectroscopy (Agung Cendekiawan *et al.*, 2025), Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) (Salamah *et al.*, 2021; Zhu *et al.*, 2023), Mass Spectrometry (Zhu *et al.*, 2023) and Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) combined with Principal Component

Analysis (PCA) (Sepminarti *et al.*, 2016). Among these techniques, LC-MS/MS offers highly specific identification through biomarker peptides and provides the lowest limits of detection for quantitative analysis in complex food matrices (Zhu *et al.*, 2023), while Near-Infrared (NIR) spectroscopy provides the rapid qualitative analysis and preserves the integrity of the sample due to its non-destructive nature (Agung Cendekiawan *et al.*, 2025).

However, protein-based methods are generally unsuitable for identifying origin of gelatine in highly processed products such as sweet jellies. During gelatine production and the final food product, protein biomarkers in the gelatine are extensively degraded (Kang *et al.*, 2018). The extreme temperature and pH treatments cause the target protein to undergo progressive denaturation (Omar *et al.*, 2018). Furthermore, several studies have demonstrated that these methods unsuitable for differentiating gelatine mixtures in food products because mammalian gelatine sources share highly similar protein sequences (Zhu *et al.*, 2023). Due to these limitations, DNA-based methods using polymerase chain reaction (PCR) are considered most reliable for detecting the origin of gelatine (Omar *et al.*, 2018).

PCR is preferred because DNA exhibits relative stability and resistance to high temperatures and chemical treatments compared to proteins (Omar *et al.*, 2018). The technique can target either nuclear DNA (nDNA) or mitochondrial DNA (mtDNA) for species identification. Nuclear DNA has lower copy numbers compared to mtDNA, rendering it less sensitive, especially when the DNA is highly degraded (Kang *et al.*, 2018; Linacre, 2021). In highly processed food, the DNA sequence is fragmented and present in low amounts. In contrast, mitochondrial DNA allows amplification of surviving fragments due to its multiple copies per cell and ability to target specific short sequences. Mitochondrial DNA exhibits a

faster evolution rate than nuclear DNA, providing sufficient sequence variation among species to support accurate species identification (Kang *et al.*, 2018).

1.2 Problem Statement

In recent years, there have been numerous reports of confectionery products containing porcine-derived ingredients despite being labelled as Halal. Some confectionery products may not directly use pork as a primary ingredient; however, certain components used in their formulation may be derived from pigs (*Sus scrofa domestica*). These porcine-derived ingredients are commonly found in the form of gelatine, glycerin, fatty acids and their derivatives, as well as enzymes or processing aids.

This issue raises major concerns among Muslim and Jewish consumers, as well as vegetarians and health-conscious individuals, who avoid pork for religious, ethical, or dietary reasons. Therefore, the detection of porcine-derived components in confectionery is essential to safeguard religious and ethical values as well as to protect consumer rights and to reduce risks of food fraud and mislabelling ingredient.

1.3 Significance of Research

The significance of this study lies in its focus on detecting the presence of porcine-derived ingredients in sweet jelly products through molecular techniques. At the same time, verify that product labels, particularly Halal certifications, are accurate and truthful. This is important because nowadays, many jelly sweets are coming from China and Thailand, where most are not Halal-certified. As we know, these products are preferred by children. Consequently, the findings of this study can contribute to the prevention of food fraud, promote

transparency in food labelling and enhance consumer confidence, especially for sweet jelly products that are not certified by JAKIM.

1.4 Objectives

1.4.1 Main Objectives

To detect the porcine-derived ingredients in sweet jelly products through PCR amplification of mtDNA ND5 gene.

1.4.2 Specific Objectives

- i. To extract and quantify the extracted genomic DNA from sweet jelly samples.
- ii. To amplify porcine DNA from the control sample using species-specific primers targeting the porcine mtDNA ND5 gene.
- iii. To identify porcine DNA from sweet jelly products using species-specific primers targeting mtDNA ND5 gene.

CHAPTER 2

LITERATURE REVIEW

2.1 Sweet Jellies and Gelatine-Based Product

Sweet jellies are popular soft, sweet, and semi-solid confectionery products with a unique texture (Astuti *et al.*, 2023; Park *et al.*, 2021). These products come in various types, including gummies, candies, marshmallows, gumdrops and pastilles (Mikhailov, 2023; Sultana *et al.*, 2018). The common ingredients in sweet jellies are sugar (sucrose), gelling agent, starch, emulsifiers, additives, flavouring and colouring agents, glucose syrups, water and citric acid (Agung Cendekiawan *et al.*, 2025; Astuti *et al.*, 2023; Sultana *et al.*, 2018). Generally, the production of these confectioneries involves several processes, which are dissolving and heating at higher temperatures, moulding and solidifying, and may also involve chemical processes (Agung Cendekiawan *et al.*, 2025; Ali and Susanto, 2024; Wang and Hartel, 2022).

The chewy, elastic and rubbery structure of sweet jelly is provided by gelling agents such as gelatine, carrageenan, starch, gum, agar or pectin (Ali and Susanto, 2024; Astuti *et al.*, 2023; Wang and Hartel, 2022). Gelatine is a protein that originates from various animal collagens found in hides, bones, skins and smooth connective tissues (Park *et al.*, 2021; Sultana *et al.*, 2018; Wang and Hartel, 2022). Meanwhile, carrageenan, starch, gum, agar and pectin are plant-based gelling agents derived from seaweeds and fruits (Ali and Susanto, 2024; Astuti *et al.*, 2023; Park *et al.*, 2021). Among these, gelatine is the widely used gelling agent (Astuti *et al.*, 2023).

Mammalian sources such as porcine and bovine dominate the gelatine production market compared to poultry and fish species (Alipal *et al.*, 2021; Rather *et al.*, 2022). Porcine

gelatine is preferable among manufacturers due to its availability, cost-effectiveness, shorter production cycle and has the greatest gelling properties compared to other gelatine sources (Abdullah Amqizal *et al.*, 2017; Hibaturrahman *et al.*, 2023; Sultana and Azlan, 2025; Sultana *et al.*, 2018). Thus, the risks of adulteration and mislabelling may arise when dishonest manufacturers conceal the presence of porcine substances on packaging labels to increase their profits while ensuring access to Muslim markets (Abdullah Amqizal *et al.*, 2017; Sultana and Azlan, 2025). This is a critical halal concern, as Islamic law strictly prohibits the consumption of any pork or its derivatives, including gelatine, lard and porcine-derived enzymes (Agung Cendekiawan *et al.*, 2025; Kang *et al.*, 2018; Kim *et al.*, 2023). This issue also affects Jewish, vegetarians and vegans, individuals with porcine allergies and health-conscious consumers (Muñoz-Colmenero *et al.*, 2016; Natonek-Wisniewska *et al.*, 2025; Sahilah *et al.*, 2016; Shahimi *et al.*, 2021; Sultana *et al.*, 2018). Hence, an analysis to verify the authentication and source origin of gelatine, especially porcine in processed food such as sweet jelly is important.

Nowadays, multiple analytical methods have been applied to identify and determine the source of gelatine. These analyses rely on protein-based and DNA-based approaches. Some of the protein-based analytical approaches are ATR-FTIR (Zhu *et al.*, 2023), ELISA (Salamah *et al.*, 2021; Zhu *et al.*, 2023), NIR Spectroscopy (Agung Cendekiawan *et al.*, 2025), Liquid Chromatography–Tandem Mass Spectrometry (Salamah *et al.*, 2021; Zhu *et al.*, 2023), Mass Spectrometry (Zhu *et al.*, 2023) and SDS-PAGE (Sepminarti *et al.*, 2016). However, DNA-based methods using polymerase chain reaction (PCR), are considered more reliable for detecting the origin of gelatine than protein-based methods. This is because DNA is often more stable than proteins and DNA biomarkers, particularly from mtDNA genes, are more suitable for processed products (Omar *et al.*, 2018). The extreme physical and chemical treatments during gelatine production and final food product cause the target protein to undergo

progressive denaturation and degradation, leaving only extremely short fragments and a low amount of DNA (Kang *et al.*, 2018; Omar *et al.*, 2018; Sultana *et al.*, 2018). Moreover, PCR can also differentiate gelatine mixtures in food products, which is challenging by protein-based analysis due to mammalian gelatine sources sharing highly similar protein sequences (Natonek-Wisniewska *et al.*, 2022; Zhu *et al.*, 2023).

2.2 Mitochondrial DNA (mtDNA)

Mitochondria are small, oval-shaped organelles located in the cytoplasm that act as cell's energy production and other metabolic functions (Chadwick, 2025; Kowalczyk *et al.*, 2021; Mohammad *et al.*, 2025). This organelle has its own DNA, which is inherited maternally and is known as mitochondrial DNA (mtDNA) (Chadwick, 2025; Figura *et al.*, 2024). Mitochondrial DNA is haploid, non-recombining and circular in shape (Kang *et al.*, 2018). This mtDNA contains a double-stranded DNA molecule, with heavy (H) and light (L) strands that form due to different guanine density (Ferreira and Rodriguez, 2024). The mitochondrial genome is approximately 16.6 Kbp and encodes for 37 genes, including 13 protein-encoding genes for oxidative respiration, 22 transfer RNA (tRNA) genes and two ribosomal RNA (rRNA) genes. The Displacement loop (D-loop) in mtDNA is a non-coding region with a length of 1.1 kb (Ferreira and Rodriguez, 2024; Garcia *et al.*, 2017).

Ferreira and Rodriguez (2024) reported that 13 protein-encoding genes in mtDNA are essential for four complexes of the oxidative phosphorylation (OXPHOS) system. Complex I consist of seven subunits of nicotinamide adenine dinucleotide (NADH) dehydrogenase (ND1, ND2, ND3, ND4, ND5, ND6, and ND4L). Complex III of 1 subunit, cytochrome b (Cyt b), while Complex IV consists of three subunits cytochrome c oxidase (COI, COII and COIII). Additionally, Complex V consists of two subunits (ATP6 and ATP8). The tRNA genes encode

22 tRNA molecules corresponding to specific amino acids for mitochondrial protein synthesis, while rRNA genes form small (12s rRNA) and large (16s rRNA) mitochondrial ribosomal subunits.

The mtDNA has several advantages over nuclear DNA (nDNA), making it preferable for species identification. The mitochondrial has 1000 to 10000 copies per cell (Figura *et al.*, 2024) while nDNA only has two copies per cell (Mohammad *et al.*, 2025). This feature increases the probability of successful analysis and is very useful for biological samples with limited DNA. Besides, the small, circular double-stranded mtDNA is more resistant to degradation compared with the linear nDNA (Al-Shaibany *et al.*, 2022; Nikzad *et al.*, 2017). Additionally, low probability of combination, absence of introns (Figura *et al.*, 2024) and rapid evolution for closely related species detection (Figura *et al.*, 2024; Kang *et al.*, 2018) are the reasons why mtDNA is preferred for species identification.

2.2.1 mtDNA Gene Regions Used in Species Authentication

Several mtDNA genes have been utilised for species authentication from various processes foods. MtDNA genes such as cytochrome c oxidase I (COI) (Natonek-Wisniewska *et al.*, 2025), cytochrome b (cytb) (Mohammad *et al.*, 2025), ribosomal RNA (Figura *et al.*, 2024), D-loop (Kang *et al.*, 2018), ATP8 (Kang *et al.*, 2018), ATP6 (Uddin *et al.*, 2021), ND1 (Ran *et al.*, 2016), ND2 (Barido *et al.*, 2023) and ND5 (Kang *et al.*, 2021) are among the DNA markers used for species identification.

DNA barcoding is the first established DNA marker proposed for species identification based on a standard mtDNA gene, known as cytochrome oxidase I (COI). The COI gene has become a standard framework established by the Barcode of Life Data System (BOLD) and

widely used due to a high level of interspecies and intraspecies variation (Linacre, 2021; Sultana *et al.*, 2018). In mammals, the COI gene is located between the nucleotide positions 5904 and 7445 (Linacre, 2021). Meanwhile, COII is useful for discriminating closely related groups when combined with COI (Natonek-Wiśniewska *et al.*, 2025). A study by Mutalib *et al.* (2015) showed that the sensitivity of the COII primer to detect porcine DNA was 0.1 ng in capsule samples.

Another mtDNA marker is the cytochrome B (cytb) gene. This gene is commonly used as a marker for phylogenetic and forensic taxonomic analyses (Figura *et al.*, 2024). A study conducted by Sultana *et al.* (2018) based on species discrimination from gelatine products and confectionery products by using cytb genes. They found that the cytb gene was successful applied for species identification, even with the DNA in the samples as low as 0.1 ng. Another study on detecting porcine DNA in sugar confectionery using qPCR targeting the cytb was also successful (Mohammad *et al.*, 2025).

Another marker used for species authenticity is ribosomal genes. The 12s rRNA has been successfully used to identify vertebrates such as big cats (pumas, jaguars and ocelots) and 12 species of wild animals in Lebanon (Figura *et al.*, 2024). The amplicon size of the 12s rRNA gene is relatively short, making it a reliable marker for environmental and degraded samples (Figura *et al.*, 2024). Meanwhile, the 16s rRNA marker is suitable for species-specific DNA fragments of different lengths according to the species in complex food products (Muflihah *et al.*, 2023). Kang *et al.* (2018) has implemented the D-loop marker for the identification of porcine gelatine from as low as 0.05% (w/w) in complex mixture samples. In the same study, the ATP8 marker can be utilised for species identification in processed foods with 0.1% (w/w) limit of detection. While the ATP8 gene alone is sufficient to distinguish specific species, the

ATP6 gene should be combined with other mtDNA markers for promising detection (Uddin *et al.*, 2021).

Mitochondrial NADH dehydrogenase subunits such as ND1, ND2 and ND5 has been used in various studies, especially for detecting porcine in processed food matrices. ND1 have been used for species-specific primer in Loop-Mediated Isothermal Amplification (LAMP) assay. The results show that it can detect pork DNA in meat samples even when mixed at 0.01% with beef or mutton, as well as after undergo the heating process (Ran *et al.*, 2016). On the other hand, a duplex-PCR targeting ND2 for bovine and porcine in meat products was validated by Barido *et al.* (2023). The assay is more sensitive than many PCR methods because it can detect as little as 0.0001 ng/ μ L of DNA from highly processed meat products. ND5 has been used to identify porcine content in skeletal muscle tissue, meatballs, and highly processed meat products (Kang *et al.*, 2021; Kusnadi and Ashari, 2022). ND5 marker has also been applied in species identification from confectionery products using the PCR methods (Kang *et al.*, 2018).

2.3 Polymerase Chain Reaction (PCR)

Polymerase chain reaction is an in-vitro method used to replicate or enzymatically amplify a specific DNA fragment. The millions of copies of template DNA are produced quickly through a series of repetitive thermal cycles. The method follows the principle of nucleic acid hybridization with combination of nucleic acid replication. First, the double-stranded DNA (dsDNA) breakdown into single-stranded DNA (ssDNA). Then, the DNA template is amplify using a specific primer before that template is replicate using the DNA polymerase enzyme (Aryal, 2025; Britannica, 2025).

The PCR assay requires a reaction mixture consisting of several essential components. A DNA template that contains a selected nucleic acid sequence is one of the components for

PCR. Another component is DNA polymerase, an enzyme that synthesizes DNA. *Taq* DNA polymerase is the most commonly use enzyme than *Vent* and *Pfu* enzymes. A pair of primers from forward and reverse strands is required from short single-stranded sequences and complementary to template DNA, acting as a starting point for DNA synthesis.

Additionally, deoxynucleotide triphosphates (dNTPs) act as building blocks for new DNA strands. The dNTPs consist of four different bases; deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP), and Deoxycytidine triphosphate (dCTP), which are sequentially added to generate a new strand of DNA complementary to the template strand. The last component for PCR is the PCR buffer solution. The common buffer used is a 10X buffer reaction with Mg^{2+} cofactor from $MgCl_2$ (Aryal, 2025).

One cycle of PCR amplification consists of three steps: denaturation, annealing and elongation. In the denaturation steps, the hydrogen bonds in the dsDNA template are broken due to thermal energy, producing two ssDNA templates. Next, the temperature in the annealing step is reduced to allow primers to anneal with the complementary region of ssDNA. In elongation steps, the temperature increases to begin synthesis of new DNA strands in the 5' to 3' direction. The dNTPs are added by DNA polymerase to 3' OH- end of the annealed primer, and two new dsDNAs will form the end of the cycle.

PCR-based techniques have been widely applied in previous studies for species detection in various highly processed food products. Numerous studies have demonstrated the effectiveness of PCR as a reliable tool to solve the issue related to adulteration and mislabelling in food product. However, the success of PCR amplification depends on several factors. The

highly processed ingredients, such as gelatines, usually contain extremely low amounts of DNA, resulting in an insufficient DNA template for amplification (Al-Shaibany *et al.*, 2022). The presence of PCR inhibitors may interfere with the enzymatic reaction during amplification (Leonard *et al.*, 2016). Moreover, the specificity and efficiency of the primer are also important. Thus, utilisation of primer targeting short DNA fragments was recommended for severely degrade DNA from food samples (Mohammad *et al.*, 2025).

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Chemicals and Reagents

All chemicals, reagents and consumables utilized in this study are listed in Table 3.1.

Table 3.1: List of chemicals and reagents and consumables.

Chemical /Reagent / Consumable Materials	Company/Supplier
CTAB	Sigma-Aldrich, USA
PVP	Sigma-Aldrich, USA
EDTA disodium salt dihydrate	Sigma-Aldrich, USA
NaCl	Sigma-Aldrich, USA
Tris base	Vivantis
Boric Acid	Merck KGaA, Germany
Proteinase K	Vivantis
Phenol	Sigma-Aldrich, USA
Chloroform	Merck KGaA, Germany
Ammonium acetate powder	HMBG
Isopropanol	HMBG
Absolute ethanol	HMBG
HCl	HMBG
NaOH pellet	Sigma-Aldrich, USA
1KB Plus DNA Allelic Ladder	Sigma-Aldrich, USA
100bp Allelic Ladder	Sigma-Aldrich, USA
Taq DNA Polymerase	Invitrogen, Brazil
dNTPs	Promega
10X Taq Buffer	Invitrogen, Brazil
MgCl ₂	Invitrogen, Brazil
Primers	Integrated DNA Technologies

Ethidium bromide	Sigma-Aldrich, USA
Orange G Loading Dye	Sigma-Aldrich, USA
Agarose powder	Matrioux (M) Sensitivity. Bhd.
Pipette tips P10, P200 and P1000	Teraslab Sdn. Bhd.
1.5 mL Microcentrifuge tubes	Teraslab Sdn. Bhd.
2.0 mL Microcentrifuge tubes	Teraslab Sdn. Bhd.
0.2mL PCR Tubes	Teraslab Sdn. Bhd.
Kimwipe	Kimberly-Clark, United States
Falcon tubes 50 mL	Teraslab Sdn. Bhd.
Parafilm	Amcor Flexibles
Nitrile glove	Glof

3.1.2 Equipment and Instruments

All laboratory equipment and scientific instruments utilized in this study are listed in Table 3.2.

Table 3.2: List of instruments and equipment.

Instrument/Equipment	Brand	Model
Pipette 2.5 μ L, 10 μ L, 20 μ L, 100 μ L, 200 μ L, and 1000 μ L	Gilson/ Eppendorf/ Dragonlab/ Transferpette S	P2.5, P20, P100, P200 and P1000
Analytical balance	Sartorius	BSA 224S-CW
Shaking waterbath	Memmert	WNB14L4
Hot air oven sterilizer	Memmert	UE600
Hotplate with magnetic stirrer	DLAB	MS7-H550-S
Class II Biosafety Cabinet	Labconco	Delta Series (BSC)
Microcentrifuge (Refrigerated)	Hettich Uniersa	Micro 22R
Vortex Mixer	ERLA	EVM-6000
Spectrafuge 24D Starter Pack	Labnet	C2400
Microwave oven	ELBA	EMO-1706
Power pack	Bio-Rad	Power-Pac 3000V
DNA Electrophoresis system	Owl	B1
Gel Documentation system	Bio-Rad	GELDOC XR+
Thermal Cycler (Veriti)	Applied Biosystems	Veriti 9902
Mini-centrifuge/vortex	BioSan	Combispin FVL-2400N
Nanodrop microvolume spectrophotomer	DeNovix	DS-11

3.1.3 Buffer and Other Solution Preparation

3.1.3.1 Cetyltrimethylammonium Bromide (CTAB) lysis buffer

A total of 8.18 g of sodium chloride (NaCl) powder was dissolved in approximately 70 mL of double-distilled wate (ddH₂O) in a 250 mL beaker. The solution was stirred using a magnetic stirrer. After NaCl salt fully dissolved, 1.21 g of tris base and 1 g of polyvinylpyrrolidone (PVP) was added. Next, 2 g of CTAB was added while heating the

solution until it cleared. Subsequently, 4 mL of 0.5M ethylenediaminetetraacetic acid (EDTA) solution was added into the beaker. The pH of the solution was adjusted to pH 8 using 1 M hydrochloric acid (HCl). The solution was transferred into 100 mL volumetric flask and ddH₂O was added to the mark and then homogenized. The CTAB lysis buffer then stored in 100 mL Schott bottle at room temperature.

3.1.3.2 Proteinase K (20mg/mL)

Approximately 20 mg of Proteinase K was dissolved in one mL of sterile deionised water (ddH₂O). Then, the solution was stored at -20 °C for further use.

3.1.3.3 7.5 M Ammonium acetate

A total of 57.81 g of ammonium acetate was weighed and dissolved in approximately 70 mL of ddH₂O. The solution was then transferred into a 100 mL volumetric flask and made up to the mark with ddH₂O. The solution was mixed thoroughly and stored at room temperature.

3.1.3.4 70% Ethanol

To prepare 70% ethanol, a total of 70 mL of absolute ethanol was measure and poured into a 100 mL volumetric flash. Then, the ddH₂O was added until calibration mark and the solution was mixed. The solution was stored at room temperature.

3.1.3.5 0.5M EDTA pH8 solution

EDTA disodium dihydrate was weighed at 186.1 g and was dissolved with approximately 800 mL of ddH₂O in 1 L beaker. The solution was stirred using a magnetic stirrer. The pH was then adjusted to 8.0 with sodium hydroxide (NaOH) pallet. After the solution cleared, transfer it into a 1 L volumetric flask. The ddH₂O was added to the mark and

then homogenized. The solution was transfer into a 1 L Schott bottle, autoclaved and stored at room temperature.

3.1.3.6 Tris-EDTA (TE) (low EDTA) buffer

A total of 0.1576 g of tris base and 0.00372 g of EDTA disodium were placed in a 100 mL beaker. Then, add ddH₂O until 70 mL. The pH of the solution was adjusted to 8. After that, transfer the solution into a 100 mL volumetric flask and top up with ddH₂O until the mark. The solution was sterilised by autoclaving and stored at room temperature.

3.1.3.7 10X Tris Borate EDTA (TBE) buffer

The stock buffer solution of TBE was prepared by dissolving 108 g of tris base, 55 g of boric acid and 40 mL of 0.5 M EDTA solution in 700 mL of distilled water. The solution was stirred using a magnetic stirrer. After the solution cleared, transfer it into a 1 L volumetric flask. The ddH₂O was added to the mark and then homogenized. The solution was sterilised by autoclaving and stored at room temperature.

3.1.3.8 0.5X Tris Borate EDTA (TBE) buffer

The working TBE solution buffer for gel electrophoresis was prepared by adding 50 mL of 10X TBE buffer to a 1L volumetric flask. Then, ddH₂O was added to the mark. The solution was sterilised by autoclaving and stored at room temperature.

3.1.3.9 Orange G loading dye

Weigh 0.125 g of orange G powder and 20 g of sucrose powder and dissolved them in 30 mL of sterile ddH₂O. The sterile ddH₂O was added to the solution until the final volume reached 50 mL. Then, the solution was aliquoted into 1.5 mL microcentrifuge tubes and stored at -20°C.

3.2 Sample Collection and Preparation

In this study, fresh pork meat (*Sus scrofa*) was used as control sample. The other seven fresh raw meat samples were used as non-target animal species. These fresh raw meats are from cow (*Bos taurus*), Mozambique tilapia fish (*Oreochromis mossambicus*), chicken (*Gallus gallus domesticus*), mallard duck (*Anas platyrhynchos*), quail (*Coturnix japonica*), lamb (*Ovis aries*) and buffalo (*Bubalus bubalis*). The control sample and non-target samples were purchased from the wet market around Kota Bharu, Kelantan. All raw meat samples were stored at -20°C prior to the DNA extraction.

A total of 20 samples including gummies, jelly beans, marshmallow, and creamy candy were bought from different store around Kota Bharu, Kelantan. Some information on the product used in this study were recorded as Table 3.3 and Appendix A. Table 3.4 was prepared to summarise the gelatine content and halal status from each type of candy. Seven out of 20 candies were gelatine labelled.

Table 3.3: Product information of candy samples used in this study.

No.	Sample	Type of candy	Gelatine type	Halal certification bodies
1	S1	Gummy	Gelatine	No information
2	S2	Creamy candy	Gelatine	No information
3	S3	Jelly bean	No information	Shanxi SSPY
4	S4	Gummy	No information	No info
5	S5	Gummy	No information	ARA Halal Certification Services Center
6	S6	Gummy	Gelatine	Shandong Halal Certification Service
7	S7	Gummy	No information	No information
8	S8	Gummy	No information	No information
9	S9	Gummy	No information	No information
10	S10	Gummy	No information	No information
11	S11	Jelly bean	No information	Shandong Halal Certification Service
12	S12	Marshmallow	Pork	No information
13	S13	Jelly bean	No information	Shandong Halal Certification Service
14	S14	Gummy	No information	ARA Halal Certification Services Center
15	S15	Gummy	No information	ARA Halal Certification Services Center
16	S16	Gummy	No information	ARA Halal Certification Services Center
17	S17	Gummy	No information	ARA Halal Certification Services Center
18	S18	Gummy	Bovine	ARA Halal Certification Services Center
19	S19	Gummy	Bovine	Shandong Halal Certification Service
20	S20	Gummy	Gelatine	Halal

Table 3.4: Gelatine content and halal status of candy products.

Product	Gelatine labelled		Non-gelatine labelled		Total
	Halal	Non-halal labelled	Halal	Non-halal labelled	
Gummy candy	4	1	5	5	15
Jelly bean	0	0	3	0	3
Creamy candy	0	1	0	0	1
Marshmallow	0	1	0	0	1
Total	4	3	8	5	20

3.3 DNA Extraction

Genomic DNA was extracted from all fresh raw meats and candy products using an organic method (CTAB-phenol-chloroform). The extraction process was performed strictly following the procedures and guidelines in the DNA analysis to avoid contamination.

3.3.1 DNA Extraction of Fresh Raw Meats

Firstly, 200 mg of the raw meat sample was weight and cut into small pieces. The sample was placed into a 2 mL sterile microcentrifuge tube. Then, 500 μ L of CTAB lysis buffer and 10 μ L of proteinase K solution were added to each tube. All tubes were vortexed thoroughly and incubated overnight at 56°C.

The mixtures were centrifuged for 5 minutes at 14,000 RPM after the samples were fully dissolved. The supernatant was then transferred into a new microcentrifuge tube. Half volume of chilled phenol and half volume of chloroform were added to the supernatant. The mixture was mixed gently for 10 seconds and the tubes were centrifuged for 3 minutes at 14,000 RPM. The upper phase was transferred into a new 2 mL centrifuge tube and the steps was repeated.

Next, one volume of chloroform was added into the tube containing the supernatant and mixed gently for 10 seconds. The tubes were centrifuged for 3 minutes at 14,000 RPM and the upper phase was transferred into a new 1.5 mL centrifuge tube. The amount of supernatant in each tube was measured.

After that, half volume of 7.5 M ammonium acetate was added into the tube and mixed gently for several seconds before the tubes were spun down for 10 sec at 6000 RPM. Then, 0.6 volume of isopropanol was added into the tubes and mixed gently by inverting the tube for 2 minutes followed by centrifuged the tube for 10 minutes at 14,000 RPM at 25 °C. The supernatant was then discarded and 1 mL of 70% ethanol was added into each tube to wash the pellet. The bottom part of each tube was flicked to wash the pellet. The tubes were centrifuge for 10 minutes at 14,000 RPM at 25 °C. The supernatant was discarded and the washing step was repeated. The DNA pellets were left to air dry overnight.

The next day, a total of 30 µL of TE buffer was added to each tubes containing the DNA pellet and incubated overnight at 37 °C to elute the DNA. The extracted DNA solutions were store at – 20 °C until further use.

3.3.2 DNA Extraction of Candy Samples Product

Firstly, 200 mg of the sample was weighed and cut using sterile scissor with 70% ethanol solution. The sample was placed into a 2 mL sterile microcentrifuge tube. Next, 500 µL of CTAB lysis buffer and 10 µL of proteinase K solution were added to each tube containing the sample. All tubes were vortexed thoroughly to mix the contents and incubated overnight at 56°C in a shaking water bath, set at a high speed.

The following day, after the samples were fully dissolved, the mixtures were centrifuged for 5 minutes at 14,000 RPM. The supernatant was then transferred into a new 2 mL centrifuge tube. Half volume of chilled phenol and half volume of chloroform were added to the supernatant. The mixture was mixed gently for 10 seconds and the tubes were centrifuged for 3 minutes at 14,000 RPM. Next, the upper phase was transferred into a new 2 mL centrifuge tube. Half volume of chilled phenol and half volume of chloroform were added to the supernatant and mixed gently for 10 seconds. The tubes were centrifuged for 3 minutes at 14,000 RPM and the upper phase was transferred into a new 1.5 mL centrifuge tube.

After that, one volume of chloroform was added into the tube containing the supernatant and mixed gently for 10 seconds. The tubes were centrifuged for 3 minutes at 14,000 RPM and the upper phase was transferred into a new 1.5 mL centrifuge tube. The amount of supernatant in each tube was measured. Subsequently, half volume of 7.5 M ammonium acetate was added into the tube and mixed gently by inverting the tube for several seconds before the tubes were spin down for 10 sec at 6000 RPM. Then, 0.6 volume of isopropanol was added into the tubes. The mixture was mixed gently by inverting the tube for 2 minutes, followed by centrifuging the tube for 10 minutes at 14,000 RPM at 25 °C.

The supernatant was then discarded and 1 mL of 70% ethanol was added into each tube. The bottom part of each tube was flicked to wash the pellet. The tubes were centrifuge for 10 minutes at 14,000 RPM at 25 °C. The supernatant was discarded and 1 mL of 70% ethanol was added to each tube. The tubes were flicked and centrifuged for 10 minutes at 14,000 RPM at 25 °C. The ethanol was then discarded and the DNA pellets were left to air dry overnight. The next day, the 30 µL of TE buffer was added to each tubes containing DNA pellet and incubated

overnight at 37 °C to elute the DNA. The extracted DNA solutions were stored at – 20 °C until further use.

3.4 Agarose Gel Electrophoresis of Genomic DNA

The casting tray for agarose was prepared by applying the cellophane tape at both ends of the casting tray. Then, the comb was disinfected by wiping with 70% ethanol before placed to the casting tray. After that, the 1% of agarose gel was prepared by adding 1 gram of agarose powder and 100 mL of 0.5X TBE buffer into a 250 mL Schott bottle. The cap was placed loosely on the bottle. The mixture was dissolved by heated in the microwave oven at medium-low temperature for 3 minutes. The agarose gel solution was allowed to cool down to 50 °C under running tap water.

A gel staining dye, 1.5 µL of ethidium bromide was added into the agarose gel solution and swirled to mix it. The solution was poured into the casting tray and at this step, it should be ensured that no bubbles were present on the surface of the gel. The solution was left to solidify at room temperature for 45 minutes.

After gel was solidified, remove the cellophane tape and place the casting tray inside the tank. A 0.5X TBE buffer was poured into the tank until it reached indicator marks. The comb was gently removed from the gel. A 2 µL of 1Kbp DNA ladder was pipetted into first well in the gel to serve as DNA marker. A 5 µL of the extracted DNA sample was mixed with 2 µL orange G dye. The mixture was loaded into the well. The electrophoresis was performed at 90 V for 1 hour. The DNA product was visualized under UV light using gel documentation system.