DEVELOPMENT OF LAMP (LOOP MEDIATED ISOTHERMAL AMPLIFICATION) BASED TEST FOR DIRECT SPECIES IDENTIFICATION IN MULTIPLE MEAT TYPES

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

MELİSA KAYA KILAVUZ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

SEPTEMBER 2026

Approval of the thesis:

DEVELOPMENT OF LAMP (LOOP MEDIATED ISOTHERMAL AMPLIFICATION) BASED TEST FOR DIRECT SPECIES IDENTIFICATION IN MULTIPLE MEAT TYPES

submitted by **MELİSA KAYA KILAVUZ** in partial fulfillment of the requirements for the degree of **Master of Science** i**n Biology, Middle East Technical U**niversity by,

Prof. Dr. Naci Emre Altun
Dean, **Graduate School of Natural and Applied Sciences** _____

Prof. Dr. Meryem Beklioğlu
Head of the Department, **Biology** _____

Prof. Dr. Hüseyin Avni Öktem
Supervisor, **Biology, METU** _____

Examining Committee Members:

Prof. Dr. Füsun Eyidoğan
Molecular Biology and Genetics Dept., Başkent University _____

Prof. Dr. Hüseyin Avni Öktem
Biology Dept., METU _____

Prof. Dr. Çağdaş Devrim Son
Biology Dept., METU _____

Date: 01.09.2026

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name Last name : Melisa Kaya Kılavuz

Signature :

ABSTRACT

DEVELOPMENT OF LAMP (LOOP MEDIATED ISOTHERMAL AMPLIFICATION) BASED TEST FOR DIRECT SPECIES IDENTIFICATION IN MULTIPLE MEAT TYPES

Kaya Kılavuz, Melisa
Master of Science, Biology
Supervisor : Prof. Dr. Hüseyin Avni Öktem

September 2026, 107 pages

Increasing concerns regarding meat adulteration have highlighted the need for rapid and reliable methods for meat species authentication. LAMP offers a hopeful alternative to conventional DNA-based methods due to its amplification speed, high specificity, and minimal equipment requirements. This study aimed to advance a colorimetric direct LAMP system with common reaction conditions for porcine, equine, and chicken identification as a basis for a future multiplex platform enabling simultaneous detection of multiple meat species. Species-specific primer sets and an animal internal control were evaluated under common reaction conditions. The optimized system consisted of reaction buffer at pH 8.75, 6 mM $MgSO_4$, 250 µM cresol red, and 50 µM hydroxy naphthol blue (HNB), with amplification performed at 65°C for 60 min. The combined use of cresol red and HNB provided clear visual discrimination of amplification results. Direct LAMP enabled amplification from heat-treated meat samples without conventional DNA isolation, simplifying sample preparation. Specificity experiments demonstrated selective detection of the target species without cross-reactivity with the other tested meat species, while the animal

internal control confirmed successful amplification from meat samples. The assay also maintained target detection following heat treatment at 98°C for up to 60 min and autoclaving at 121°C for up to 45 min. Overall, the establishment of common reaction conditions for three species-specific assays, together with the dual colorimetric detection approach, provides a foundation for the development of a rapid, visually interpretable, and potentially multi-LAMP platform that reduces the need for advanced equipment, supports field applicability, and may contribute to more accessible meat authentication, improved food safety, and consumer protection.

Keywords: Multiple Meat Species Detection, Dual Colorimetric Detection, LAMP, Direct LAMP.

ÖZ

ÇOKLU ET TÜRLERİNDE DOĞRUDAN TÜR TAYİNİ İÇIN LAMP (İLMİĞE DAYALI İZOTERMAL ÇOĞALTMA) TEMELLİ TEST GELİŞTİRİLMESİ

Kaya Kılavuz, Melisa
Yüksek Lisans, Biyoloji
Tez Yöneticisi: Prof. Dr. Hüseyin Avni Öktem

Eylül 2026, 107 sayfa

Et tağşişine ilişkin artan endişeler, et türlerinin doğrulanmasına yönelik hızlı ve güvenilir yöntemlere duyulan ihtiyacı artırmıştır. İlmiğe dayalı izotermal çoğaltma (LAMP), hızlı amplifikasyon sağlaması, yüksek özgüllüğü ve minimum ekipman gereksinimi nedeniyle geleneksel DNA temelli yöntemlere umut vadeden bir alternatif sunmaktadır. Bu çalışmada, birden fazla et türünün eş zamanlı tespitine olanak sağlayacak gelecekteki bir çoklu platforma temel oluşturmak üzere, domuz, at ve tavuk türlerinin tanımlanması için ortak reaksiyon koşullarına sahip kolorimetrik direkt LAMP sisteminin geliştirilmesi amaçlanmıştır. Türlere özgü primer setleri ve hayvansal internal kontrol, ortak reaksiyon koşulları altında değerlendirilmiştir. Optimize edilen sistem; pH 8,75 reaksiyon tamponu, 6 mM $MgSO_4$, 250 µM krezol kırmızısı ve 50 µM hidroksi naftol mavisi (HNB) içerecek şekilde oluşturulmuş ve amplifikasyon 65°C'de 60 dakika süreyle gerçekleştirilmiştir. Krezol kırmızısı ve HNB'nin birlikte kullanımı, amplifikasyon sonuçlarının görsel olarak belirgin şekilde ayırt edilmesini sağlamıştır.

Direkt LAMP yöntemi, geleneksel DNA izolasyonuna ihtiyaç duyulmadan ısıl işlem görmüş et örneklerinden amplifikasyona olanak sağlayarak örnek hazırlama sürecini basitleştirmiştir. Özgüllük deneyleri, hedef türlerin test edilen diğer et türleriyle çapraz reaksiyon göstermeksizin seçici olarak tespit edildiğini ortaya koyarken, hayvana özgü iç kontrol et örneklerinden başarılı amplifikasyon gerçekleştirildiğini doğrulamıştır. Ayrıca yöntem, 98°C'de 60 dakikaya kadar uygulanan ısıl işlem ve 121°C'de 45 dakikaya kadar uygulanan otoklav işlemi sonrasında da hedef türleri tespit etme yeteneğini korumuştur. Genel olarak, üç türe özgü analiz için ortak reaksiyon koşullarının oluşturulması ve dual kolorimetrik tespit yaklaşımının kullanılması, ileri düzey ekipman gereksinimini azaltan, saha uygulamalarına uygun, hızlı, görsel olarak değerlendirilebilir ve potansiyel çoklu LAMP platformunun geliştirilmesi için temel oluşturmaktadır. Çalışma, et türü doğrulama analizlerinin daha erişilebilir hale getirilmesine, gıda güvenliğinin iyileştirilmesine ve tüketicinin korunmasına katkı sağlama potansiyeli sunmaktadır.

Anahtar Kelimeler: Çoklu Et Tür Tayini, İkili Kolorimetrik Tanı, LAMP, Doğrudan LAMP.

To my family and friends…

ACKNOWLEDGEMENTS

I would like to extend my sincere appreciation to my supervisor, Prof. Dr. Hüseyin Avni Öktem, for his academic guidance, scientific supervision, and valuable contributions throughout the course of this thesis. His mentorship has played a crucial role in the development and completion of this study.

I would also like to extend my sincere gratitude to the members of my thesis evaluation committee, Prof. Dr. Füsun Eyidoğan and Prof. Dr. Çağdaş Devrim Son, for their time, careful evaluation, constructive feedback, and valuable academic contributions to this thesis.

I am also grateful to all members of our research group and laboratory. I would especially like to thank Dilara Özden for her valuable contributions and support throughout the experimental and thesis preparation process. I would like to extend my warmest thanks to my colleagues, İpek Öktem, Yiğit Aksoy, Gizem Burduroğlu, Doğa İlke Yanıkdoruğu, Serpil Apaydın, and Özge Kalman, who continuously supported, and motivated me throughout this journey. I am deeply thankful to my dear friends, Selen Akçakoca, Deniz and Ömürcan Çeken, Zeynep and Mehmetcan Onaran, Sena Kayaoğlu, Arda Çeken, Elif and Ada Akın, Selin Kaplan, Fadıl Özler and Halil Onur Altay, for their encouragement, and friendship. My deepest gratitude goes to my family, Dilek Kaya, Menderes Kaya, Aslıhan Kaya, Ayşe Burcu Taner, Ali Emre Taner, and our beloved cat Marvin, for their endless support, unconditional love, understanding, and encouragement throughout my academic journey. I would also like to sincerely thank Ayşe Kılavuz and Ahmet Kılavuz for their kind support. I would like to express my most special and heartfelt gratitude to my husband, Burhan Kerem Kılavuz, whose endless support, motivation, understanding, and constant presence gave me strength throughout this entire process. His encouragement and belief in me were invaluable, and I am sincerely grateful for having his support throughout every step of the way.

This thesis research was funded by the METU Scientific Research Projects Coordination Unit under the Master's Thesis Project Support Program (Project No. TEZ-YL-108-2025-11595). Furthermore, Nanobiz Technology Inc. contributed to this study.

TABLE OF CONTENTS

ABSTRACT v

ÖZ vii

ACKNOWLEDGEMENTS x

TABLE OF CONTENTS xii

LIST OF TABLES xvii

LIST OF FIGURES xviii

LIST OF ABBREVIATIONS xxiv

LIST OF SYMBOLS xxvi

Chapters 1

1 INTRODUCTION 1

1.1 Detection of Meat Species 1

1.1.1 Protein Based Methods 3

1.1.1.1 Electrophoresis 4

1.1.1.2 Chromatography 4

1.1.1.3 ELISA 5

1.1.1.4 Spectroscopy 5

1.1.1.5 Lateral Flow Tests 6

1.1.2 DNA-Based Detection Methods 6

1.1.2.1 PCR 6

1.1.2.2 Real-Time PCR 8

1.1.2.3 Digital PCR 8

1.1.2.4 RFLP 9

1.1.2.5 RAPD 9

1.1.2.6 DNA Barcoding-Based Identification 10
1.1.2.7 NGS-Based DNA Metabarcoding 10
1.1.2.8 DNA Hybridization 11
1.1.2.9 DNA Biosensors 11
1.2 Loop Mediated Isothermal Amplification (LAMP) 12
1.2.1 LAMP Mechanism 12
1.2.2 Applications of LAMP 14
1.2.3 Meat Detection with LAMP 15
1.2.4 LAMP Reaction Detection 16
1.2.4.1 Gel Electrophoresis 16
1.2.4.2 Turbidity 17
1.2.4.3 Colorimetric Detection 17
1.2.4.3.1 Metal-Ion Binding Dyes 18
1.2.4.3.2 DNA Intercalating Dyes 18
1.2.4.3.3 pH-Sensitive Dyes 20
1.2.4.3.4 Mixed-Dye Detection 20
1.2.4.4 Annealing/Melting Curve Analysis 21
1.2.4.5 Real-Time LAMP 22
1.2.4.6 Lateral Flow Assay 22
1.2.5 Direct LAMP 24
1.3 Advantages and Disadvantages of Detection Methods 25
1.4 Research Objective 26
2 MATERIALS & METHODS 27
2.1 Materials 28

2.1.1 Chemicals and Solutions 28
2.1.2 Meat samples 29
2.1.2.1 Unprocessed Meat Samples 29
2.1.2.2 Processed Meat Samples 29
2.1.2.3 Mixed Meat Samples 30
2.1.3 Primers 30
2.2 Methods 33
2.2.1 Primer Design and In Silico Analysis 33
2.2.2 Modified Salt DNA Extraction Method 33
2.2.3 Meat Sample Preparation for Direct LAMP Analysis 34
2.2.4 LAMP Reaction Optimization 35
2.2.4.1 Buffer preparation and pH Optimization 37
2.2.4.2 $MgSO_4$ Concentration 38
2.2.4.3 Colorimetric Detection 38
2.2.4.3.1 Cresol Red 39
2.2.4.3.2 Dual Colorimetric Detection 39
2.2.4.4 Reaction Temperature 39
2.2.4.5 Reaction Time 40
2.2.5 Sensitivity of the Optimized Method 40
2.2.6 Specificity of the Optimized Method 41
2.2.7 Analysis of Processed Meat Samples 41
2.2.8 Shelf-life Stability at Room Temperature 42
3 RESULTS AND DISCUSSION 43
3.1 DNA Isolation with Modified Salt Method 43

3.2 LAMP Assay Optimization for Meat Identification 45
3.2.1 Optimization of the Buffer 45
3.2.2 Optimization of the Magnesium Concentration 48
3.2.3 Colorimetric Detection Optimization 50
3.2.3.1 Optimization of Cresol Red Concentration 50
3.2.3.2 Optimization of Dual Colorimetric Detection 52
3.2.4 Reaction Temperature 53
3.2.5 Optimization of Reaction Time 55
3.3 Sensitivity of the Optimized Method 59
3.3.1 Sensitivity of Porcine Specific Primers 59
3.3.2 Sensitivity of Equine Specific Primers 60
3.3.3 Sensitivity of Chicken Specific Primers 62
3.3.4 Sensitivity of Animal Internal Control Primers 63
3.4 Specificity of the Optimized Method 64
3.4.1 Specificity of Porcine Specific Method 64
3.4.2 Specificity of Equine Specific Method 65
3.4.3 Specificity of Chicken Specific Method 66
3.4.4 Validation of Specificity with Animal Internal Control Method 67
3.5 Analysis of Processed Meat Samples 68
3.5.1 Heat Treatment Analysis of Horse Meat 68
3.5.2 Heat Treatment Analysis of Chicken Meat 71
3.6 Shelf-Life Stability at Room Temperature 74
4 CONCLUSION 79
REFERENCES 83

APPENDICES 95

A. Buffer and Solution Preparations 95

B. *In Silico* Analysis of Primers 97

C. Commercial Products and News Reports on Meat Adulteration 106

LIST OF TABLES

TABLES

Table 2.1 Porcine-specific primer sequences 31
Table 2.2 Equine-specific primer sequences 31
Table 2.3 Chicken-specific primer sequences 32
Table 2.4 Animal internal control primer sequences 32
Table 2.5 LAMP reaction preparation according to Özden (2024) 36
Table 2.6 Primer mix preparation for porcine and animal internal control primers 36
Table 2.7 Primer mix preparation for equine primers 37
Table 2.8 Primer mix preparation for chicken primers 37
Table 3.1 DNA concentration and purity values of genomic DNA isolated from raw meat samples. 44
Table 3.2 Optimized multi-LAMP reaction components 58
Table A.1 TE buffer preparation. 95
Table A.2 Betaine preparation 95
Table A.3 Lysis buffer preparation 95
Table A.4 PBS preparation 96
Table A.5 Cresol red preparation 96
Table A.6 HNB preparation 96
Table B.1 Summary of the *in silico* cross-reaction analysis for the porcine, equine, and chicken-specific primers 105

LIST OF FIGURES

FIGURES

Figure 1.1 Schematic representation of LAMP primers. The inner primers FIP-BIP are composed of F2 (B2) and F1c (B1c). The outer primers are F3 and B3. The loop primers are LB and LF (Mori & Notomi, 2009). 13

Figure 1.2 Schematic overview of direct and indirect detection methods used for LAMP amplification. (A) DNA polymerization results in the formation of pyrophosphate, which reacts with divalent metal ions (Me^{2+}). (B) Indirect detection based on amplification by-products, including magnesium pyrophosphate formation, the Mg^{2+}-dependent color change of hydroxy naphthol blue (HNB), and calcein fluorescence resulting from changes in Mn^{2+} and Mg^{2+} availability. (C) Direct detection of amplified double-stranded DNA using intercalating dyes such as SYBR Green I, EvaGreen, and berberine. (Fischbach et al., 2015). 19

Figure 1.3 LAMP reaction coupled with lateral flow assay (Becherer et al., 2020). 23

Figure 2.1 Meat sample preparation for direct LAMP analysis (created with Canva.com). 35

Figure 3.1 Visualization of LAMP results under the white light. NTC: non-template control, N: negative control containing beef meat sample, P: positive test containing 1% pork meat sample. 1: 10X Thermopol Buffer-Vazyme, 2: 10X Isothermal Buffer-Vazyme, 3: NBZ 10X Isothermal buffer (pH 8.70), 4: NBZ 10X Isothermal buffer (pH 8.75), 5: NBZ 10X Isothermal buffer (pH 8.80), 6: NBZ 10X Isothermal buffer (pH 8.85), 7: NBZ 10X Isothermal buffer (pH 8.90). 47

Figure 3.2 Visualization of LAMP results under the white light. Results of increasing magnesium concentration (4 mM to 10 mM). NTC: non-template control, N: negative control containing beef meat sample, P: positive test containing 1% pork meat sample. 49

Figure 3.3 Visualization of LAMP results under the white light. Effect of increasing cresol red concentration (100 µM to 500 µM). NTC: non-template control, N:

negative control containing beef meat sample, P: positive test containing 1% pork meat sample. 51
Figure 3.4 Visualization of LAMP results under the white light. Effect of increasing HNB concentration (0 μM to 100 μM) on dual colorimetric method. NTC: non-template control, N: negative control containing beef meat sample, P: positive test containing 1% pork meat sample. 52
Figure 3.5 Visualization of LAMP results under the white light. Results of the Equine specific direct LAMP. NTC: non-template control,1: negative control containing 1% pork meat sample, 2: negative control containing beef meat sample, 3: positive test containing horse meat sample. 54
Figure 3.6 Visualization of LAMP results under the white light. Results of the chicken specific direct LAMP. NTC: non-template control,1: negative control containing 1% pork meat sample, 2: negative control containing beef meat sample, 3: positive test containing chicken meat sample. 54
Figure 3.7 Visualization of LAMP results under the white light. Effect of reaction duration on porcine-specific LAMP reaction. 30 to 60 minutes reaction durations were tested. NTC: non-template control, N: negative control containing beef sample, P: positive test containing pork meat sample. 56
Figure 3.8 Visualization of LAMP results under the white light. Effect of reaction duration on equine-specific LAMP reaction. 30 to 60 minutes reaction durations were tested. NTC: non-template control, N: negative control containing beef sample, P: positive test containing horse meat sample. 56
Figure 3.9 Visualization of LAMP results under the white light. Effect of reaction duration on chicken-specific LAMP reaction. 30 to 60 minutes reaction durations were tested. NTC: non-template control, N: negative control containing beef sample, P: positive test containing chicken meat sample. 57
Figure 3.10 Visualization of LAMP results under the white light. Effect of reaction duration on animal internal control LAMP reaction. 30 to 60 minutes reaction durations were tested. NTC: non-template control, P: positive test containing beef sample. 57

Figure 3.11 Visualization of LAMP results under the white light. Analytical sensitivity of the porcine-specific LAMP assay using DNA concentrations ranging from 1 ng to 0.01 pg. A: LAMP reactions containing porcine DNA, B: LAMP reactions containing beef DNA as a negative control. NTC: non- template control, reactions 1–6 contained 1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1 pg, and 0.01 pg DNA, respectively. 60
Figure 3.12 Visualization of LAMP results under the white light. Analytical sensitivity of the equine-specific LAMP assay using DNA concentrations ranging from 1 ng to 0.01 pg. A: LAMP reactions containing equine DNA, B: LAMP reactions containing beef DNA as a negative control. NTC: non- template control, reactions 1–6 contained 1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1 pg, and 0.01 pg DNA, respectively. 61
Figure 3.13 Visualization of LAMP results under the white light. Analytical sensitivity of the chicken-specific LAMP assay using DNA concentrations ranging from 1 ng to 0.01 pg. A: LAMP reactions containing chicken DNA, B: LAMP reactions containing beef DNA as a negative control. NTC: non- template control, reactions 1–6 contained 1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1 pg, and 0.01 pg DNA, respectively. 62
Figure 3.14 Visualization of LAMP results under the white light. Analytical sensitivity of the animal internal control LAMP assay using beef DNA concentrations ranging from 1 ng to 0.01 pg. NTC: non- template control, reactions 1–6 contained 1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1 pg, and 0.01 pg DNA, respectively. 63
Figure 3.15 Visualization of LAMP results under the white light. Specificity test of the porcine-specific direct LAMP assay. Lane 1: 1% pork meat sample, 2: 0.1% pork meat sample, 3: raw pork meat sample, 4: pork-prosciutto, 5: pork-bacon, 6: horse meat sample, 7: chicken meat sample, 8: beef sample, 9: fish meat sample, 10: turkey meat sample, 11: duck meat sample, 12: lamb meat sample, 13: goat meat sample. NTC: non-template control. 65

Figure 3.16 Visualization of LAMP results under the white light. Specificity test of the equine-specific direct LAMP assay. 1: pork meat sample, 2: pork-prosciutto, 3: pork-bacon, 4: horse meat sample, 5: chicken meat sample, 6: beef meat sample, 7: fish meat sample, 8: turkey meat sample, 9: duck meat sample, 10: lamb meat sample, 11: goose meat sample, 12: goat meat sample. NTC: non-template control. 66
Figure 3.17 Visualization of LAMP results under the white light. Specificity test of the chicken-specific direct LAMP assay. 1: pork meat sample, 2: pork-prosciutto, 3: pork-bacon, 4: horse meat sample, 5: chicken meat sample, 6: beef meat sample, 7: fish meat sample, 8: turkey meat sample, 9: duck meat sample, 10: lamb meat sample, 11: goose meat sample, 12: goat meat sample. NTC: non-template control. 67
Figure 3.18 Visualization of LAMP results under the white light. Validation of the porcine-specific, equine-specific and chicken-specific direct LAMP assays using animal internal control primers. 1: pork meat sample, 2: pork-prosciutto, 3: pork-bacon, 4: horse meat sample, 5: chicken meat sample, 6: beef meat sample, 7: fish meat sample, 8: turkey meat sample, 9: duck meat sample, 10: lamb meat sample, 11: goose meat sample, 12: goat meat sample. NTC: non-template control. 68
Figure 3.19 Visualization of LAMP results under the white light. Results of the direct LAMP reaction using samples autoclaved at 121°C for 15, 30, and 45 minutes. A: Negative samples containing beef. B: Positive samples containing horse meat. Lanes 1, 2, and 3 correspond to samples autoclaved for 15, 30, and 45 min, respectively. NTC: non-template control. 69
Figure 3.20 Visualization of LAMP results under the white light. Results of the direct LAMP reaction using samples heated at 98°C for different time intervals. A: Negative samples containing beef. B: Positive samples containing horse meat. Lanes 1–7 correspond to samples heated at 98°C for 2, 5, 10, 15, 30, 45, and 60 min, respectively. NTC: non-template control. 70
Figure 3.21 Visualization of LAMP results under the white light. Results of the direct LAMP reaction using samples autoclaved at 121°C for 15, 30, and 45 minutes. A:

Negative samples containing beef. B: Positive samples containing chicken meat. Lanes 1, 2, and 3 correspond to samples autoclaved for 15, 30, and 45 min, respectively. NTC: non-template control. 72
Figure 3.22 Visualization of LAMP results under the white light. Results of the direct LAMP reaction using samples heated at 98°C for different time intervals. A: Negative samples containing beef. B: Positive samples containing chicken meat. Lanes 1–7 correspond to samples heated at 98°C for 2, 5, 10, 15, 30, 45, and 60 min, respectively. NTC: non-template control. 73
Figure 3.23 Visualization of LAMP results under white light during the shelf-life study at room temperature for porcine-specific method. A: day zero, B: day three, C: day six, D: day nine, E: day twelve, and F: day fifteen. N: beef meat sample (negative control), P: 1% pork meat sample (positive sample), NTC: non-template control. 75
Figure 3.24 Visualization of LAMP results under white light during the shelf-life study at room temperature for equine-specific method. A: day zero, B: day three, C: day six, D: day nine, E: day twelve, and F: day fifteen. N: beef meat sample (negative control), P: horse meat sample (positive sample), NTC: non-template control. 76
Figure 3.25 Visualization of LAMP results under white light during the shelf-life study at room temperature for chicken-specific method. A: day zero, B: day three, C: day six, D: day nine, E: day twelve, and F: day fifteen. N: beef meat sample (negative control), P: chicken meat sample (positive sample), NTC: non-template control. 76
Figure 3.26 Visualization of LAMP results under white light during the shelf-life study at room temperature for animal internal control test. A: day zero, B: day three, C: day six, D: day nine, E: day twelve, and F: day fifteen. P: beef meat sample (positive sample), NTC: non-template control. 77
Figure B.1 *In silico* binding analysis of the Sus scrofa specific forward-reverse primer set. 98
Figure B.2 *In silico* binding analysis of the Equus caballus specific forward-reverse primer set. 99

Figure B.3 *In silico* binding analysis of the Gallus gallus specific forward-reverse primer set. 100
Figure B.4 *In silico* binding analysis of the IC primers with the Sus scrofa genome. 101
Figure B.5 *In silico* binding analysis of the IC primers with the Equus caballus genome 102
Figure B.6 In silico binding analysis of the IC primers with the Gallus gallus genome. 103
Figure B.7 Example of a potential amplicon formed between the porcine-specific primers and the cattle genome. 104

LIST OF ABBREVIATIONS

ABBREVIATIONS

BIP: backward inner primer

bp: base pair

CR: Cresol red

COI: Cytochrome c oxidase subunit I

DNA: Deoxyribonucleic acid

dNTP: Deoxynucleotide triphosphate

D-loop: displacement loop

ELISA: Enzyme-linked immunosorbent assay

FDA: food and drug administration

fg: femtogram

FIP: Forward inner primer

FTIR: Fourier-transform infrared

GC (content): guanine-cytosine

HCl: Hydrochloric acid

HNB: Hydroxy naphthol blue

KCl: Potassium chloride

LAMP: Loop mediated amplification

LOD: limit of detection

$MgSO_4$: Magnesium sulfate

mM: Millimolar

N: Negative control

NaOH: Sodium hydroxide

$(NH_4)_2SO_4$: Ammonium sulfate

ng: Nanogram

NGS: Next-generation sequencing

NIR: Near-Infrared

NTC: non-template control

PAGE: Polyacrylamide gel electrophoresis

PCR: Polymerase chain reaction

pH: potential of hydrogen

qPCR: Quantitative polymerase chain reaction

RAPD: Random amplified polymorphic DNA

RFLP: Restriction fragment length polymorphism

SDS: sodium dodecyl sulfate

TE: Tris-EDTA

Tp: time-to-positive value

TnI: troponin I

LIST OF SYMBOLS

SYMBOLS

Δ: Delta

%: percent

°: degree

CHAPTERS

CHAPTER 1

INTRODUCTION

Concerns about food authenticity, traceability, and safety in contemporary food supply chains have grown as a result of the world's population growth and consequent rise in demand for animal-derived protein.

1.1 Detection of Meat Species

Accurate identification of meat species is critical for ensuring food safety, sustaining consumer confidence, meeting religious dietary requirements, and safeguarding against fraud in the meat industry, while also helping regulators validate label claims and uphold fair trade standards (Ballin, 2010; Ali et al., 2015). Meat is among the food categories most targeted by economically motivated adulteration, with substitution of declared, higher-value species by cheaper, undeclared ones (commonly pork or poultry replacing beef) remaining one of the most reported forms of fraud (Cavin et al., 2018). Such substitution becomes undetectable by visual control once meat is minced or processed, making fraud both easier to commit and harder for consumers to detect, underscoring the need for reliable species information. These risks make it clear that consumers must be reliably informed about the true species content of the meat they consume (Abbas et al., 2018).

The fundamental logic behind meat-species legislation is essentially the same wherever it is written. When a product moves out of a single manufacturer's control and enters a supply chain with multiple agents, verification becomes the sole remaining assurance for the consumer. In Turkiye, this principle is codified through

the Regulation on Special Hygiene Rules for Foodstuffs of Animal Origin, which requires activities involving pork and equine to be carried out and stored only in separately approved or registered establishments and prohibits the production of gelatin or collagen from pig skins, hides, or bones in establishments processing materials from other animal species (Official Gazette of the Republic of Turkiye, 2011). This framework reflects a strict approach to species segregation in food production, particularly with respect to pork and derivatives. Beyond national borders, the same traceability logic is formalized differently. While the European Union addresses this through mandatory ingredient labeling under Regulation (EU) No. 1169/2011 (European Parliament and Council, 2011), in the United States it falls under the FDA's broader food-code enforcement authority rather than a species-specific labeling regulation (FDA, 2025). The divergence in legal instruments illustrates that there is no single regulatory template for meat authenticity, what is constant across judicial systems is the assumption that declared species content can, and periodically must, be independently confirmed.

That such regulations exist does not mean that violations are rare. Some of the most damaging food fraud incidents in modern history have involved undeclared meat species: the 2013 European horse-meat incident showed that even tightly regulated markets are not immune, after products labeled and sold as beef were found in several countries to consist substantially of undeclared horse meat (Brooks et al., 2017). Such cases are not limited to the past or to Europe. In February 2026, an electronic identification microchip belonging to a registered racehorse was detected in a meat dish distributed by a municipal public kitchen in Mersin, Türkiye. Following an inspection, laboratory analyses confirmed the presence of meat from an equine animal, and 213 kg of the prepared meat was subsequently destroyed (GZT, 2026a). Later that same year, further inspections by the Turkish Ministry of Agriculture and Forestry identified horse, donkey, and mule meat sold as beef in additional products from Adana and Mersin, and equine meat was reported in a *pide* filling in İzmir (GZT, 2026b; Turkish Post, 2026). These cases are forming a pattern that indicates

species mislabeling remains an active and recurring problem in the Turkish market rather than an isolated event.

Cases of this kind underline why meat species identification cannot rely on labeling and self-declaration alone. Analytical detection capacity is therefore treated as a core component of food control systems, a necessity made especially clear in contexts such as halal and kosher certification. There, verified species composition rather than a label claim alone is what gives the certification meaning for the communities that depend on it (Martuscelli et al., 2020). Over the past several decades, a wide range of laboratory techniques has been developed for this purpose. These techniques can be broadly grouped into two categories according to the biological target they exploit. Protein-based methods rely on differences in muscle and serum proteins between species, differences well established even at the level of individual serum proteins such as albumin, whose species-specific structure has been shown to underlie cross-reactivity patterns between species (Hilger et al., 1997). DNA-based methods exploit species-specific variation in nuclear or mitochondrial genetic sequences and have been extended to multiplex formats capable of simultaneously tracing several species with high efficiency in both raw and heat-processed meat materials (Zhou et al., 2022). The following sections briefly review the principal techniques within each category, before turning in greater depth to loop-mediated isothermal amplification (LAMP) and its colorimetric detection formats, which form the methodological basis of this study.

1.1.1 Protein Based Methods

Protein-based approaches use immunological or biochemical methods to identify antigenic structures or species-specific proteins. Enzyme linked immunosorbent assay (ELISA), electrophoresis, chromatography, spectroscopy, and lateral flow assays are common methods. Despite the widespread use of these techniques in food authentication, target epitope structural degradation and protein denaturation during heat processing frequently impair their effectiveness (Ballin, 2010).

1.1.1.1 Electrophoresis

Electrophoresis techniques separate biomolecules based on charge, size, or isoelectric point under an electric field. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) enables molecular weight-based separation, while isoelectric focusing provides separation according to isoelectric points (Montowska & Pospiech, 2007). Despite their utility in protein profiling, electrophoretic methods exhibit limited discriminatory power for closely related species due to overlapping protein patterns. Moreover, thermal processing induces protein denaturation, significantly reducing analytical reliability in processed meat matrices (Zia et al., 2020).

1.1.1.2 Chromatography

Chromatographic approaches identify species-specific proteins or peptide fragments based on their differential retention or electrochemical behavior during separation. Liquid chromatography coupled with electrochemical detection using copper nanoparticle–plated electrodes has been shown to differentiate meat from fifteen animal species based on characteristic amino acid and peptide signatures (Chou et al., 2007). More recent glycomic profiling approaches, combining liquid chromatography with mass spectrometry, have further demonstrated that chicken, pork, and beef carry distinguishable glycosylation patterns that can serve as an additional authentication marker beyond amino acid composition (Chia et al., 2022). While highly informative, these techniques require costly instrumentation and specialized expertise, restricting their routine use to well-equipped reference laboratories.

1.1.1.3 ELISA

One of the most used immunological techniques for identifying meat species is ELISA (enzyme-linked immunosorbent test), which uses antigen–antibody interactions to identify species-specific proteins. The technique is appropriate for routine food product screening. Nevertheless, because thermal processing can denature proteins, decreasing their solubility and changing antigenic epitopes necessary for antibody recognition, its use in highly processed meat products is restricted. Additionally, the inability of traditional ELISA assays to differentiate meat proteins from other animal-derived substances like milk, eggs, or whey proteins may lower analytical specificity in complex dietary matrices. The performance of commercially available ELISA kits has been observed to vary with respect to the target analyte and sample structure, despite the proposal for tissue-specific protein markers to improve discrimination (Hitchcock & Crimes, 1985; Ballin, 2010). ELISA offers high throughput and does not require sophisticated instrumentation, but antibody cross-reactivity between closely related species and sensitivity to protein denaturation during extensive cooking remain recognized limitations.

1.1.1.4 Spectroscopy

Vibrational spectroscopic techniques, including near-infrared (NIR), Fourier-transform infrared (FTIR), and Raman spectroscopy, exploit the distinct molecular fingerprints produced by lipid, protein, and water content across species to enable rapid, non-destructive authentication. Raman spectroscopy combined with chemometric modeling has been used to discriminate beef from horsemeat with high classification accuracy (Boyacı et al., 2014), while FTIR spectroscopy has been applied specifically to detect pork, horse, or donkey meat adulteration within beef-based formulations (Candoğan et al., 2020). These methods require no reagents and can deliver results within minutes, but their accuracy is highly sensitive to sample

heterogeneity, and reliable classification typically depends on large, well-curated reference spectral libraries.

1.1.1.5 Lateral Flow Tests

Lateral flow immunoassays translate the antibody-based recognition principle of ELISA into a single-use, strip-format device that produces a visible line within minutes and requires no laboratory equipment, making it attractive for on-site meat inspection. A lateral flow immunoassay targeting skeletal troponin I (TnI) has been developed to detect the addition of beef, pork, lamb, and horse muscle into raw meat mixtures and finished meat products, exploiting the thermostability of troponin I as a marker that survives moderate processing (Zvereva et al., 2020). Although lateral flow tests are a fast, equipment-free, and easily portable alternative to methods such as LAMP, they compromise sensitivity and quantitative accuracy.

1.1.2 DNA-Based Detection Methods

DNA-based methods are considered the most reliable approaches for meat species authentication due to the high stability of DNA under processing conditions. These methods offer sensitivity and specificity compared to protein-based assays and are widely applied in food forensic analysis.

1.1.2.1 PCR

Polymerase chain reaction (PCR) amplifies short, species-specific DNA regions, commonly targeting mitochondrial genes such as *cytochrome b, COI,* and *D-loop* because of their high copy number and variation between species. For this reason, PCR is one of the most widely validated molecular methods for meat authentication. Compared with the protein-based methods discussed above, PCR has important advantages in species identification. DNA is more resistant to heat than most muscle

and serum proteins, allowing species information to be detected even in cooked, cured, or highly processed meat where protein-based methods may no longer provide reliable results. Multiplex PCR can further improve this approach by detecting several species in a single reaction. For example, a validated hexaplex PCR assay was developed to simultaneously identify pork, horse, chicken, lamb/mutton, ostrich, and beef without prior DNA extraction, and was shown to remain effective in degraded and highly processed food samples (Kitpipit et al., 2014). This combination of high specificity, sensitivity, and the ability to screen for several forbidden or economically substituted species in one reaction is precisely what has made PCR the preferred confirmatory method for pork detection in halal and kosher enforcement, as well as for horse-meat screening following the 2013 European incident.

These advantages also have some limitations for meat species detection. Thermal processing can fragment DNA into shorter pieces, and when DNA fragments become shorter than the target region of the primers, the species may not be detected even when it is present in the sample. This is particularly important for detecting horse and pork in highly processed products, such as canned meats, where increased heat exposure has been shown to cause DNA fragmentation and reduce amplification of beef, pork, and chicken targets (Şakalar et al., 2012). Similar effects have also been reported for meat subjected to combined heat and pressure processing, such as canning (Hird et al., 2006). Conventional PCR also requires a thermal cycler and post-amplification gel electrophoresis, which limits its use outside laboratory settings and increases the analysis time. This can be a limitation when rapid screening is required, such as in the Mersin case, where a faster confirmatory test could have detected the substituted horsemeat before distribution. In addition, multiplex PCR can be affected by differences in amplification efficiency between primer pairs. As a result, a species present at a low proportion may be preferentially amplified less efficiently and remain undetected, leading to a false-negative result (Markoulatos et al., 2002). Finally, conventional PCR generally provides qualitative or semi-quantitative results from an endpoint reaction. Accurate determination of the proportion of an undeclared meat species usually requires real-time or digital PCR,

which have been developed for the quantitative detection of meat species in raw and processed food products (Ren et al., 2017).

1.1.2.2 Real-Time PCR

Real-time quantitative PCR combines DNA amplification with fluorescent probe or dye-based detection, enabling species identification and relative quantification in a single closed-tube assay while eliminating the need for post-PCR processing. A TaqMan probe–based multiplex real-time PCR assay has been developed for the simultaneous identification of pork, chicken, and beef in commercial processed meat products within 40 minutes, validated against a panel of twenty animal species including horse with no observed cross-reactivity (Kim & Kim, 2019). Real-time PCR offers substantially higher throughput and sensitivity than gel-based PCR, but instrument and reagent costs remain considerably higher, and accurate quantification still depends on species-specific standard curves that must be constructed separately for each target species.

1.1.2.3 Digital PCR

Digital PCR divides a single reaction into thousands of small droplets before amplification. This allows the absolute quantification of target DNA copies based on end-point Poisson statistics, without the need for a standard curve. A droplet digital PCR assay has been used for the simultaneous detection and quantification of beef, pork, horse, sheep, chicken, and turkey in commercial meat products. The assay achieved 100% accuracy and detected undeclared traces of meat species in several samples (Basanisi et al., 2020). Although digital PCR reduces the need for calibration compared with real-time PCR, it requires specialized equipment for droplet generation and detection. The high cost and limited availability of these instruments make the method less suitable for field-level screening and mainly applicable to well-equipped reference laboratories.

1.1.2.4 RFLP

PCR-restriction fragment length polymorphism (PCR-RFLP) adds a discriminatory step after amplification, in which a conserved gene fragment is digested with restriction endonucleases to generate species-characteristic band patterns. The original application of this principle amplified a 359 bp *cytochrome b* fragment and successfully differentiated pork, cattle, wild boar, buffalo, sheep, goat, horse, chicken, and turkey using four separate restriction enzymes (Meyer et al., 1995). However, the additional digestion step increases the analysis time and cost compared with conventional PCR and may result in unclear banding patterns when mixed meat samples are analyzed rather than single-species samples. This is an important limitation because pork, horse, and chicken adulteration generally occurs through partial substitution in mixed meat products.

1.1.2.5 RAPD

Random amplified polymorphic DNA (RAPD) analysis uses short, arbitrary primers to generate species-characteristic fingerprint patterns without requiring prior knowledge of the target genome sequence. RAPD fingerprinting has been used to differentiate ten red meat species, including pig and horse, using a panel of short GC content rich primers (Koh et al., 1998), and a separate protocol successfully discriminated pork, chicken, duck, turkey, and goose components in poultry pâté at detection levels as low as 250 pg of DNA (Calvo et al., 2001). Because amplification depends on non-specific primer annealing, RAPD patterns are highly sensitive to minor changes in reaction conditions and are often poorly reproducible between laboratories, which has limited its adoption for the kind of routine regulatory testing pork or horse-meat enforcement requires.

1.1.2.6 DNA Barcoding-Based Identification

DNA barcoding is a sequencing-based method for species identification that commonly targets a standardized region such as mitochondrial *COI* gene. The obtained sequences are compared with reference databases, such as the Barcode of Life Database (BOLD) and GenBank, to determine the species present in a sample. Unlike species-specific PCR methods, DNA barcoding is not restricted to a predefined set of target species, allowing a broader range of species to be identified. This approach has been applied to meat and poultry products, where both full-length (658 bp) and mini-barcoding (127 bp) regions of the *COI* gene have been evaluated for species identification (Hellberg et al., 2017). However, DNA degradation during food processing can reduce sequencing success, particularly for full-length barcodes. In addition, conventional DNA barcoding has limited applicability for complex mixed samples because the sequencing result may be dominated by the most abundant DNA template. Therefore, although DNA barcoding is useful for species identification and food authentication, its application to highly processed and mixed meat products can be challenging.

1.1.2.7 NGS-Based DNA Metabarcoding

DNA metabarcoding overcomes the single-species limitation of conventional barcoding by combining barcode amplification with next-generation sequencing (NGS), enabling the simultaneous identification of multiple species in mixed or complex meat samples. This approach can also detect species that are not specifically anticipated by the analyst through comparison with reference barcode databases. NGS-based metabarcoding has been successfully applied to 27 meat and poultry products sold in China, identifying animal components across all tested samples. NGS of a short segment of the 16S ribosomal RNA (16S rRNA) mitochondrial gene was performed to validate meat and poultry species achieved detection and differentiation (Xing et al., 2019). Despite its broad detection capacity, NGS

metabarcoding remains more costly than targeted PCR methods and requires specialized sequencing instrumentation and bioinformatic expertise for data analysis. In addition, the lack of standardized protocols for primer selection and quality control limits its routine regulatory application (Giusti et al., 2023).

1.1.2.8 DNA Hybridization

DNA hybridization-based methods identify species through the complementarity of a labeled species-specific probe to target DNA, with signal intensity after hybridization indicating both the presence and, in some formats, the relative amount of a given species. An early application used a radiolabeled porcine genomic DNA probe to quantify pork adulteration in beef, with detection limits of approximately 0.1% in raw meat and 0.5% in heat-treated samples (Ebbehøj & Thomsen, 1991). More recently, the approach was adapted to array-based formats for the simultaneous detection of multiple species. Study combined PCR with a commercial DNA macro-array containing meat-specific capture probes, enabling the simultaneous identification of 32 meat species and achieving a detection limit of 1% (w/w) (Cottenet et al., 2016). Reviews have reported detection limits ranging from 0.1% to below 0.01%, depending on the target species (Rahmati et al., 2016). However, the method requires precise control of hybridization conditions and, outside multi-target array formats, is generally limited to one species per reaction, reducing its practicality for simultaneous screening of multiple meat species.

1.1.2.9 DNA Biosensors

DNA biosensors combine a species-specific nucleic acid probe with an electrochemical or optical transducer to convert probe–target hybridization into a measurable signal without gel electrophoresis. In this sense, biosensors can be understood as a technological evolution of the classical hybridization principle described above. A label-free electrochemical biosensor targeting the porcine

cytochrome b gene was developed using gold-modified screen-printed carbon electrodes and successfully detected pork in mixed meat samples containing 5% pork (Hartati et al., 2023). In another study, electrochemical biosensor based on graphene biochips was developed for meat species identification by combining DNA–redox interactions with electrochemical detection. The system integrated LAMP amplification with square-wave voltammetry and successfully identified chicken and pork, with detection limits of 1 pg/µL and 100 pg/µL, respectively (Roy et al., 2016). Although DNA biosensors offer potential for portable, rapid, and low-cost testing, issues such as probe stability, electrode reproducibility, and interference from complex meat matrices remain challenges for their widespread field application

1.2 Loop Mediated Isothermal Amplification (LAMP)

Among nucleic acid-based diagnostic methods, loop-mediated isothermal amplification (LAMP) is notable for its high sensitivity in detecting target DNA sequences. Due to its practical advantages over many conventional amplification methods, LAMP has been increasingly used for meat species identification. Its advantages compared with other detection methods have also led to its widespread use in various diagnostic and detection applications.

1.2.1 LAMP Mechanism

Loop-mediated isothermal amplification (LAMP) was first introduced by Notomi and colleagues (2000) as a method that can amplify DNA with high specificity, efficiency, and speed under constant temperature conditions. Unlike conventional and real-time PCR, LAMP does not require repeated heating and cooling cycles for DNA denaturation. Instead, the reaction is performed at a constant temperature, typically around 60–65°C, using Bst DNA polymerase, which has strong strand-displacement activity. Bst enzyme can synthesize a new DNA strand while

displacing the strand ahead of it, allowing DNA amplification without repeated thermal denaturation.

The main feature of LAMP is its unique primer design, which contributes to its high specificity. A standard LAMP reaction uses four primers: two outer primers (F3 and B3) and two inner primers (FIP and BIP). Together, these primers recognize six distinct regions of the target DNA, whereas conventional PCR uses two primers that recognize two target regions (Notomi et al., 2000). Each inner primer contains two target-specific sequences. FIP consists of the F1c and F2 sequences, while BIP contains the B1c and B2 sequences. The positions and structures of these primers within the target DNA are illustrated in Figure 1.1 (Mori and Notomi, 2009). In addition to the four core primers, loop primers (LF and LB) can be used to bind to the loop regions of the amplification products and increase the amplification rate (Mori & Notomi, 2009).

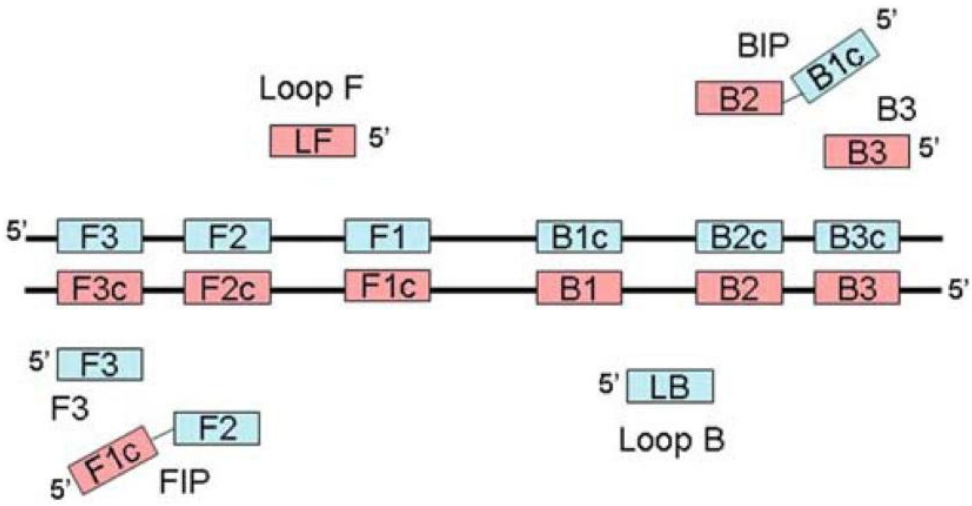

Figure 1.1 Schematic representation of LAMP primers. The inner primers FIP-BIP are composed of F2 (B2) and F1c (B1c). The outer primers are F3 and B3. The loop primers are LB and LF (Mori & Notomi, 2009).

The LAMP reaction begins with an initial step that generates a stem-loop DNA structure, followed by cycling amplification and elongation/recycling reactions

(Notomi et al., 2000; Mori & Notomi, 2009). In the initial stage, the inner and outer primers act sequentially to generate a single-stranded DNA with self-complementary ends. This strand forms a dumbbell-shaped structure, which serves as the starting structure for the cycling stage. During this stage, the inner primers bind to the loop regions and initiate strand-displacement DNA synthesis, producing new stem-loop structures and continuously amplifying the target DNA. The final products consist of stem-loop DNA structures with different stem lengths and cauliflower-like structures containing multiple inverted repeats of the target sequence (Notomi et al., 2000).

The continuous strand-displacement amplification in LAMP allows approximately 10^9 copies of the target DNA to accumulate in less than one hour (Notomi et al., 2000). The amplification can be further accelerated by adding two loop primers, LF and LB, which bind to the loop regions of the amplification products and provide additional sites for DNA synthesis. Nagamine and colleagues (2002) showed that the use of loop primers reduced the reaction time to less than half that of the original LAMP method. During DNA synthesis, pyrophosphate ions are also released and can react with magnesium ions in the reaction mixture to form insoluble magnesium pyrophosphate, contributing to increased turbidity and providing a visible indication of amplification (Mori & Notomi, 2009).

1.2.2 Applications of LAMP

LAMP has been applied across a wide range of diagnostic fields because of its rapid amplification, simple operation, and limited equipment requirements. In medical diagnostics, colorimetric RT-LAMP was developed for SARS-CoV-2 detection using RNA extracted from nasopharyngeal and throat swabs, with amplification performed at 65°C for 30 min and visual detection using hydroxy naphthol blue (HNB) or cresol red (Hongjaisee et al., 2023). In veterinary diagnostics, LAMP has also shown potential for field-based testing. An assay for African swine fever virus was verified using clinical samples during an active outbreak in Timor-Leste,

supporting its use in resource-limited areas with limited access to conventional molecular laboratories (Mee et al., 2020).

LAMP has also been widely investigated in food analysis, including the detection of foodborne pathogens, allergens, genetically modified organisms, and other food-related targets (Huang et al., 2020). In meat analysis, its rapid amplification and low instrumentation requirements have supported applications in both microbiological quality assessment and animal species identification (Kumar et al., 2017). Species-specific LAMP assays targeting mitochondrial DNA have been developed for a broad range of meat species. For example, Cho et al. (2014) developed a panel of LAMP assays capable of identifying and discriminating cattle, pig, horse, goat, sheep, chicken, duck, and turkey. The assays were completed within 30 min and detected species in raw and cooked meat mixtures at levels ranging from 0.01% to 0.0001%, depending on the target (Cho et al., 2014). These findings demonstrate the suitability of LAMP for rapid meat species authentication and adulteration screening.

1.2.3 Meat Detection with LAMP

Species-specific LAMP assays have been developed for each of the three species targeted in the present study. For pork, a method developed a direct real-time LAMP assay targeting the mitochondrial *D-loop* region, detecting 0.1% pork in meat mixtures within 30 min without nucleic acid extraction (Lee et al., 2016). For horse, Zahradnik and colleagues (2015) reported a mitochondrial LAMP assay with no cross-reactivity against beef, pork, or chicken and reliable detection of 0.1% horse meat in model sausages, independent of cooking time. A later colorimetric LAMP assay targeting the mitochondrial *COI* gene also detected horse meat at 0.1% in mixed samples (Wang et al., 2019). For chicken, Sul and colleagues (2019) developed a direct LAMP assay targeting the mitochondrial 16S rRNA gene, with a DNA detection limit of 10 fg and successful application to raw and processed meat products without DNA isolation.

Multi-species LAMP approaches have also been reported. Cho and colleagues (2014) developed LAMP assays for eight meat species and differentiated the targets by their characteristic annealing temperatures. More recently, a method has been developed with a real-time fluorescent microfluidic LAMP platform for the simultaneous detection of pork, beef, sheep, and duck. Species-specific primers were precoated in separate reaction chambers, allowing detection of 0.1% (w/w) adulteration within 30 min (Zhang et al., 2023). Similarly, a handheld colorimetric microfluidic LAMP system was developed for six meat species, including pork and chicken, using separate chambers containing pre-lyophilized species-specific primers (Xiao et al., 2023).

These studies demonstrate the potential of LAMP for multi-species meat authentication but also highlight the difficulty of performing true multiplex detection in a single reaction condition (Zhang et al., 2023). In the present study, pork, horse, and chicken-specific primer sets were therefore optimized separately under the same reaction composition and amplification conditions. Rather than performing simultaneous identification within a single tube, this approach establishes a common LAMP reaction system for the detection of the three target species and provides a basis for the future development of a multiplex single-tube LAMP platform.

1.2.4 LAMP Reaction Detection

LAMP amplification can be detected using several methods, ranging from conventional gel electrophoresis to turbidity, fluorescence, and colorimetric approaches.

1.2.4.1 Gel Electrophoresis

LAMP products can be detected by agarose gel electrophoresis, where successful amplification typically produces a characteristic ladder-like pattern. This pattern results from the formation of stem-loop DNA products of different lengths

containing repeated target sequences, in contrast to the single discrete amplicon commonly obtained by conventional PCR (Notomi et al., 2000). Gel electrophoresis is therefore useful for confirming amplification during assay development and primer evaluation. However, it requires opening the reaction tubes and handling amplified products, which increases the risk of carry-over contamination. It also requires electrophoresis and imaging equipment, limiting its suitability for rapid field-based testing.

1.2.4.2 Turbidity

LAMP amplification can be detected through the turbidity generated during DNA synthesis. As the reaction proceeds, pyrophosphate ions released during nucleotide incorporation bind to magnesium ions and form a white precipitate of magnesium pyrophosphate, increasing the turbidity of the reaction mixture (Mori et al., 2004). Turbidity can also be monitored in real time using a turbidimeter. Mori and colleagues (2004) showed that the time required to reach a defined turbidity level was dependent on the initial template concentration, allowing quantitative analysis over a wide concentration range. Although turbidity provides a simple closed-tube detection method without additional dyes, visual interpretation may be less clear than colorimetric detection, while quantitative measurement requires a dedicated turbidimeter.

1.2.4.3 Colorimetric Detection

Colorimetric detection is widely used for LAMP because it converts DNA amplification changes into a color signal that can often be evaluated by the naked eye. This reduces the need for gel electrophoresis or specialized detection instruments and makes LAMP particularly suitable for rapid and field-based applications. Dye-based detection methods can operate through different chemical mechanisms, including direct interaction with amplified DNA, changes in free

metal-ion concentration, and changes in reaction pH (Chatterjee & Sulabh, 2026). These approaches have been increasingly used in food authentication and meat adulteration studies because they provide a simple visual distinction between positive and negative reactions.

1.2.4.3.1 Metal-Ion Binding Dyes

Metal-ion indicators detect changes in the concentration of divalent metal ions during LAMP amplification. Hydroxy naphthol blue (HNB) is one of the most used indicators in this group. During DNA synthesis, pyrophosphate is released and reacts with Mg^{2+} to form magnesium pyrophosphate, thereby reducing the concentration of free Mg^{2+} in the reaction mixture. HNB responds to this change with a visible color transition from violet to sky blue in positive reactions. Importantly, HNB can be added before amplification, allowing the reaction tube to remain closed. Goto and colleagues (2009) demonstrated that HNB at a concentration of 120 μM provided clear visual detection without inhibiting LAMP amplification.

Calcein is another metal-ion indicator used for visual LAMP detection, although its mechanism differs from that of HNB. Before amplification, calcein fluorescence is quenched by Mn^{2+}. As amplification proceeds, the generated pyrophosphate binds Mn^{2+}, releasing calcein, which subsequently interacts with Mg^{2+} and produces strong green fluorescence (Tomita et al., 2008). Thus, both HNB and calcein respond to changes in metal-ion availability during DNA synthesis, but HNB produces a visible color change, whereas calcein generates green fluorescence. Schematic overview of metal-ion binding dyes used for LAMP amplification illustrated in Figure 1.2 (B).

1.2.4.3.2 DNA Intercalating Dyes

DNA-intercalating dyes detect amplification by binding directly to the double-stranded DNA produced during the LAMP reaction. Common examples include SYBR Green I, EvaGreen, propidium iodide, and PicoGreen, and their binding to

amplified DNA produces an increased fluorescent or visible signal (Chatterjee & Sulabh, 2026). Although intercalating dyes can provide sensitive detection, their compatibility with LAMP depends on the type and concentration of the dye. SYBR Green-I may inhibit amplification when added before the reaction and is therefore often added after amplification. This requires opening the reaction tube and increases the risk of carry-over or aerosol contamination (Moon et al., 2022). For this reason, indicators that can be added before amplification, including metal-ion and pH-sensitive dyes, are particularly useful for closed-tube visual detection. Schematic overview of DNA intercalating dyes used for LAMP amplification illustrated in Figure 1.2 (C).

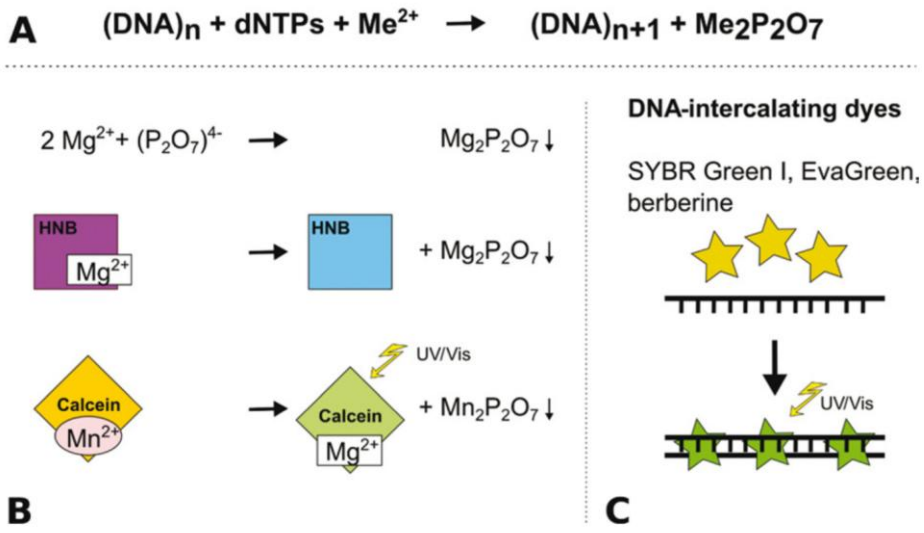

Figure 1.2 Schematic overview of direct and indirect detection methods used for LAMP amplification. (A) DNA polymerization results in the formation of pyrophosphate, which reacts with divalent metal ions (Me^{2+}). (B) Indirect detection based on amplification by-products, including magnesium pyrophosphate formation, the Mg^{2+}-dependent color change of hydroxy naphthol blue (HNB), and calcein fluorescence resulting from changes in Mn^{2+} and Mg^{2+} availability. (C) Direct detection of amplified double-stranded DNA using intercalating dyes such as SYBR Green I, EvaGreen, and berberine. (Fischbach et al., 2015).

1.2.4.3.3 pH-Sensitive Dyes

A different colorimetric approach is based on the decrease in pH that occurs during DNA amplification. DNA polymerization releases protons as nucleotides are incorporated into the growing DNA strand. When LAMP is performed in a reaction mixture with low buffering capacity, the accumulation of these protons produces a measurable decrease in pH. pH-sensitive indicators can therefore convert successful DNA amplification into a visible color change (Tanner et al., 2015). Phenol red and cresol red typically change from red or pink to yellow as the pH decreases, whereas neutral red changes from a yellow/orange state toward pink or red. Because this detection mechanism depends directly on changes in pH, careful control of the initial reaction pH and buffering capacity is important for obtaining a clear distinction between positive and negative reactions.

pH-sensitive detection has also been successfully applied to meat species authentication. One study developed a one-step colorimetric LAMP assay using neutral red for chicken identification. Positive reactions showed a clear yellow-to-pink/magenta color change, and the assay detected chicken DNA without cross-reactivity with other tested meat species. The method achieved detection limits of 1 pg of chicken DNA and 0.01% (w/w) chicken in binary meat mixtures and was also applied to commercial processed food products (Thangsunan et al., 2021). Similarly, Wang et al. (2019) evaluated both HNB and neutral red for colorimetric detection of horse-specific LAMP products and showed that both indicators allowed clear naked-eye interpretation despite relying on different chemical mechanisms.

1.2.4.3.4 Mixed-Dye Detection

The use of more than one indicator in the same LAMP reaction has also been investigated to improve visual discrimination between positive and negative results. Pang and colleagues (2019), for example, developed a mixed-dye system combining HNB and calcein and reported a clear detection of LAMP results, including weak-

positive reactions, compared with individual visual indicators. Such approaches suggest that dyes responding to amplification-associated chemical changes can be combined to improve visual interpretation, provided that their concentrations and compatibility with the amplification reaction are carefully optimized (Zhang et al., 2022). Based on this principle, the present study investigates a dual-colorimetric approach to increase visual discrimination and sensitivity combining HNB, which responds to changes in free Mg^{2+} concentration, with cresol red, which responds to the decrease in pH during amplification.

1.2.4.4 Annealing/Melting Curve Analysis

Annealing or melting curve analysis provides an additional method for confirming LAMP amplification based on the characteristic thermal behavior of the amplified DNA. After amplification, the reaction is subjected to a controlled temperature change while fluorescence is monitored, producing a characteristic annealing or melting peak for the target amplicon. This can help distinguish the expected product from non-specific amplification and provides additional confirmation beyond a simple positive or negative signal (Becherer et al., 2020; Moehling et al., 2021).

This approach has been applied directly to meat species identification. Cho et al. (2014) developed LAMP assays for eight meat species and differentiated the amplified targets according to their characteristic annealing temperatures. For example, pork, horse, and chicken produced mean annealing temperatures of 84.96°C, 85.99°C, and 86.31°C, respectively. Although annealing curve analysis can improve confirmation of LAMP products and may support target differentiation, it requires fluorescence-compatible instrumentation capable of controlled temperature ramping. Closely spaced peaks may also complicate interpretation in multiplex assays, making this approach less suitable for simple visual field testing than colorimetric detection (Moehling et al., 2021).

1.2.4.5 Real-Time LAMP

Real-time LAMP allows amplification to be monitored continuously by measuring fluorescence throughout the reaction. A fluorescent dye is included in the reaction mixture, and the fluorescence signal is recorded at regular intervals to generate an amplification curve. The results can be expressed as a time-to-positive (Tp) value, representing the time required for the fluorescence signal to reach a defined threshold. Higher initial template concentrations generally result in shorter Tp values, allowing the initial amount of target DNA to be estimated when a suitable standard curve is established (Tomlinson et al., 2007).

Real-time LAMP has been successfully applied to meat species authentication using portable fluorescence instruments. Lee et al. (2016) developed a direct real-time LAMP assay for pork detection using a portable Genie II fluorometer, identifying pork in processed meat products within 30 min without conventional DNA extraction. Similarly, Sul et al. (2019) used a portable fluorescence device for direct detection of chicken in raw and processed meat products, also obtaining results within approximately 30 min. The main advantage of real-time LAMP is that fluorescence measurement provides an objective and continuous indication of amplification and can also be followed by annealing curve analysis for further confirmation of the amplified product. However, this approach requires a fluorescence-compatible instrument and a power source. Although portable systems are available, these requirements increase cost and reduce the simplicity of the assay compared with naked-eye colorimetric detection, particularly for low-resource or field applications (Becherer et al., 2020).

1.2.4.6 Lateral Flow Assay

Lateral flow assays provide a simple visual method for detecting LAMP products using paper-based test strips. LAMP amplicons are commonly detected through labeled primers or probes, such as biotin and FITC, which interact with capture

reagents on the strip to generate visible test and control lines. This approach allows results to be interpreted by the naked eye without fluorescence or electrophoresis equipment and is therefore suitable for point-of-care and field applications (Becherer et al., 2020). LAMP reaction coupled with lateral flow assay schematically illustrated in Figure 1.3.

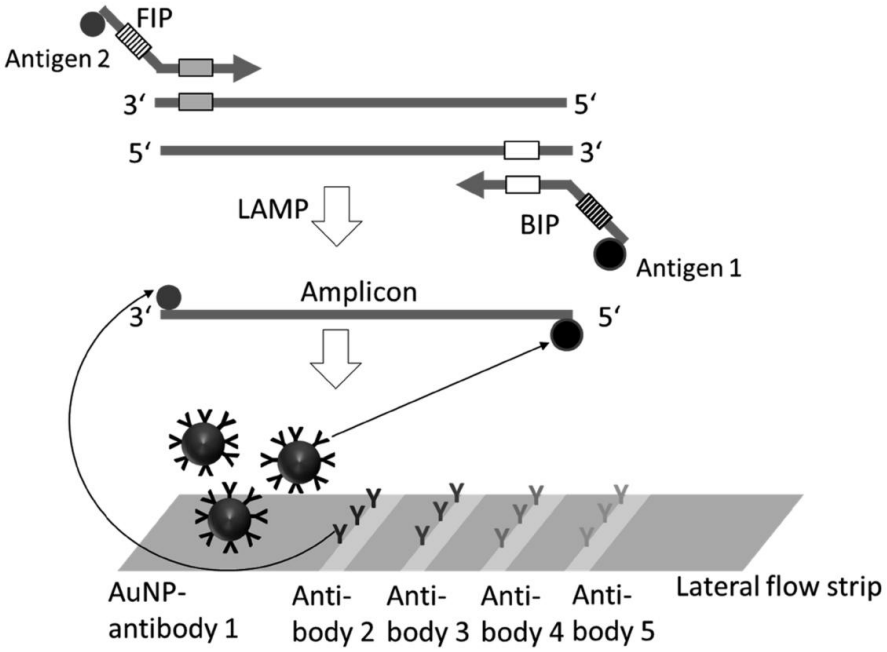

Figure 1.3 LAMP reaction coupled with lateral flow assay (Becherer et al., 2020).

LAMP coupled with lateral flow detection has also been applied directly to meat authentication. Jawla et al. (2021) developed a paper-based LAMP–lateral flow assay targeting the mitochondrial *COI* gene for the identification of pork-derived tissues. The assay was designed as a ready-to-use format for field applications. Although lateral flow detection provides a simple and visual results, many formats require transfer of amplified products to the strip after amplification,

which introduces may increase the risk of carry over contamination. In addition, strip results are primarily qualitative or semi-quantitative, unlike real-time fluorescence-based detection.

1.2.5 Direct LAMP

A major practical advantage of LAMP is its relatively high tolerance to inhibitors present in raw biological samples. Previous studies have shown that LAMP can be less affected by components of complex samples than PCR which DNA purification to be reduced or completely omitted (Kaneko et al., 2007). This property has supported the development of direct LAMP**,** in which raw DNA obtained by simple procedures such as short heat treatment, alkaline lysis, or sample dilution is used directly for amplification. Direct LAMP has already been successfully applied to meat species identification. Direct real-time LAMP assays have enabled pork detection without conventional DNA extraction and chicken detection in both raw and processed meat products without DNA purification (Lee et al., 2016; Sul et al., 2019). The broader value of simplified sample preparation has also been demonstrated using direct multiplex PCR, which identified six meat species without prior DNA extraction and achieved a 97.4% success rate across 115 commercial food products using a dilution-based sample preparation approach (Kitpipit et al., 2014).

Eliminating DNA extraction can considerably reduce analysis time, reagent use, cost, and sample handling, making direct LAMP favorable for rapid and field-based meat authentication. However, raw samples still contain matrix components that may delay amplification, reduce product formation, or effect detection signals (Nwe et al., 2024). Therefore, the performance of direct LAMP depends strongly on sample preparation and matrix composition, requiring a balance between simplified processing and reliable analytical sensitivity.

1.3 Advantages and Disadvantages of Detection Methods

Across the methods reviewed, a general shift is observed from laboratory-based analytical techniques toward faster and more field-applicable approaches. Protein-based methods such as electrophoresis and ELISA are relatively simple and inexpensive, but their performance may decrease in heat-processed products because protein markers can lose their solubility during heating (Hitchcock & Crimes, 1985). DNA-based methods are generally more suitable for processed foods because DNA is comparatively stable and provides species-specific information. Conventional PCR offers high specificity and the ability to detect several target species, while more advanced approaches such as digital PCR and NGS metabarcoding provide absolute quantification. However, these methods require increasingly specialized instrumentation and, particularly for NGS, more complex data analysis (Kitpipit et al., 2014; Basanisi et al., 2020; Giusti et al., 2024). Within this range of methods, LAMP provides a favorable balance between analytical performance and practical applicability. Its isothermal operation removes the need for repeated thermal cycling, while recognition of six distinct target regions by four core primers contributes to high amplification specificity (Notomi et al., 2000). LAMP also shows relatively high tolerance to some biological inhibitors, supporting direct or minimally processed sample preparation approaches (Kaneko et al., 2007; Lee et al., 2016; Sul et al., 2019). In addition, visual colorimetric detection can further reduce instrumentation requirements. Dual-colorimetric methods, such as the combination of cresol red and HNB, can improve visual detection with respect to different chemical changes associated with amplification (Zhang et al., 2022). Nevertheless, LAMP requires more complex primer design than conventional PCR and remains sensitive to non-specific amplification (Becherer et al., 2020; Wang et al., 2025). Overall, these characteristics make LAMP particularly suitable for cheap, rapid and field-applicable meat species screening, while careful primer design and reaction optimization are crucial for reliable detection.

1.4 Research Objective

In this study, a colorimetric direct LAMP platform was developed for the rapid identification of porcine, equine, and chicken meat species. The primary aim was to establish a common reaction system in which species-specific primer sets could be used under the same optimized reaction composition and amplification conditions. The study also aimed to simplify sample preparation through direct LAMP method without conventional DNA extraction and to enable clear visual detection using a dual-colorimetric system based on HNB and cresol red. In addition, an animal-specific internal control was incorporated to confirm the presence of amplifiable animal DNA and to support the reliable interpretation of negative results. By harmonizing the reaction conditions for all three target species and the endogenous control for validation, the developed platform aimed to provide a practical, rapid, and cost-effective approach for meat species authentication.

CHAPTER 2

MATERIALS & METHODS

The materials, biological samples, reagents, primer sets, and experimental techniques utilized for developing and analysis of a colorimetric multiplex LAMP assay for the simultaneous identification of pork, horse, and chicken meat are described in this chapter. The aim of the study was to develop a species-specific amplification technique that could be assessed using both unprocessed, processed meat samples and purified DNA templates. To support the evaluation of amplification performance and the presence of amplified animal DNA, the study included a positive internal control in addition to the target-specific assays. Sample collection and preparation, primer selection and in silico analysis, DNA extraction and quantification, optimization of LAMP reaction conditions, analysis of individual and paired colorimetric detection systems, development of a direct LAMP approach, and determination of the method's specificity and sensitivity formed the experimental workflow. Raw meat samples, processed pork products, laboratory prepared meat mixtures, and verified mixed samples from food control laboratories were used to evaluate the developed method. Heat treatment experiments were also performed on horse and chicken meat to assess the effect of heat processing on the performance of species-specific LAMP detection.

Throughout the study, particular attention was paid to minimizing the risk of contamination, a crucial consideration in high-throughput nucleic acid amplification methods such as LAMP. Sample preparation and reaction preparation areas were completely separated, using different laboratory equipment (such as micropetettes and tubes). In the workflow, reaction mixtures were first prepared and stored at +4°C, then DNA and/or meat samples were prepared. Subsequently, the tubes containing the reaction mixture were removed from +4°C, and the reaction was started by adding the sample. After the reaction was complete, the amplified reaction

tubes were carefully removed from the heat block and photographed. To prevent the release of amplified products into the laboratory environment, the tubes were not opened after amplification; they were sealed in a zip-lock bags and disposed of as biological waste.

2.1 Materials

2.1.1 Chemicals and Solutions

The components necessary for LAMP reaction, colorimetric detection, DNA extraction, sample preparation, and buffer preparation were among the chemicals and reagents used in this study. Cresol Red (CR) was obtained from BioShop and Hydroxy naphthol Blue (HNB), a metal-ion indicator used for colorimetric LAMP detection, was obtained from Merck. Betaine was purchased from Sigma-Aldrich. dNTP mix, Bst DNA Polymerase Large Fragment, and magnesium sulfate ($MgSO_4$) used in the LAMP reactions were obtained from Vazyme Biotech. The reaction buffer used in the study was obtained from NANObiz Technology Inc., while the remaining chemical components were obtained from Sigma-Aldrich.

Betaine solutions and colorimetric dye solutions were prepared using nuclease-free distilled water. The prepared betaine solution passed through a 0.2 µm membrane filter before use, and stored at -20°C. HNB and CR solutions were prepared at appropriate stock concentrations according to experimental requirements and stored at +4°C to maintain reagent stability. TE buffer and phosphate-buffered saline (PBS) were used for DNA extraction and direct LAMP sample preparation, respectively. Proteinase K, sodium dodecyl sulfate (SDS), sodium chloride (NaCl), isopropanol, and other chemicals required for the modified salt-based DNA extraction procedure were used according to the relevant experimental protocol. All solutions were prepared using appropriate laboratory practices to minimize contamination and decomposition. Appendix A provides a detailed composition of the prepared buffers and solutions.

2.1.2 Meat samples

The method's analytical performance was assessed using meat samples from both target and non-target animal species. In addition to commercially available samples, composition-controlled mixed meat samples were obtained from the food control laboratories.

Both raw and processed meat materials were included in the study. The use of samples from different sources and under different physical conditions allowed the performance of the developed method to be evaluated under various sample matrices. To reduce cross-contamination, each meat sample was processed independently. Before analysis, all the samples were kept frozen.

2.1.2.1 Unprocessed Meat Samples

Raw meat samples included pork, horse meat, chicken, beef, turkey, and other animal species used for specificity and comparative analyses. Except for the horse meat obtained from the Turkish Jockey Club Equine Hospital, meat samples were purchased from local chain supermarkets were obtained from facilities approved by the relevant governmental authority. Raw meat samples were used for DNA extraction, direct LAMP analysis, specificity testing, and sensitivity studies. The meat samples were individually and carefully packaged to ensure that the package is completely sealed and free of any openings. Then, they were labeled and stored at -20°C to minimize contamination.

2.1.2.2 Processed Meat Samples

Processed pork products were included in the study to determine the applicability of the developed test to processed food matrices. Processed pork products analyzed included bacon and prosciutto, purchased from supermarkets with verified ingredients listed on the product labels. Additionally, chicken and horse meat

samples were subjected to heat treatment through boiling and autoclaving. All these processed meat samples were included in the test using the developed direct LAMP sample preparation procedure. Inclusion of processed meat samples was important because heat treatment, salting, and other processing-related conditions can affect DNA integrity and the performance of molecular detection.

2.1.2.3 Mixed Meat Samples

The method's capacity to identify target animal DNA in complicated meat matrices was tested using mixed meat samples with predetermined species proportions. The Republic of Turkey Ministry of Agriculture and Forestry Afyonkarahisar Food Control Laboratory and the Republic of Turkey Ministry of Agriculture and Forestry Ankara Food Control Laboratory provided percentage-defined pork meat samples. These samples served as independently produced meat mixtures with a fixed percentage. They were only considered as composition-controlled samples provided by food control laboratory resources because no specific certification documentation was included with them. These percentage-defined pork meat mixtures were subsequently used for direct LAMP analysis and sensitivity assessment.

2.1.3 Primers

Species-specific LAMP primer sets targeting pork, horse, and chicken DNA were used in this study. In addition, a primer set targeting an internal animal DNA control was included.

The porcine-specific primer set was obtained from the primer design reported by Tasrip et al. (2021). The primer set targeted the mitochondrial *cytochrome b* region (GenBank: PZ534484.1) and consisted of F3, B3, FIP, BIP, and LB primers. The equine-specific primer set was obtained from Zahradnik et al. (2014). The primer set targeted the mitochondrial NADH dehydrogenase subunit 1 (GenBank: PZ147901.1) consisted of F3, B3, FIP, BIP, LF, and LB primers. The chicken-specific primer set

was obtained from Thangsunan et al. (2022). The primer set targeted the mitochondrial *cytochrome b* region (GenBank: PZ672148.1) consisted of F3, B3, FIP, and BIP primers. The positive internal control primer set was obtained from Xiao et al. (2023). The internal control targeted an animal 18S ribosomal RNA-related sequence and consisted of F3, B3, FIP, BIP, and LB primers. The primer sequences are given below (Table 2.1- Table 2.4)

Table 2.1 Porcine-specific primer sequences

Primer	Sequence	Reference
F3	5'-TCATAGGCTACGTCCTGC-3'	(Tasrip et al., 2021)
B3	5'-GCGGTAATGATGAATGGCA-3'	
FIP	5'-TCCGATATAAGGGATAGCTGATAGT-AGGACAAATATCATTCTGAGGAG-3'	
BIP	5'-ACAGACCTCGTAGAATGAATCTGA-GGATAAAGTGAAAGGCGAAGA-3'	
LB	5'-TTTTCCGTCGACAAAGCAACCCTC-3'	

Table 2.2 Equine-specific primer sequences

Primer	Sequence	Reference
F3	5'-CGAGCTCCATTTGACCTAAC-3'	(Zahradnik et al., 2014)
B3	5'- AAGGAGAGCTTTAATGGTGA-3'	
FIP	5'-CTGCTAGGAAGAATAGAGCAAATGG-AGAAGGAGAATCAGAACTCGT-3'	
BIP	5'-ACATCTTCACAACAACCCTATT TCT-ATTAATTGAGTAGAGTTCTGGC-3'	
LF	5'-TGCGTATTCAACGTTGAATCCAG-3'	
LB	5'-AGGAGCATTTCACAACCCCT-3'	

Table 2.3 Chicken-specific primer sequences

Primer	Sequence	Reference
F3	5’-ATCCTCCTCCTCACACTCAT-3’	
B3	5’-AGGTGAGGTGGATGATAGTAATA-3’	
FIP	5’-TGATAACGGTGGCCCCTCAGAGCCTTTGTGGGCTATGTTCT-3’	(Thangsunan et al., 2022)
BIP	5’-TAGTAGAGTGAGCCTGAGGGGGAAGCGAAGAATCGGGTAAGG-3’	

Table 2.4 Animal internal control primer sequences

Primer	Sequence	Reference
F3	5’-CCGGCGGCTTTGGTGA-3’	
B3	5’-TTTGCGCGCCTGCTG-3’	
FIP	5’-CAGACGTTCGAATGGGTCGTCGCTCTAGATAACCTCGGGCCG-3’	(Xiao et al., 2023)
BIP	5’-CGTGCCTACCATGGTGACCACGTGGATGTGGTAGCCGTTTCT-3’	
LB	5’-GTGACGGGGAATCAGGGTTCG-3’	

2.2 Methods

2.2.1 Primer Design and In Silico Analysis

The primer sets used in this study were selected from previously reported and experimentally evaluated species-specific LAMP primer sets. Target sequences were evaluated to determine their potential to distinguish the target species from other animal species commonly encountered in meat products in order to confirm species-specific amplification. Multiple sequence comparisons were used to assess sequence conservation and species-specific regions. Primer sequences were evaluated using MFE Primer 4.0 and MFE Primer 4.1.0 tools to assess potential primer interactions and possible cross-reactivity with non-target animal species. In silico analysis included the assessment of non-specific primer binding and the potential for primer-primer interactions. The detailed findings of the in silico analysis, including multiple sequence comparisons, evaluation of species-specific regions, and potential cross-reactivity with non-target species, are presented in Appendix B.

2.2.2 Modified Salt DNA Extraction Method

A modified salt precipitation approach based on the protocol described by Yalçınkaya et al. (2017) was used to isolate DNA from meat samples. After transferring about 5 g of the meat sample into an appropriate container, 2 mL of TE buffer was added. A glass homogenizer was used to homogenize the sample. After the sample was homogenized, 50 mg of it was put into a 1.5 mL microcentrifuge tube. The material was mixed with 400 µL of lysis buffer and 40 µL of 20% SDS. To guaranty sufficient mixing, the mixture was vortexed. Next, 2 µL of a 20 mg/mL Proteinase K solution was added. To promote cellular disruption, the samples were incubated for 60 minutes at 65°C. The samples were then centrifuged at 10,000 × g for 30 min. Carefully, the supernatant was moved to a clean microcentrifuge tube. The collected supernatant was mixed with an equal volume of isopropanol. The

samples were incubated for 10 minutes at -20°C. After that, the samples were centrifuged for 20 minutes at 16,000 × g. Then supernatant was discarded. After discarding the supernatant, the resultant DNA pellet was given a quick drying period. After that, 100 µL of TE buffer was used to dissolve the pellet. A Nanodrop spectrophotometer was used to assess the extracted DNA's concentration and purity. DNA concentration was measured in ng/µL, and DNA quality was evaluated using absorbance measurements, such as A260, A260/A280, and A260/A230 ratios. Samples of extracted DNA were kept at -20°C until they were examined further.

2.2.3 Meat Sample Preparation for Direct LAMP Analysis

For the direct LAMP reaction, the sample preparation technique described by Özden (2024) was applied. Samples weighing between 200 and 300 mg have been taken from meats and meat products with a sterile biopsy punch or bistoury. The taken samples were put into a sterile 1.5 ml lab tube and dissolved in 100 µL of PBS (phosphate-buffered saline). 2 µL of the supernatant were immediately used as the template for the LAMP reaction following a two-minute incubation period at 98°C (Lee et al., 2016). Meat sample preparation for direct LAMP analysis illustrated in Figure 2.1.

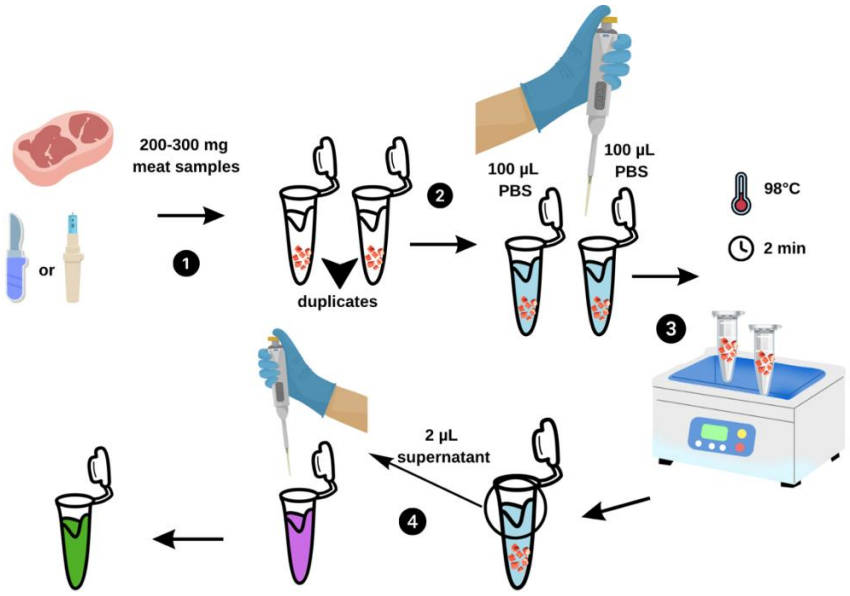

Figure 2.1 Meat sample preparation for direct LAMP analysis (created with Canva.com).

2.2.4 LAMP Reaction Optimization

LAMP reactions were optimized to identify standard reaction conditions for colorimetric detection and amplification that targets multiple species. The optimization studies were carried out based on the pork-specific LAMP protocol optimized by Özden (2024), and the optimized conditions were adapted for other target species. In this context, the pH of the reaction buffer, $MgSO_4$ concentration, reaction temperature, and duration were systematically optimized. Bst DNA Polymerase Large Fragment enzyme was used to prepare LAMP reactions in a final volume of 25 µL. The reaction mix contained the appropriate primer set, dNTPs, $MgSO_4$, betaine, reaction buffer, enzyme, colorimetric indicator, template DNA, and nuclease-free water. Table 2.5 displays the reaction mix's composition.

Table 2.5 LAMP reaction preparation according to Özden (2024)

Components	Volume	Final concentration
10X ThermoPol Buffer	2.5 µL	1 x
MgSO4 (100 mM)	1.5 µL	6 mM (8 mM in total)
dNTP Mix (10 mM)	3.5 µL	1.4 mM
FIP/BIP Primers (25 x)	1 µL	1.6 µM
F3/B3 Primers (25 x)	1 µL	0.2 µM
Loop B Primer/ LB/ LF Primers (25 x)	1 µL	0.8 µM
Bst DNA Polymerase Large Fragment (8 U/µL)	1 µL	320 U/mL
Betaine (5 M)	3.5 µL	0.7 M
Cresol Red (5 mM)	1.25 µL	250 µM
HNB (3 mM)	0.42 µL	50 µM
DNA/ Direct LAMP Template	x µL/ 2 µL	1 ng/ -
DNase/ RNase free water	up to 25 µL	-

The preparation of FIP/BIP, F3/B3, and LB/LF 25 x primer stocks were prepared at the specified concentrations shown in Table 2.6, Table 2.7, Table 2.8. Primer mixes were prepared as 100 µL and stored at -20°C.

Table 2.6 Primer mix preparation for porcine and animal internal control primers

Primer mix (25X)	Primers	Final concentration
FIP/BIP	FIP	40 µM
	BIP	40 µM
F3/B3	F3	5 µM
	B3	5 µM
Loop	Loop B Primer	20 µM

Table 2.7 Primer mix preparation for equine primers

Primer mix (25X)	Primers	Final concentration
FIP/BIP	FIP	40 µM
	BIP	40 µM
F3/B3	F3	5 µM
	B3	5 µM
Loop	LB	10 µM
	LF	10 µM

Table 2.8 Primer mix preparation for chicken primers

Primer mix (25X)	Primers	Final concentration
FIP/BIP	FIP	40 µM
	BIP	40 µM
F3/B3	F3	5 µM
	B3	5 µM

In LAMP reactions carried out using template DNA obtained through DNA isolation, an initial denaturation was performed at 97°C for 5 minutes, and the reaction was run at 65°C for 60 minutes. In direct LAMP applications, the sample preparation was done at 98°C for 2 minutes, and then the reaction was run at 65°C for 60 minutes.

2.2.4.1 Buffer preparation and pH Optimization

The buffer is obtained from NANObiz Technology Inc. at a concentration of 50X. Afterwards, it is diluted to 10X with nuclease-free water to make it ready for use in the LAMP reaction mixture. The 10X NBZ Isothermal buffer pH was adjusted to the 8.70, 8.75, 8.80, 8.85, and 8.90 respectively. Hydrochloric acid (HCl) and sodium

hydroxide (NaOH) solutions were used to adjust the pH of the buffer. The pH-adjustment solutions were prepared using nuclease-free water. Following preparation, dilution, and pH adjustment the reaction buffer was passed through a 0.2 µm membrane filter before use, aliquoted into 1 ml portions, and stored at +4°C.

LAMP reactions carried out using meat sample, the sample preparation was done at 98°C for 2 minutes, and then the reaction was run at 65°C for 60 minutes on heat block.

2.2.4.2 $MgSO_4$ Concentration

4 mM, 6 mM, 8 mM, and 10 mM $MgSO_4$ concentrations were tested while keeping the remaining reaction components constant. pH of the reaction buffer was kept at 8.75. Concentrations were tested using direct LAMP method and the reactions were carried out 65°C for 60 minutes on heat block.

2.2.4.3 Colorimetric Detection

Colorimetric detection was used to visually evaluate the amplification products produced by the LAMP test. The dyes were evaluated individually and in combination under optimized LAMP reaction conditions. First, only the cresol red concentration was tested, followed by gradient HNB concentrations in combination with the determined cresol red concentration. Color changes were assessed by comparing negative, positive, and NTC tubes. Photographs were taken under the same lighting conditions and against a white background to facilitate visual comparison between reactions. Each experiment included a non-template control (NTC) containing nuclease free water. A negative control consisting of a non-target beef meat sample was also included to assess the possibility of non-specific

amplification originating from the meat matrix. Positive reactions were prepared using a 1% pork meat sample obtained from The Republic of Turkey Ministry of Agriculture and Forestry Afyonkarahisar Food Control Laboratory.

2.2.4.3.1 Cresol Red

To evaluate the effect of buffer and pH variation on the color change in the LAMP reaction performed at the optimized cresol red concentration, a LAMP reaction containing only cresol red was generated using a pork sample. In this context, cresol red concentrations of 100 µM, 125 µM, 250 µM, and 500 µM were tested. Concentration adjustment was made by adding volume to the reaction mixture (for 100 µM, 0.5 µL of 5 mM cresol red stock solution; for 125 µM, 0.63 µL of 5 mM cresol red stock solution). The buffer pH was kept constant at 8.75 and the $MgSO_4$ concentration at 6 mM. The LAMP reaction was carried out on a heat block at 65°C for 60 minutes.

2.2.4.3.2 Dual Colorimetric Detection

To evaluate the effect of buffer and pH variation on color change in the LAMP reaction performed with an optimized dual dye system, 0 µM, 50 µM, 75 µM, and 100 µM HNB concentrations were tested while keeping the cresol red concentration constant at 250 µM. For this purpose, LAMP reactions with gradient HNB concentrations were tested using a pork sample. In the reactions, the buffer pH was kept constant at 8.75 and the $MgSO_4$ concentration at 6 mM. The LAMP reaction was carried out on a heat block at 65°C for 60 minutes.

2.2.4.4 Reaction Temperature

After optimizing the buffer and pH value, $MgSO_4$ concentration, and dual colorimetric method, a study was conducted to determine the reaction temperature.

Since the aim of this project was to perform all species detection on the same platform, the temperature of 65°C, determined by Özden (2024) and used in other optimization experiments, where the porcine-specific primer worked efficiently, was chosen. Horse and chicken primers were tested at this temperature. The reaction conditions were kept constant as buffer pH at 8.75, 6 mM $MgSO_4$, 250 µm cresol red, and 50 µm HNB. Beef meat sample and pork meat sample were used as negative controls, and primer-specific chicken and horse meat samples were used as positive tests. Each experiment included a non-template control (NTC) containing nuclease free water. The reaction was carried out on a heat block at 65 °C for 60 minutes.

2.2.4.5 Reaction Time

30, 45, and 60 minutes were tested as reaction duration. The buffer pH was set at 8.75, 6 mM MgSO4, 250 µm cresol red, and 50 µm HNB. Target meat samples were used as the positive control for each species-specific test, while beef samples were used as the negative control for the assays involving pork, horse, and chicken. Every reaction contained a non-template control (NTC). A sample of beef meat served as the positive control for the animal internal control test. LAMP reactions were carried out 65°C for 60 minutes on heat block.

2.2.5 Sensitivity of the Optimized Method

Genomic DNA extracted from pork, horse, chicken, and beef tissue using the modified salt-based extraction protocol was serially diluted in TE buffer to generate a dilution series of 1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1 pg, and 0.01 pg. Each concentration within this dilution series served as a positive template under the previously optimized reaction parameters. A non-template control was prepared with nuclease-free water. For every assay, beef-derived DNA was additionally included, following the same dilution sequence, functioning as a negative control across all species-specific reactions while simultaneously serving as the positive reference for the

internal amplification control targeting the animal genome. All remaining reaction components were held at their fixed, optimized concentrations throughout testing. Amplification was carried out isothermally at 65°C for 60 minutes using a heat block.

2.2.6 Specificity of the Optimized Method

To determine the specificity of the method, ten different types of meat (pork, horse, chicken, beef, fish, turkey, duck, lamb, goose, and goat); two different processed pork products (bacon (87% pork) and prosciutto (98% pork) were tested. All meats were prepared as specified in Section 2.2.3. A non-template control was prepared with nuclease-free water. The direct LAMP reaction method was used with optimized conditions for specificity experiments. Optimized reaction components were kept constant. Each species-specific reactions were tested independently. Direct LAMP reactions were carried out 65°C for 60 minutes on heat block.

2.2.7 Analysis of Processed Meat Samples

To evaluate the performance of the direct colorimetric LAMP test in heat-processed meat samples, horse and chicken meat samples were used as positive tests and beef samples as negative controls. These heat treatments were autoclaving and prolonged boiling of meat samples. For autoclaving, duplicate samples prepared according to the direct LAMP sample preparation protocol were processed in a laboratory autoclave at 121°C for 15, 30, and 45 minutes. For prolonged boiling, similarly prepared samples were heated in a heat block at 98°C for 2, 5, 10, 15, 30, 45, and 60 minutes. After heat treatment, all samples were analyzed under optimized direct LAMP conditions, and the amplification results were evaluated by dual colorimetric observation. The processes were performed in two separate batches with standard optimized LAMP mixtures prepared separately with chicken and horse primers. A non-template control (NTC) was included in all experiments.

2.2.8 Shelf-life Stability at Room Temperature

Optimized reaction mixes were stored without adding any template (DNA or meat samples). All the other components including enzyme and primers were added. Dual colorimetric direct LAMP method was used to detect the amplification results.

Aliquoted reaction mixtures were stored at room temperature for 15 days. Reaction performance was evaluated at 3-day intervals during storage. At each time point, 3 tubes were taken from the stored reaction mixture for pork, horse, and chicken, and 2 tubes for animal internal control. A species-specific meat sample was added as a positive test for each species. For the animal internal control specific method, a beef sample was used as a positive test. Nuclease-free water was included in all tests as NTC. A beef meat sample was used as a negative control in each test for the pork, horse, and chicken specific methods. Reactions were performed simultaneously on a heat block at 65°C for 60 minutes. Reaction results were regularly controlled and photographed for 15 days.

CHAPTER 3

RESULTS AND DISCUSSION

The LAMP method was optimized for identifying pork, horse, and chicken meat from meat samples in the same reaction parameters. To enhance visual diagnosis, a dual colorimetric detection method was tested. Meat samples were subjected to direct LAMP using the optimal technique. Tests for sensitivity and specificity were also performed.

3.1 DNA Isolation with Modified Salt Method

Genomic DNA was extracted from raw pork, horse, chicken and beef meat samples using the modified salt extraction method described by Yalçınkaya et al. (2017). This extraction protocol was selected because it has been reported to provide high DNA yield and quality from meat tissues and was also employed in the optimization study conducted by Özden (2024). Following DNA extraction, DNA concentration (ng/µL) and purity were determined using a spectrophotometer by measuring the A260/A280 and A260/A230 absorbance ratios. The concentration and purity values obtained from the isolated pork, horse, chicken and beef DNA samples are presented in Table 3.1.

Table 3.1 DNA concentration and purity values of genomic DNA isolated from raw meat samples.

Meat Samples	ng/ µL	A260/ A230	A260/A280
Porcine	83.729	0.50	1.65
Equine	49.756	0.46	1.52
Chicken	226.923	1.03	1.80
Beef	19.866	0.78	1.86

The concentration and purity of the isolated genomic DNA were evaluated using the A260/A280 and A260/A230 absorbance ratios (Table 3.1). DNA concentrations ranged from 19.866 ng/µL in the beef sample to 226.923 ng/µL in the chicken sample. The A260/A280 ratio is widely used to assess DNA purity, with values around 1.8 generally indicating relatively pure DNA, whereas lower values suggest the presence of protein or other organic contaminants (Manchester, 1996; Wilfinger et al., 1997). In this study, chicken and beef samples exhibited A260/A280 values close to the expected range, while porcine and equine samples showed lower ratios, indicating residual contamination.

All samples showed A260/A230 values below the optimal range, suggesting the presence of residual salts or other extraction-related contaminants (Manchester, 1996; Wilfinger et al., 1997). Similar purity profiles have been reported for the modified salt extraction method by Yalçınkaya et al. (2017), and these contamination levels were not found to interfere with downstream amplification. The same DNA isolation protocol was also employed in the study of Özden (2024), and the extracted DNA was considered suitable for subsequent colorimetric direct LAMP analysis.

3.2 LAMP Assay Optimization for Meat Identification

Firstly, in the optimization of the LAMP reaction targeting the pork specific mitochondrial *cytochrome b (cytb)* gene, the reaction conditions determined in the thesis study conducted by Dilara Özden and the conditions recommended in the Vazyme Bst DNA Polymerase Large Fragment manufacturer's guide were used as a basis (Özden, 2024). However, the prior buffer formulation was requested from the company because the manufacturer discontinued the 10X Thermopol buffer used with the enzyme and switched to the 10X Isothermal buffer with a different chemical composition. Because this change affects reaction efficiency, a reaction buffer from NANObiz Technology Inc., mentioned in the Materials and Methods section of Chapter 2, was used and its performance was evaluated by adjusting it to different pH values. Following buffer optimization, parameters such as magnesium sulfate ($MgSO_4$) concentration, dye concentration (CR and HNB), and reaction time were systematically changed and optimized to improve reaction conditions. Optimization results were evaluated visually based on color changes in the tubes. Each optimization experiment included a non-template control (NTC) and a negative control (N) which involving non-targetted species DNA.

3.2.1 Optimization of the Buffer

The optimization of the pork-specific colorimetric direct loop-mediated isothermal amplification (LAMP) assay was previously established in the master's thesis conducted by Dilara Özden. In the present study, the reaction conditions optimized by Özden were adopted as the initial experimental conditions. However, a significant change in the reaction system occurred because the manufacturer discontinued the 10X Thermopol buffer originally recommended for use with the Vazyme Bst DNA Polymerase Large Fragment enzyme and replaced it with a 10X Isothermal buffer of different composition. This modification resulted in noticeable differences in reaction performance. Since LAMP reactions are highly sensitive to buffer

composition, ionic strength, and pH, changes in buffer formulation can substantially affect amplification efficiency as well as the reliability and visual discrimination of colorimetric reactions (Notomi et al., 2000; Tanner et al., 2015).

As the original Thermopol Buffer was discontinued by the manufacturer, the buffer solution supplied by NANObiz Technology Inc. (NBZ Isothermal Buffer) was adopted for use in subsequent studies. The buffer was obtained at a stock concentration of 50X and subsequently diluted to a working concentration of 10X using nuclease-free water. To adapt this buffer formulation to the LAMP reaction format, pH optimization was performed by adjusting the diluted 10X buffer to five different values (8.70, 8.75, 8.80, 8.85, and 8.90), and the performance of the assay was evaluated comparatively across these conditions to determine the pH yielding the most efficient amplification. Optimization of buffer pH was considered a critical parameter because the colorimetric detection system employed in this study relied on pH-sensitive indicator dyes. Cresol red and hydroxy naphthol blue (HNB) undergo distinct color changes depending on their protonation and deprotonation states. Cresol red exhibits a clear color transition within the neutral-to-alkaline pH range, while HNB is an ion and pH responsive indicator widely used in colorimetric LAMP assays because of its ability to provide direct visual detection of amplification products (Goto et al., 2009; Tanner et al., 2015). Consequently, the pH of the reaction buffer influences not only the amplification process itself but also the visual contrast between positive and negative reactions. Therefore, establishing an appropriate buffer pH was regarded as an essential step in optimizing the assay for reliable visual colorimetric detection.

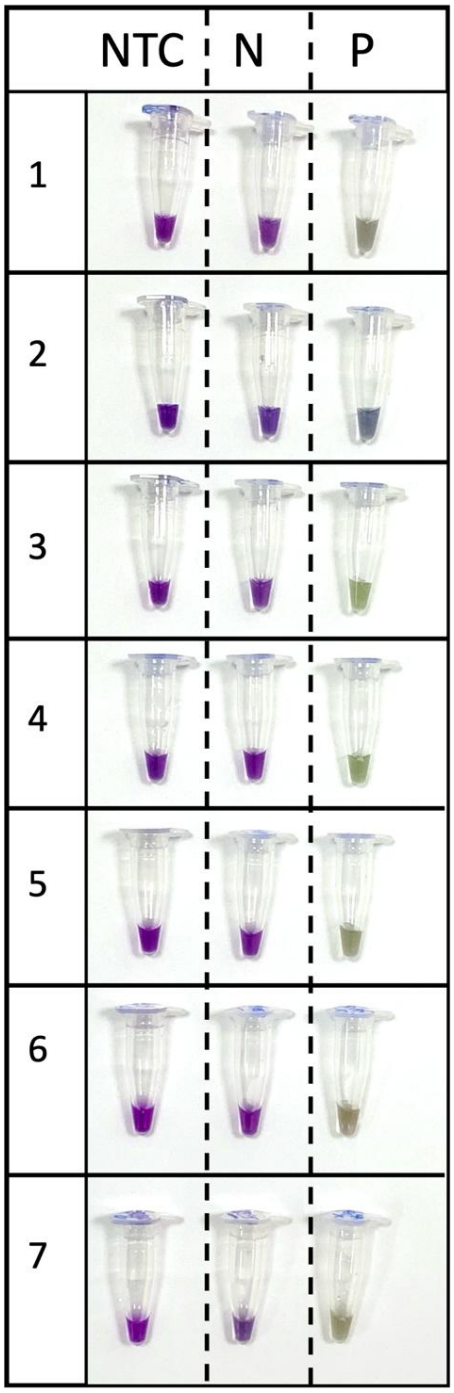

Figure 3.1 Visualization of LAMP results under the white light. NTC: non-template control, N: negative control containing beef meat sample, P: positive test containing 1% pork meat sample. 1: 10X Thermopol Buffer-Vazyme, 2: 10X Isothermal Buffer-Vazyme, 3: NBZ 10X Isothermal buffer (pH 8.70), 4: NBZ 10X Isothermal buffer (pH 8.75), 5: NBZ 10X Isothermal buffer (pH 8.80), 6: NBZ 10X Isothermal buffer (pH 8.85), 7: NBZ 10X Isothermal buffer (pH 8.90).

As shown in Figure 3.1, the trademark 10X Isothermal buffer (Vazyme) produced less distinct color differentiation between positive and negative reactions compared with the NBZ reaction buffers. According to the manufacturer, the Vazyme 10X Thermopol buffer is formulated at pH 8.80. However, among the tested NBZ 10X Isothermal buffer conditions, the buffer adjusted to pH 8.75 (Figure 3.1, lane 4) produced the clearest visual discrimination, with a consistent green color in the positive reaction and a stable purple color in both the negative control and the non-template control. In contrast, the buffer prepared at pH 8.80 also generated successful amplification, but the color contrast between negative and positive reactions was less distinct than that observed at pH 8.75.

Based on these findings, the NBZ 10X Isothermal buffer (pH 8.75) was selected for all following experiments. This result highlights the sensitivity of colorimetric LAMP reactions to small variations in buffer and pH, which directly affect the performance of pH-responsive indicator dyes and the visual interpretation of amplification results.

3.2.2 Optimization of the Magnesium Concentration

Following the selection of the NBZ 10X Isothermal buffer at pH 8.75, the $MgSO_4$ concentration was optimized to determine the most suitable reaction condition for the colorimetric direct LAMP assay. Magnesium ions are essential cofactors for Bst DNA polymerase and directly influence strand displacement and DNA synthesis efficiency (Tanner et al., 2015). In addition, hydroxy naphthol blue (HNB) is a metal ion-sensitive indicator whose color change depends on the reduction of free Mg^{2+} ions during DNA amplification. Consequently, $MgSO_4$ concentration affects both amplification performance and the visual colorimetric response of the assay (Goto et al., 2009). The direct LAMP reaction was performed at 65°C for 60 minutes using

dual colorimetric method (50 µM HNB, 250 µ M cresol red), while $MgSO_4$ concentrations of 4, 6, 8, and 10 mM were compared (Figure 3.2).

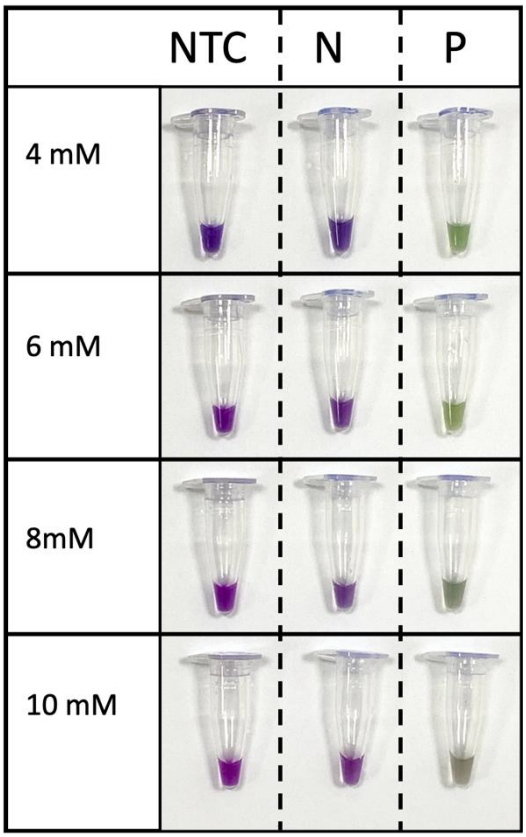

Figure 3.2 Visualization of LAMP results under the white light. Results of increasing magnesium concentration (4 mM to 10 mM). NTC: non-template control, N: negative control containing beef meat sample, P: positive test containing 1% pork meat sample.

As shown in Figure 3.2, both 4 mM and 6 mM $MgSO_4$ produced clear color changes and visually distinguishable positive and negative reactions. However, 6 mM was selected for subsequent experiments because lower magnesium concentrations may reduce the efficiency of LAMP amplification. Magnesium ions are required for the catalytic activity of Bst DNA polymerase, and insufficient Mg^{2+} availability can limit strand displacement activity, resulting in reduced DNA synthesis (Notomi et al.,

2000; Tanner et al., 2015). Although the colorimetric response at 4 mM was comparable to that at 6 mM, selecting 6 mM provided a benefit for maintaining amplification efficiency while preserving clear visual color discrimination. This concentration was therefore considered the most appropriate condition for the standardized colorimetric direct LAMP assay.

3.2.3 Colorimetric Detection Optimization

After establishing the optimized reaction conditions using the NBZ 10X Isothermal buffer at pH 8.75 and 6 mM $MgSO_4$, subsequent experiments were performed to evaluate first the cresol red-based colorimetric detection system and then the dual colorimetric detection system combining cresol red and hydroxy naphthol blue (HNB) under the same reaction conditions.

3.2.3.1 Optimization of Cresol Red Concentration

Following optimization of the reaction buffer (pH 8.75) and 6 mM $MgSO_4$, the concentration of cresol red was optimized to determine the most suitable condition for visual colorimetric detection. Cresol red (CR) is a pH-sensitive indicator that changes color according to the pH changes during the LAMP reaction, and its concentration directly influences the intensity and distinguishability of the color transition between positive and negative reactions (Tanner et al., 2015). Therefore, cresol red concentrations of 100, 125, 250, and 500 µM were evaluated under identical reaction parameters (Figure 3.3).

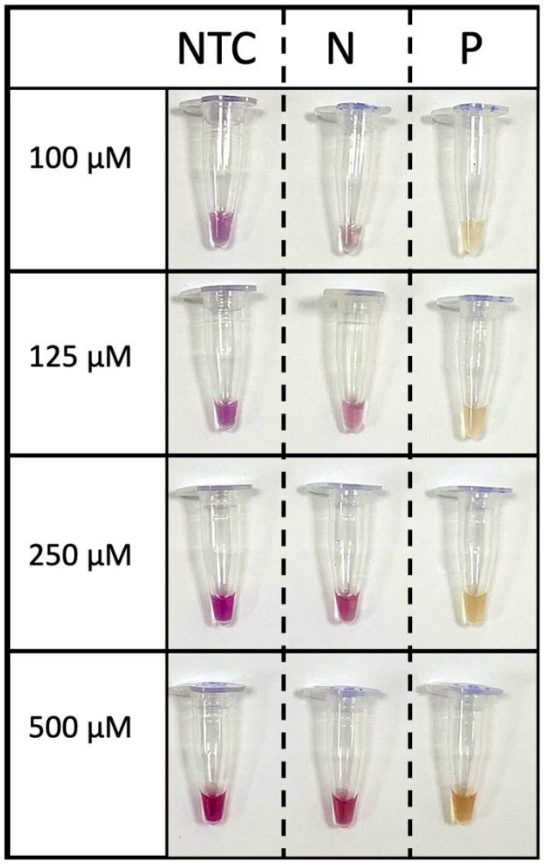

Figure 3.3 Visualization of LAMP results under the white light. Effect of increasing cresol red concentration (100 µM to 500 µM). NTC: non-template control, N: negative control containing beef meat sample, P: positive test containing 1% pork meat sample.

As shown in Figure 3.3, the positive reactions were successfully distinguished from the non-template controls at all tested concentrations. However, at 100 µM and 125 µM concentrations, the difference in color between the negative and positive reactions was very weak, whereas 500 µM generated a darker background, resulting in reduced overall color contrast. On the other hand, 250 µM cresol red produced the clearest and most balanced color contrast, providing a distinct yellow positive reaction while maintaining a stable pink-purple color in the negative reactions. Based on these observations, 250 µM was selected for all subsequent experiments, as it provided the most reliable visual detection.

3.2.3.2 Optimization of Dual Colorimetric Detection

After determining the optimal cresol red concentration (250 µM), the concentration of hydroxy naphthol blue (HNB) was optimized for the dual colorimetric detection system. HNB concentrations of 0, 50, 75, and 100 µM were evaluated while maintaining all other reaction conditions constant, including buffer (pH 8.75), $MgSO_4$ (6 mM) and CR (250 µM). All reactions were carried out on a heat block for 60 minutes at 65°C.

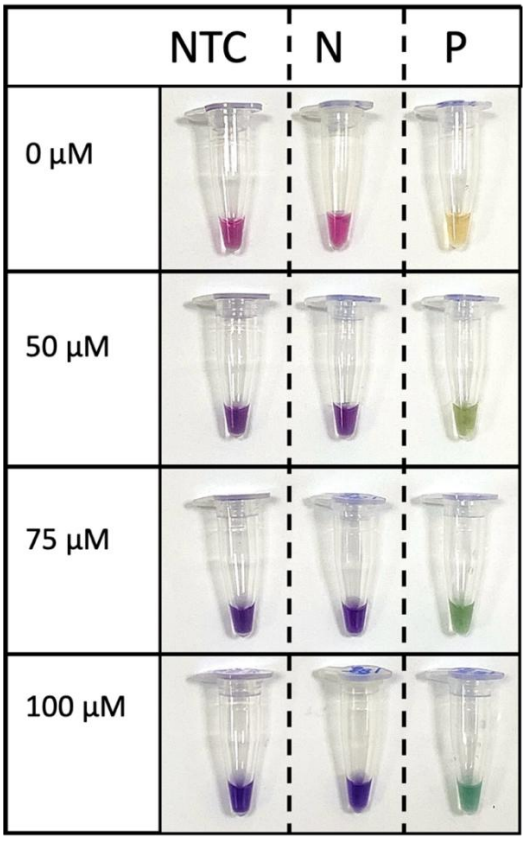

Figure 3.4 Visualization of LAMP results under the white light. Effect of increasing HNB concentration (0 µM to 100 µM) on dual colorimetric method. NTC: non-template control, N: negative control containing beef meat sample, P: positive test containing 1% pork meat sample.

HNB addition enhanced the visual colorimetric response of the test. The most distinct color contrast was observed with 50 µM HNB, producing the clearest and most vibrant color difference between negative and positive reaction tubes, improving the visual distinction of the amplification results. In contrast, 75 µM and 100 µM HNB resulted in darker color transitions which could reduce sensitivity. Therefore, 50 µM HNB was selected as the optimal concentration for the dual colorimetric LAMP test, as it provided the best combination of color contrast and visual clarity.

3.2.4 Reaction Temperature

The pork-specific LAMP assay has previously been shown to perform efficiently at 65°C (Özden, 2024). As the main objective of this study was to establish a platform allowing all reactions to be conducted under uniform conditions, the chicken and equine-specific primer sets were evaluated at 65°C, 60 minutes. All reactions were carried out using the optimized buffer conditions, including buffer (pH 8.75), $MgSO_4$ (6 mM), CR (250 µM) and 50 µM hydroxy naphthol blue (HNB). This approach enabled the assessment of whether the equine (Figure 3.5) and chicken (Figure 3.6) primer sets could be successfully amplified under the same reaction composition and temperature conditions established for the pork-specific assay.

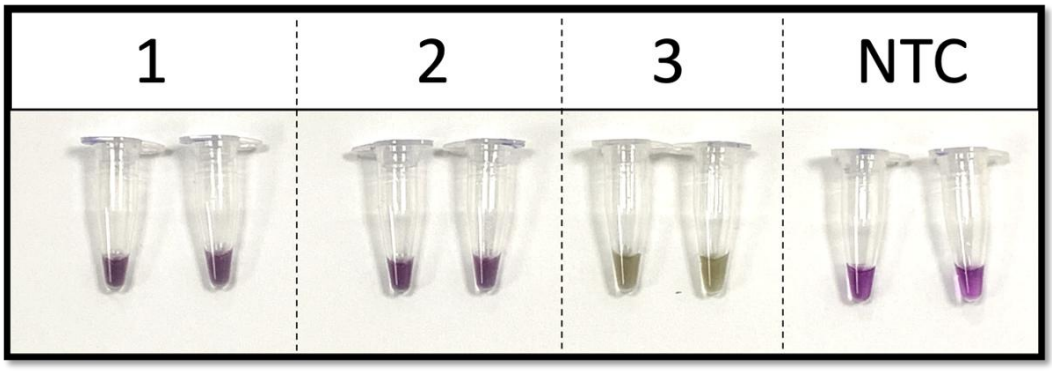

Figure 3.5 Visualization of LAMP results under the white light. Results of the Equine specific direct LAMP. NTC: non-template control,1: negative control containing 1% pork meat sample, 2: negative control containing beef meat sample, 3: positive test containing horse meat sample.

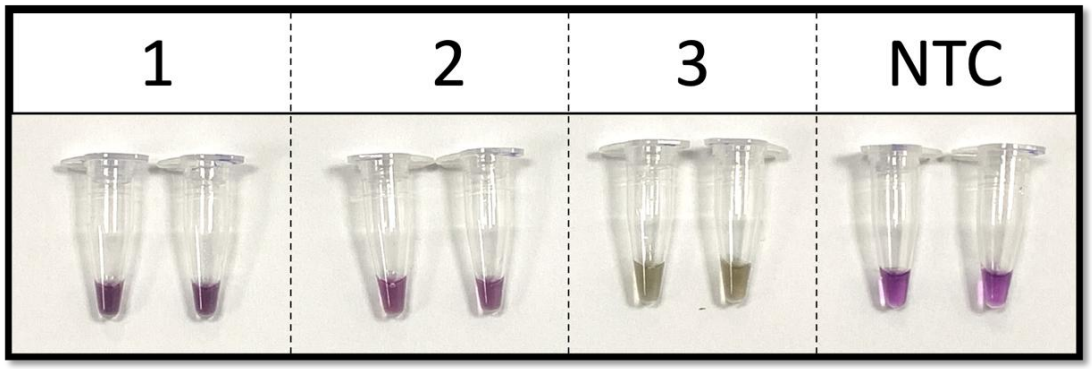

Figure 3.6 Visualization of LAMP results under the white light. Results of the chicken specific direct LAMP. NTC: non-template control,1: negative control containing 1% pork meat sample, 2: negative control containing beef meat sample, 3: positive test containing chicken meat sample.

The optimized reaction temperature was successfully applied to both the equine and chicken specific methods, producing clear and reproducible color differentiation between positive and negative reactions shown in figures above (Figure 3.5 and Figure 3.6). The different visual contrast observed for all three species indicates that the selected reaction conditions were compatible with different primer sets.

Establishing a common reaction temperature and composition is particularly advantageous for multiplex assay development and routine laboratory applications, as it eliminates the need for species-specific reaction optimization, reduces detection time and costs, and simplifies the overall workflow. These findings demonstrate that the optimized conditions provide a potent platform for colorimetric LAMP based multiple meat species identification.

3.2.5 Optimization of Reaction Time

To determine the optimum reaction duration for the multi-LAMP platform, the pork, equine, chicken-specific, and animal internal control primer sets were evaluated at 65°C for 30, 45, and 60 minutes on a heat block. All reactions were performed under the optimized reaction conditions consisting of the reaction buffer (pH 8.75), $MgSO_4$ (6 mM), CR (250 µM) and 50 µM hydroxy naphthol blue (HNB). For each species-specific assay, target meat sample was used as the positive control, while beef sample served as the negative control for the pork, equine, and chicken assays. Nuclease free water was added in all reactions as NTC. For the animal internal control assay, beef meat sample was used as the positive control together with an NTC. The corresponding results are presented below (Figures 3.7–3.10).

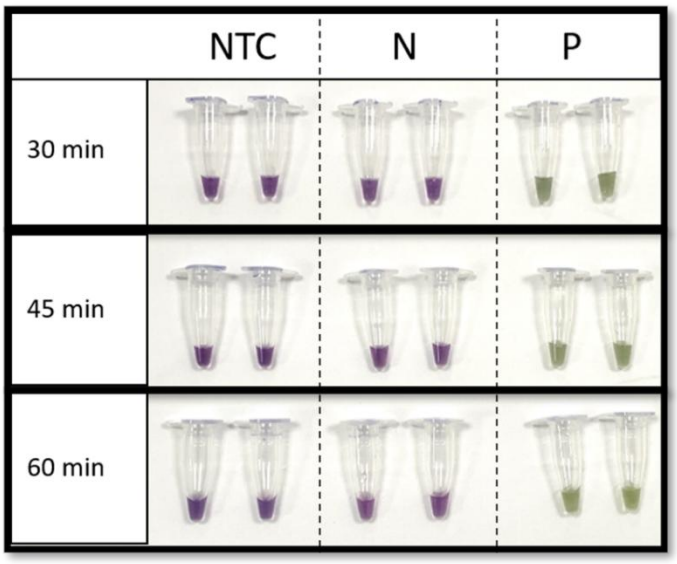

Figure 3.7 Visualization of LAMP results under the white light. Effect of reaction duration on porcine-specific LAMP reaction. 30 to 60 minutes reaction durations were tested. NTC: non-template control, N: negative control containing beef sample, P: positive test containing pork meat sample.

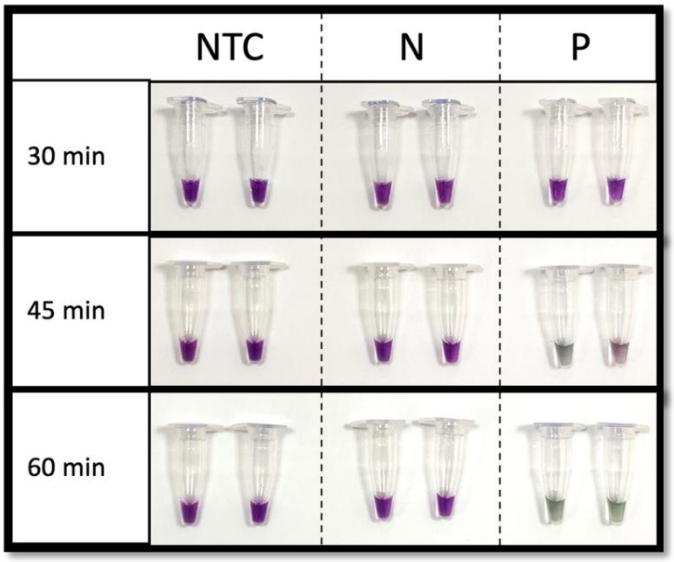

Figure 3.8 Visualization of LAMP results under the white light. Effect of reaction duration on equine-specific LAMP reaction. 30 to 60 minutes reaction durations were tested. NTC: non-template control, N: negative control containing beef sample, P: positive test containing horse meat sample.

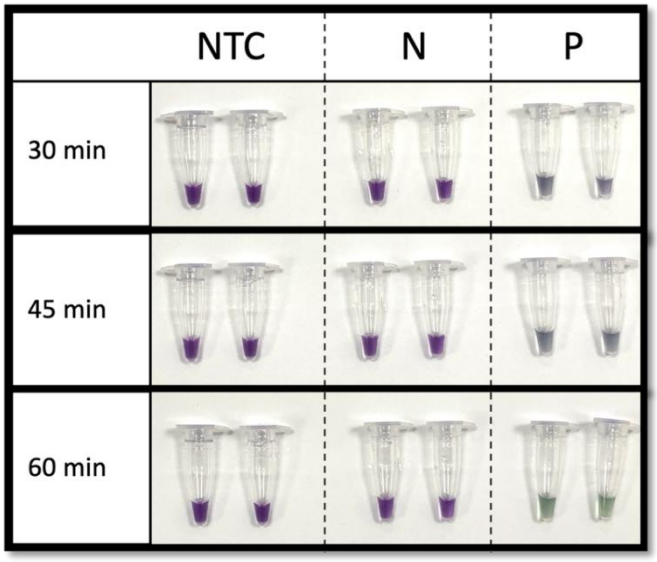

Figure 3.9 Visualization of LAMP results under the white light. Effect of reaction duration on chicken-specific LAMP reaction. 30 to 60 minutes reaction durations were tested. NTC: non-template control, N: negative control containing beef sample, P: positive test containing chicken meat sample.

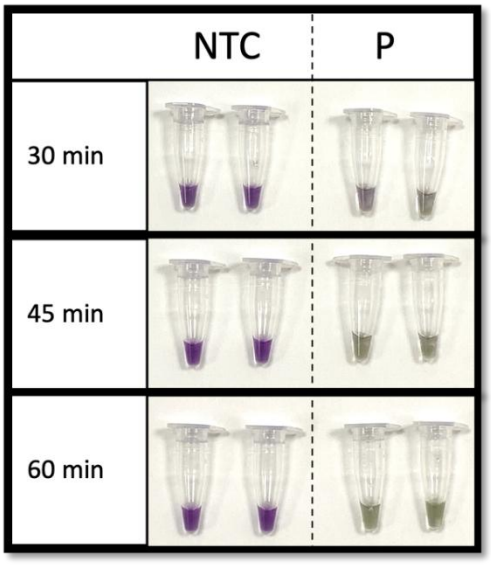

Figure 3.10 Visualization of LAMP results under the white light. Effect of reaction duration on animal internal control LAMP reaction. 30 to 60 minutes reaction durations were tested. NTC: non-template control, P: positive test containing beef sample.

The pork-specific test began to show a positive color change within 30 minutes under optimized conditions. In contrast, equine, chicken, and animal internal control assays showed only weak color changes at 45 minutes, while a clear and easily distinguishable color difference between negative and positive reaction tubes was consistently observed at 60 minutes. To provide all species-specific detections to be performed under a common reaction platform, a reaction duration of 60 minutes was selected as optimal. This duration provided reliable amplification and reproducible visual discrimination in all tests, establishing a protocol for subsequent experiments.

Finally, optimized LAMP reaction mixture ingredients for multi-LAMP analysis for pork, horse, chicken and animal internal control primers are given in Table 3.2. Reactions are carried out at 65°C for 60 minutes on heat block.

Table 3.2 Optimized multi-LAMP reaction components.

Components	Volume	Final concentration
10X NBZ Isothermal Buffer (pH 8.75)	2.5 µL	1 x
MgSO4 (100 mM)	1.5 µL	6 mM (8 mM in total)
dNTP Mix (10 mM)	3.5 µL	1.4 mM
FIP/BIP Primers (25 x)	1 µL	1.6 µM
F3/B3 Primers (25 x)	1 µL	0.2 µM
LB/ LF Primers (25 x)	1 µL	0.8 µM
Bst DNA Polymerase Large Fragment (8 U/µL)	1 µL	320 U/mL
Betaine (5 M)	3.5 µL	0.7 M
Cresol Red (5 mM)	1.25 µL	250 µM
HNB (3 mM)	0.42 µL	50 µM
DNA/ Direct LAMP Template	x µL/ 2 µL	1 ng/ -
DNase/ RNase free water	up to 25 µL	-

3.3 Sensitivity of the Optimized Method

To assess the analytical sensitivity of the developed multi-LAMP platform, serial DNA dilutions ranging from 1 ng to 0.01 pg were evaluated using the pork, horse, chicken, and animal internal control specific methods. The detection performance of each assay was determined based on the color change following amplification. The results showed that all four primer sets could detect low DNA concentrations, although their detection limits are different. Dual-colorimetric detection enhanced the visual differentiation between negative and positive reaction results, enabling weak amplification signals to be identified at low template concentrations. These findings indicate that the developed platform provides sensitive detection across all four primer sets while allowing the assays to be performed under identical reaction conditions.

3.3.1 Sensitivity of Porcine Specific Primers

The analytical sensitivity performance of the porcine-specific colorimetric LAMP assay was determined by testing a ten-fold dilution series of porcine genomic DNA, covering concentrations from 1 ng to 0.01 pg, using the optimized reaction conditions (Figure 3.11). Positive amplification was observed from 1 ng to 0.1 pg DNA, whereas 0.01 pg DNA did not produce a detectable color change and remained comparable to the negative control. Beef DNA was included as the negative control for all tested DNA concentrations, and all negative reactions remained purple, indicating the absence of non-specific amplification. Based on these results, the LOD (limit of detection) of the pork-specific LAMP method was determined to be 0.1 pg/reaction DNA, consistent with the findings reported by Özden (2024), thereby confirming the previously established detection limit under the optimized assay conditions.

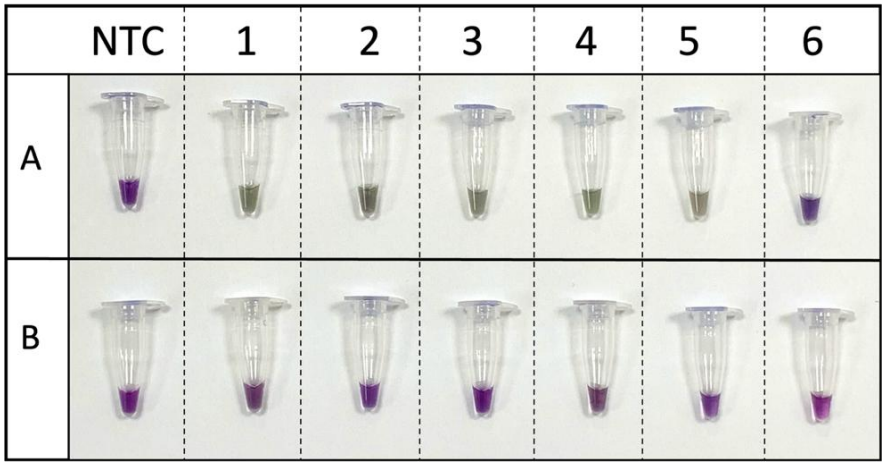

Figure 3.11 Visualization of LAMP results under the white light. Analytical sensitivity of the porcine-specific LAMP assay using DNA concentrations ranging from 1 ng to 0.01 pg. A: LAMP reactions containing porcine DNA, B: LAMP reactions containing beef DNA as a negative control. NTC: non- template control, reactions 1–6 contained 1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1 pg, and 0.01 pg DNA, respectively.

The assay produced a clear and distinguishable color transition across a broad range of DNA concentrations, demonstrating that the optimized reaction conditions maintained efficient amplification even at very low template levels. The ability to detect 0.1 pg DNA indicates a high level of analytical sensitivity, which is a characteristic advantage of LAMP-based amplification methods. These findings suggest that the developed dual colorimetric multi-LAMP method is suitable for low concentrations of pork DNA detection.

3.3.2 Sensitivity of Equine Specific Primers

The analytical sensitivity performance of the equine-specific colorimetric LAMP assay was determined by testing a ten-fold dilution series of porcine genomic DNA, covering concentrations from 1 ng to 0.01 pg, using the optimized reaction conditions (Figure 3.12). Positive amplification was observed from 1 ng to 1 pg DNA**,** whereas 0.1 pg and 0.01 pg DNA did not produce a detectable color change

and remained comparable to the negative control. Beef DNA was included as the negative control for all tested DNA concentrations, and all negative reactions remained purple, indicating the absence of non-specific amplification. Based on these results, LOD of the equine-specific colorimetric LAMP assay was determined to be 1 pg DNA.

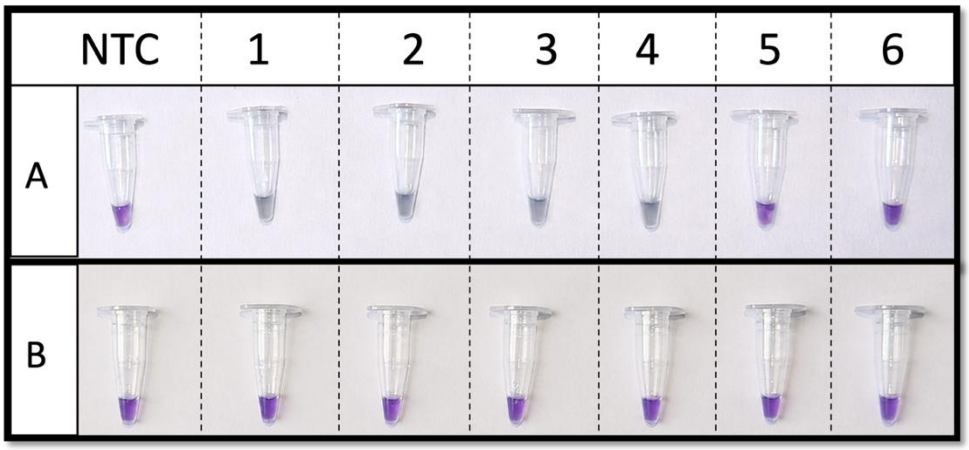

Figure 3.12 Visualization of LAMP results under the white light. Analytical sensitivity of the equine-specific LAMP assay using DNA concentrations ranging from 1 ng to 0.01 pg. A: LAMP reactions containing equine DNA, B: LAMP reactions containing beef DNA as a negative control. NTC: non- template control, reactions 1–6 contained 1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1 pg, and 0.01 pg DNA, respectively.

The assay produced a visual discrimination between negative and positive reaction results over a broad range of DNA concentrations, demonstrating that the optimized reaction conditions supported efficient amplification down to the picogram level. The equine-specific assay exhibited lower sensitivity than the porcine-specific assay. Nevertheless, its ability to detect genomic DNA at a concentration as low as 1 pg demonstrates a high degree of analytical sensitivity. Differences in detection limits among LAMP assays are commonly associated with primer characteristics, target sequence properties, and amplification efficiency under identical reaction conditions

(Park, 2022). These findings demonstrate that the optimized reaction platform is suitable for sensitive detection of equine DNA while maintaining high specificity and reliable visual colorimetric interpretation.

3.3.3 Sensitivity of Chicken Specific Primers

The analytical sensitivity of the chicken-specific colorimetric LAMP assay was evaluated using ten-fold serial dilutions of horse DNA ranging from 1 ng to 0.01 pg under the optimized reaction conditions (Figure 3.13). Positive amplification was observed from 1 ng to 1 pg DNA**,** whereas 0.1 pg and 0.01 pg DNA did not produce a detectable color change and remained comparable to the negative control. Beef DNA was included as the negative control for all tested DNA concentrations, and all negative reactions remained purple, indicating the absence of non-specific amplification. Based on these results, LOD of the chicken-specific colorimetric LAMP assay was determined to be 1 pg DNA**.**

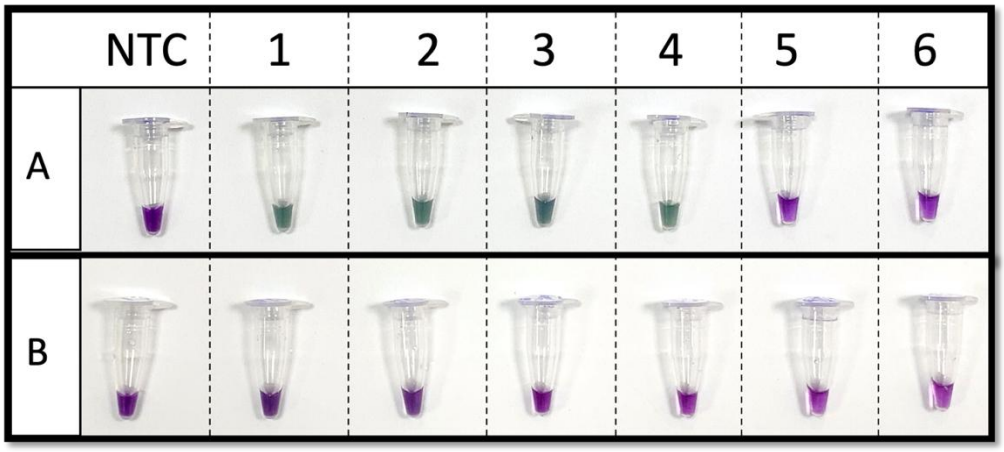

Figure 3.13 Visualization of LAMP results under the white light. Analytical sensitivity of the chicken-specific LAMP assay using DNA concentrations ranging from 1 ng to 0.01 pg. A: LAMP reactions containing chicken DNA, B: LAMP reactions containing beef DNA as a negative control. NTC: non- template control, reactions 1–6 contained 1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1 pg, and 0.01 pg DNA, respectively.

The assay produced a noticeable color difference between negative and positive reaction tubes over a broad range of DNA concentrations, indicating that the optimized reaction conditions supported efficient amplification down to the picogram level. Although the sensitivity of the chicken-specific method was lower than that observed for the pork-specific method, the ability to detect 1 pg genomic DNA still indicates a high level of analytical sensitivity. These results show that the optimized reaction system can detect chicken DNA having high specificity and reliable visual colorimetric interpretation.

3.3.4 Sensitivity of Animal Internal Control Primers

The analytical sensitivity of the animal internal control LAMP assay was evaluated using ten-fold serial dilutions of beef genomic DNA (from 1 ng to 0.01 pg) under the optimized reaction conditions (Figure 3.14). Positive amplification was observed from 1 ng to 0.01 ng DNA. However, the color change was very low for 0.1 ng and 0.01 ng because the amount of DNA was low for this primer to efficiently amplify. Moreover, 1 pg, 0.1 pg, and 0.01 pg DNA did not produce a color change and remained similar to the non-template control. Based on these results, LOD of the animal internal control LAMP method was identified as 10 pg DNA.

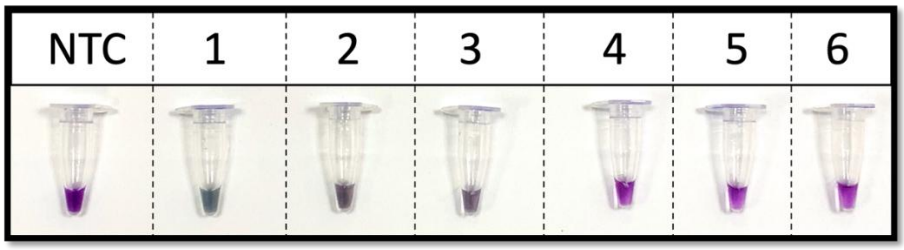

Figure 3.14 Visualization of LAMP results under the white light. Analytical sensitivity of the animal internal control LAMP assay using beef DNA concentrations ranging from 1 ng to 0.01 pg. NTC: non- template control, reactions 1–6 contained 1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1 pg, and 0.01 pg DNA, respectively.

Compared with the pork, equine and chicken-specific assays, the animal internal control assay exhibited a lower analytical sensitivity. An explanation for this observation is that the internal control primers target the 18S rRNA gene, which may display different amplification characteristics under the optimized reaction conditions. The analytical sensitivity of LAMP assays is strongly influenced by primer design, primer binding efficiency, target sequence characteristics, and amplification efficiency, and these factors can result in different detection limits even when identical reaction conditions are used (Wong et al., 2018; Park, 2022). Therefore, relatively low sensitivity observed for the internal control assay may be associated with the amplification characteristics of the 18S rRNA target region rather than a limitation of the optimized reaction platform itself.

3.4 Specificity of the Optimized Method

3.4.1 Specificity of Porcine Specific Method

The specificity of the porcine-specific direct LAMP method was assessed using raw and processed pork samples together with non-target animal species (Figure 3.15). all the samples prepared at the same reaction preparation conditions indicated in Chapter 2. Positive amplification was observed in all pork-containing samples, including 1% pork, 0.1% pork (obtained from The Republic of Turkey Ministry of Agriculture and Forestry Afyonkarahisar Food Control Laboratory), raw pork meat, pork prosciutto, and pork bacon, whereas no color change was detected in horse, chicken, beef, fish, turkey, duck, lamb, or goat samples. The non-template control also remained negative. The results confirmed that the porcine-specific primer set selectively amplified porcine DNA, with no evidence of cross-reactivity among the other animal species tested.

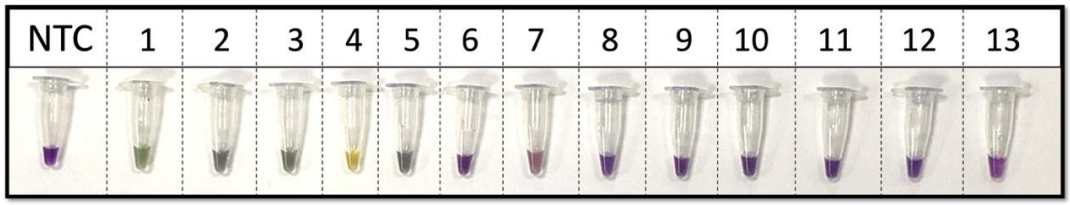

Figure 3.15 Visualization of LAMP results under the white light. Specificity test of the porcine-specific direct LAMP assay. Lane 1: 1% pork meat sample, 2: 0.1% pork meat sample, 3: raw pork meat sample, 4: pork-prosciutto, 5: pork-bacon, 6: horse meat sample, 7: chicken meat sample, 8: beef sample, 9: fish meat sample, 10: turkey meat sample, 11: duck meat sample, 12: lamb meat sample, 13: goat meat sample. NTC: non-template control.

The absence of amplification in non-target species indicates a high level of analytical specificity under the optimized reaction conditions. The ability to specifically identify pork in the presence of multiple animal species is particularly important for meat authentication and food fraud detection, where false-positive amplification could compromise the reliability of species identification assays.

3.4.2 Specificity of Equine Specific Method

The specificity of the equine-specific direct colorimetric LAMP assay was evaluated using horse meat together with pork, processed pork products, chicken, beef, fish, turkey, duck, lamb, goose, and goat samples (Figure 3.16). Positive amplification was observed only in the horse meat sample (column 4), whereas all other tested species remained negative and demonstrated no detectable color change comparable with the non-template control. These results show that the equine-specific primer set selectively amplified equine DNA with no evidence of cross-reactivity among the other tested animal species.

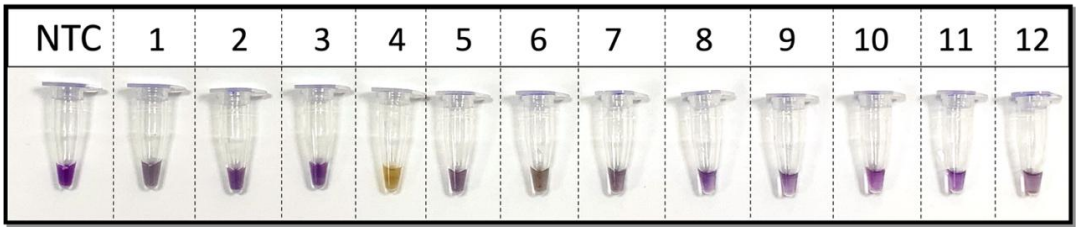

Figure 3.16 Visualization of LAMP results under the white light. Specificity test of the equine-specific direct LAMP assay. 1: pork meat sample, 2: pork-prosciutto, 3: pork-bacon, 4: horse meat sample, 5: chicken meat sample, 6: beef meat sample, 7: fish meat sample, 8: turkey meat sample, 9: duck meat sample, 10: lamb meat sample, 11: goose meat sample, 12: goat meat sample. NTC: non-template control.

The absence of amplification in pork products, and the remaining non-target species indicates that the equine-specific assay possesses high specificity under the optimized reaction conditions. This result is particularly important for meat authentication, where closely related mammalian species may increase the risk of non-specific amplification. The differentiation between the equine sample and all non-target samples indicates that the primer set can reliably detect equine DNA in meat samples.

3.4.3 Specificity of Chicken Specific Method

The specificity of the chicken-specific direct LAMP method was examined using chicken meat together with pork, processed pork products, horse, beef, fish, turkey, duck, lamb, goose, and goat samples (Figure 3.17). Positive amplification was observed only in the chicken meat sample (column 5), whereas all other tested species remained negative and demonstrated no detectable color change comparable with the non-template control. These results show that the chicken-specific primer set selectively amplified chicken DNA with no evidence of cross-reactivity among the other tested animal species.

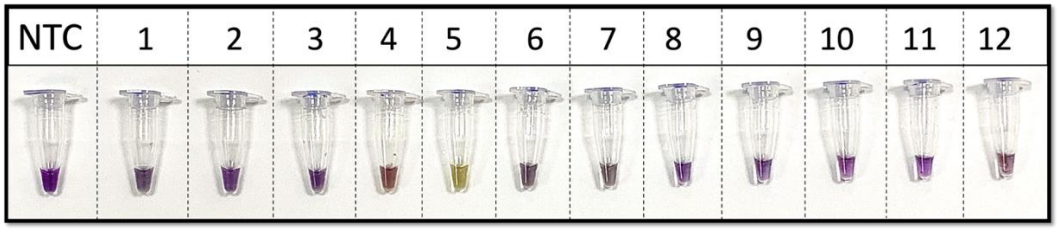

Figure 3.17 Visualization of LAMP results under the white light. Specificity test of the chicken-specific direct LAMP assay. 1: pork meat sample, 2: pork-prosciutto, 3: pork-bacon, 4: horse meat sample, 5: chicken meat sample, 6: beef meat sample, 7: fish meat sample, 8: turkey meat sample, 9: duck meat sample, 10: lamb meat sample, 11: goose meat sample, 12: goat meat sample. NTC: non-template control.

The chicken-specific assay has great specificity under the optimum reaction conditions, evidenced by the lack of amplification in horse meat, pork products, and the other non-target species. Because closely related species may raise the risk of non-specific amplification, this conclusion is especially crucial for meat authentication. The primer set seems to be appropriate for the accurate detection of chicken DNA in meat samples, according to the distinct separation between the chicken sample and all non-target samples.

3.4.4 Validation of Specificity with Animal Internal Control Method

To verify that the negative results obtained in the porcine, equine, and chicken-specific LAMP assays were due to primer specificity rather than amplification failure, all meat samples that had served as negative controls in the species-specific assays were re-evaluated using the animal internal control primer set (Figure 3.18). Positive amplification was observed in all tested animal samples, while the non-template control remained negative, confirming that amplifiable animal DNA was present in each sample.

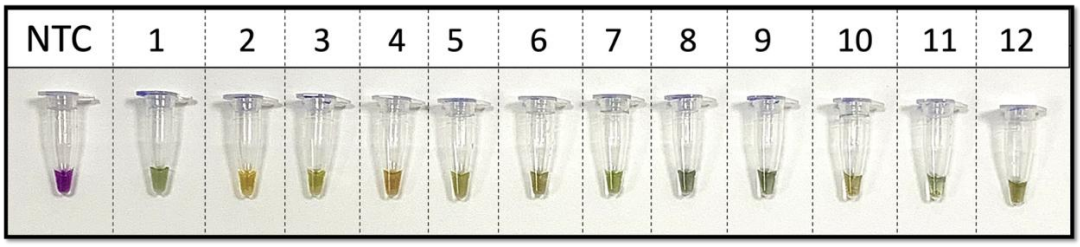

Figure 3.18 Visualization of LAMP results under the white light. Validation of the porcine-specific, equine-specific and chicken-specific direct LAMP assays using animal internal control primers. 1: pork meat sample, 2: pork-prosciutto, 3: pork-bacon, 4: horse meat sample, 5: chicken meat sample, 6: beef meat sample, 7: fish meat sample, 8: turkey meat sample, 9: duck meat sample, 10: lamb meat sample, 11: goose meat sample, 12: goat meat sample. NTC: non-template control.

The successful amplification of all samples with the animal internal control primers demonstrates that the negative reactions observed in the species-specific assays were true negatives and were not caused by poor DNA quality, reaction inhibition, or failure of the direct LAMP reaction. These results confirm that the porcine, equine, and chicken-specific primer sets did not exhibit cross-reactivity with non-target animal species and that the optimized direct colorimetric LAMP platform provides reliable species-specific detection. The inclusion of the animal internal control therefore strengthens the analytical validity of the assay by confirming that each negative sample remained amplifiable under the same reaction conditions.

3.5 Analysis of Processed Meat Samples

3.5.1 Heat Treatment Analysis of Horse Meat

To examine the performance of the direct LAMP assay with highly heat-processed samples, horse and beef meat samples were heated to 121°C for 15, 30, and 45 minutes in autoclave prior to amplification (Figure 3.19). Prepared samples were tested with equine-specific LAMP assay. Positive amplification was observed in all autoclaved horse samples, whereas the autoclaved beef samples remained negative

at all treatment times comparable with the non-template. These results demonstrate that the equine-specific direct LAMP assay successfully detected horse DNA even after thermal processing.

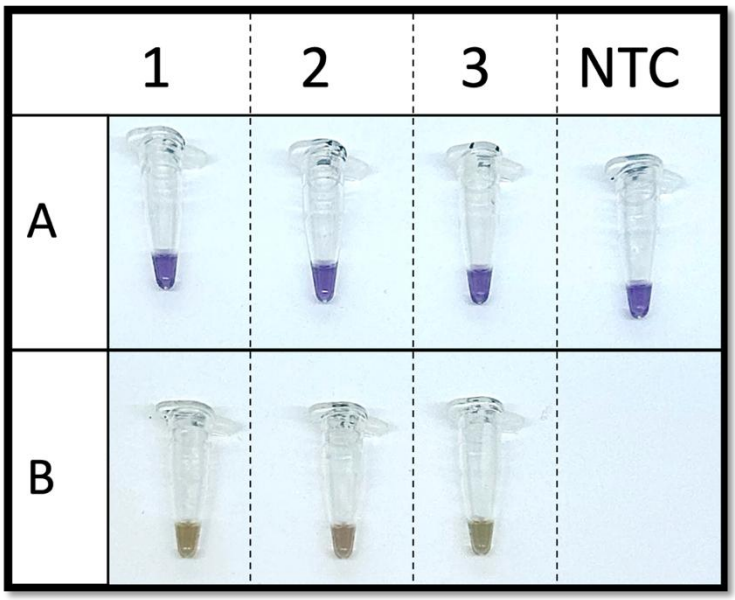

Figure 3.19 Visualization of LAMP results under the white light. Results of the direct LAMP reaction using samples autoclaved at 121°C for 15, 30, and 45 minutes. A: Negative samples containing beef. B: Positive samples containing horse meat. Lanes 1, 2, and 3 correspond to samples autoclaved for 15, 30, and 45 min, respectively. NTC: non-template control.

To investigate the effect of prolonged heat processing on the performance of the direct LAMP assay, horse and beef meat samples were heated in PBS solution at 98°C for 2, 5, 10, 15, 30, 45, and 60 min prior to amplification (Figure 3.20). Positive amplification was observed in all horse samples, the assay maintained clear visual discrimination between positive and negative reactions after heating for 2–45 min. However, the color change observed in the 60-min heated positive chicken sample was less strong than that observed at the shorter heating intervals, suggesting a DNA damage and reduction in amplification efficiency resulting from thermal exposure

longer than 60 minutes. The non-template control and all beef samples showed no color change throughout the experiment, indicating the absence of non-specific amplification.

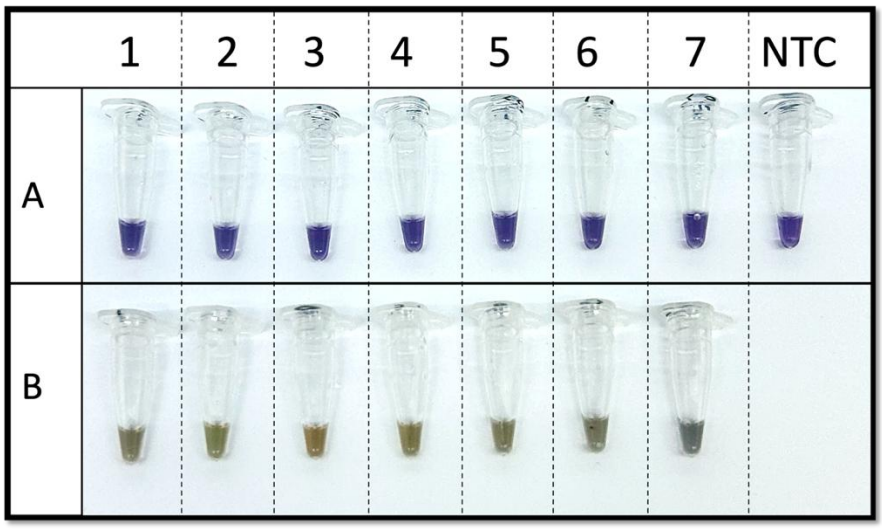

Figure 3.20 Visualization of LAMP results under the white light. Results of the direct LAMP reaction using samples heated at 98°C for different time intervals. A: Negative samples containing beef. B: Positive samples containing horse meat. Lanes 1–7 correspond to samples heated at 98°C for 2, 5, 10, 15, 30, 45, and 60 min, respectively. NTC: non-template control.

The successful amplification of horse DNA after both autoclaving at 121°C and prolonged heating at 98°C demonstrates that the developed direct colorimetric LAMP assay is highly tolerant to thermal processing. High-temperature treatment may cause DNA fragmentation and reduce the amount of intact amplifiable template. However, LAMP remains suitable for detecting short DNA targets and has been successfully applied to partially degraded samples (Notomi et al., 2000; Mori & Notomi, 2009). The absence of amplification in heat-treated beef samples further confirms that thermal processing did not compromise assay specificity. While positive amplification was maintained under all tested heating conditions, the reduced color intensity observed after 60 min at 98°C suggests that prolonged heat

exposure may decrease amplification efficiency due to partial degradation of the target DNA. Nevertheless, the positive reaction remained clearly distinguishable from the negative controls, supporting the applicability of the assay for species identification in highly heat-treated meat matrices.

3.5.2 Heat Treatment Analysis of Chicken Meat

To examine the performance of the direct LAMP assay with highly heat-processed samples, chicken and beef meat samples were heated to 121°C for 15, 30, and 45 minutes in autoclave prior to amplification (Figure 3.21). Prepared samples were tested with chicken-specific LAMP assay. Positive amplification was observed in all autoclaved chicken samples. However, the color intensity at the 45minute time point was slightly darker compared to the 15 minute and 30 minute samples, which may indicate DNA damage and a consequent decrease in amplification efficiency resulting from prolonged thermal exposure, although the sample was still clearly detected as positive. The autoclaved beef samples remained negative at all treatment times comparable with the non-template control. These results demonstrate that the chicken-specific direct LAMP assay successfully detected chicken DNA even after extensive thermal processing.

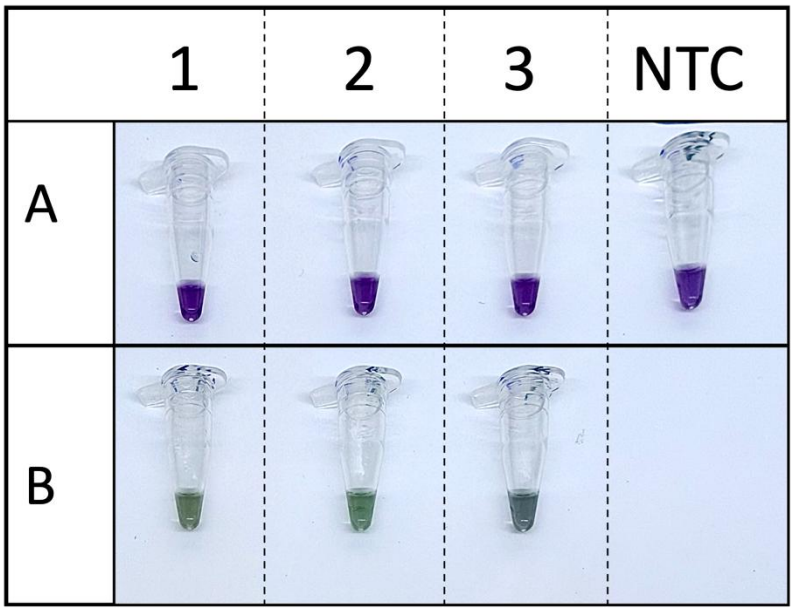

Figure 3.21 Visualization of LAMP results under the white light. Results of the direct LAMP reaction using samples autoclaved at 121°C for 15, 30, and 45 minutes. A: Negative samples containing beef. B: Positive samples containing chicken meat. Lanes 1, 2, and 3 correspond to samples autoclaved for 15, 30, and 45 min, respectively. NTC: non-template control.

To investigate the effect of prolonged heat processing on the performance of the direct LAMP assay, chicken and beef meat samples were boiled in PBS at 98°C for 2, 5, 10, 15, 30, 45, and 60 min prior to amplification (Figure 3.22). Positive amplification was observed in all chicken samples, and the assay maintained clear visual discrimination between negative and positive reaction tubes after heating for 2–45 min. However, the color change observed in the chicken sample heated for 60 min was less pronounced than that observed at shorter heating intervals, suggesting that prolonged thermal exposure may have caused increased DNA fragmentation and consequently reduced amplification efficiency. This interpretation is consistent with previous studies demonstrating that both the temperature and duration of heat treatment can significantly reduce the amount of amplifiable DNA in meat samples. Şakalar et al. (2012), for example, reported a progressive decrease in detectable mitochondrial DNA copy number in beef, pork, and chicken with increasing boiling

time and temperature. Despite this thermal degradation, DNA-based species identification methods targeting relatively short mitochondrial regions may retain their effectiveness in processed meat products. Previous LAMP studies have successfully identified the types of meat in cooked samples, including chicken and other animal species, and have shown that the LAMP can maintain its high sensitivity after heat treatment. The continuation of detectable amplification in chicken samples even after 60 minutes at 98° C indicates that the target region remains intact enough for LAMP amplification. However, weaker color development may suggest a possible decrease in reaction efficiency. The non-template control sample and all beef samples showed no color change throughout the experiment, which confirmed the absence of detectable nonspecific amplification under the tested heat treatment conditions.

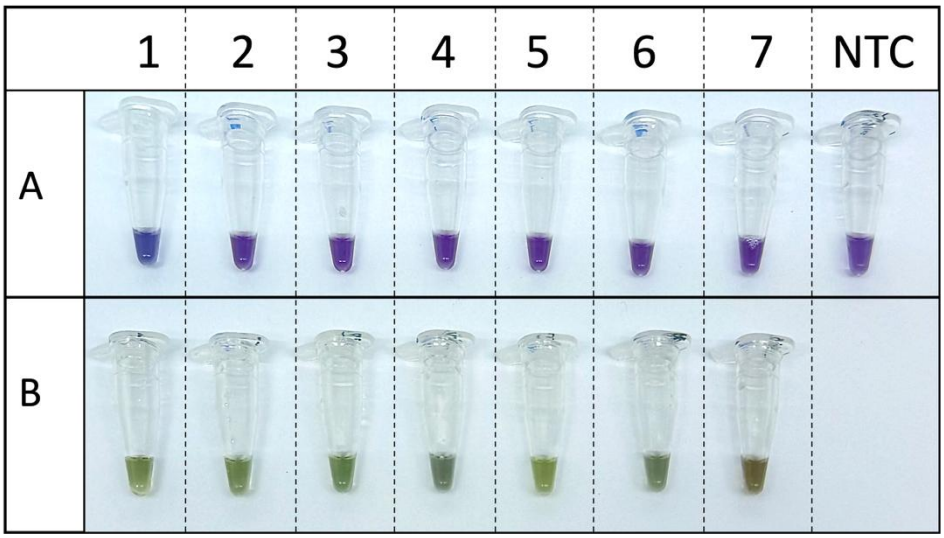

Figure 3.22 Visualization of LAMP results under the white light. Results of the direct LAMP reaction using samples heated at 98°C for different time intervals. A: Negative samples containing beef. B: Positive samples containing chicken meat. Lanes 1–7 correspond to samples heated at 98°C for 2, 5, 10, 15, 30, 45, and 60 min, respectively. NTC: non-template control.

The successful amplification of chicken DNA after both autoclaving at 121°C and prolonged heating at 98°C demonstrates that the developed direct colorimetric multi-LAMP test is highly tolerant to heat processing, similar to the performance observed for horse DNA. The absence of amplification in heat-treated beef samples further confirms that heat treatment does not affect the specificity of the test. While positive amplification was maintained under all tested heating conditions, the decreased color intensity observed after 45 minutes at 121°C and 60 minutes at 98°C suggests that prolonged heat exposure may reduce amplification efficiency due to partial degradation of the target DNA. However, the positive reaction remained clearly distinguishable from negative controls, supporting the applicability of the chicken-specific direct LAMP test for species identification in highly heat-processed meat matrices.

3.6 Shelf-Life Stability at Room Temperature

Although the manufacturer recommends a storage temperature of −20°C for Bst DNA polymerase, the fact that the cold chain is not always sustainable and that suitable temperature conditions cannot be maintained in facilities or laboratories due to technical reasons necessitates the evaluation of the stability of reaction components under alternative storage conditions. In this context, LAMP reaction mixtures for all four primer sets, including porcine, equine, chicken, and animal internal control, were prepared under the optimized reaction conditions without template DNA and stored at room temperature for 15 days to evaluate their stability under conditions relevant to reaction tube storage and transportation.

Consistent with the findings of Özden (2024), who demonstrated that species-specific LAMP reaction mixtures remained stable for up to 14 days when stored at room temperature, the storage period in the present study was extended to 15 days. For this purpose, reaction mixtures prepared under the same optimized conditions using the porcine, equine, and chicken specific primer sets and the animal internal control primer set were stored at room temperature and evaluated by direct LAMP

at 3-day intervals. For each species-specific assay, the corresponding meat sample was used as the positive control, beef meat was included as the negative control, and nuclease-free water was used as the non-template control (NTC). For the animal internal control primer set, beef sample was used as a positive control, and NTC were included in reactions. The results are presented in Figures 3.23–3.26.

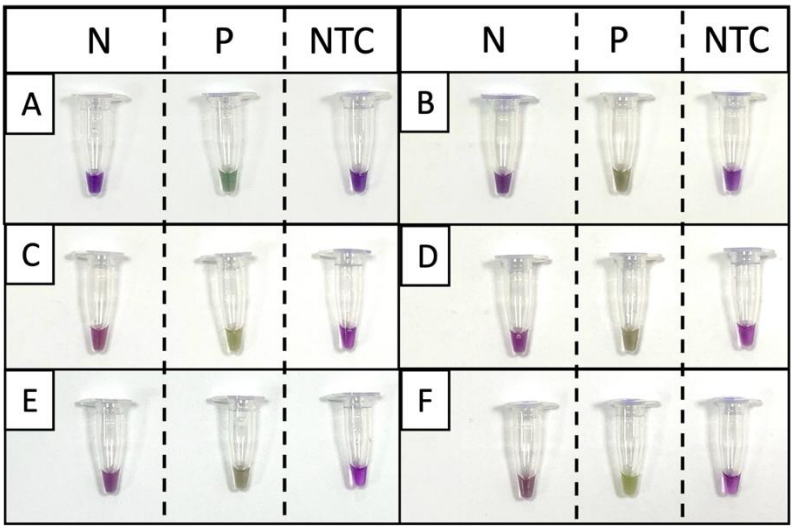

Figure 3.23 Visualization of LAMP results under white light during the shelf-life study at room temperature for porcine-specific method. A: day zero, B: day three, C: day six, D: day nine, E: day twelve, and F: day fifteen. N: beef meat sample (negative control), P: 1% pork meat sample (positive sample), NTC: non-template control.

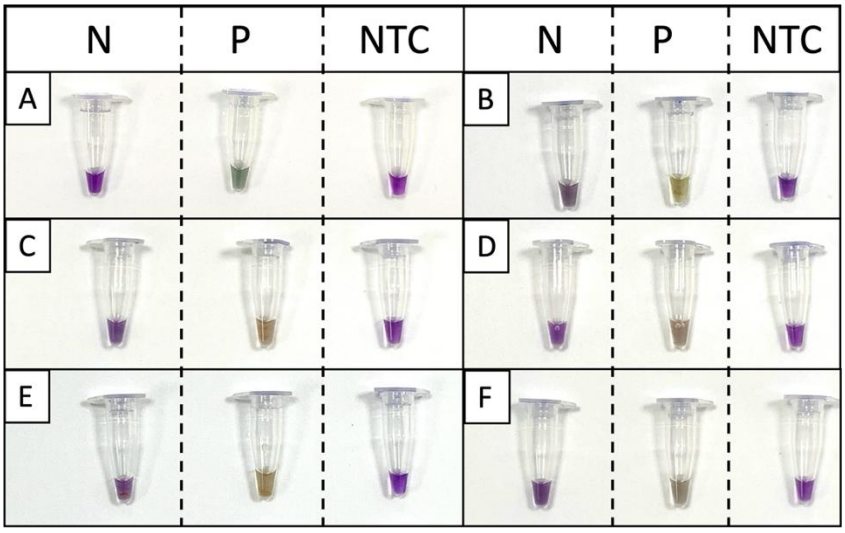

Figure 3.24 Visualization of LAMP results under white light during the shelf-life study at room temperature for equine-specific method. A: day zero, B: day three, C: day six, D: day nine, E: day twelve, and F: day fifteen. N: beef meat sample (negative control), P: horse meat sample (positive sample), NTC: non-template control.

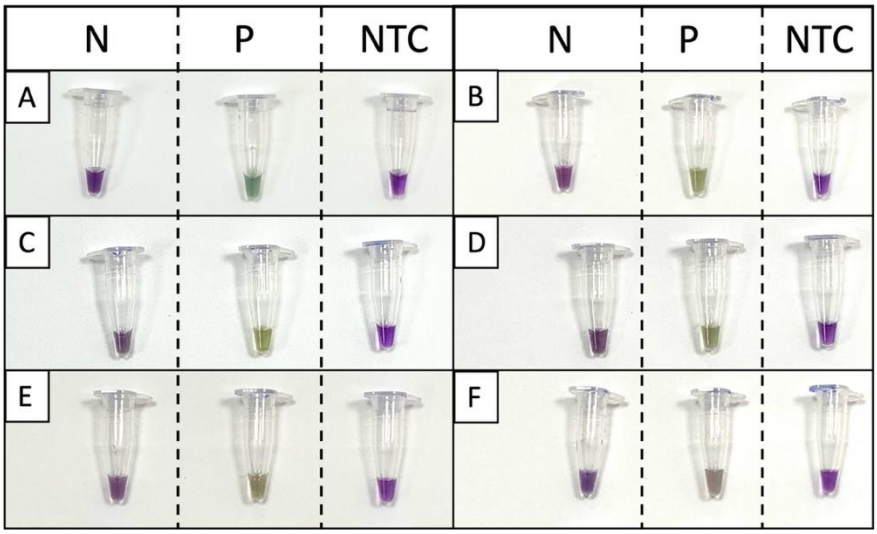

Figure 3.25 Visualization of LAMP results under white light during the shelf-life study at room temperature for chicken-specific method. A: day zero, B: day three, C: day six, D: day nine, E: day twelve, and F: day fifteen. N: beef meat sample (negative control), P: chicken meat sample (positive sample), NTC: non-template control.

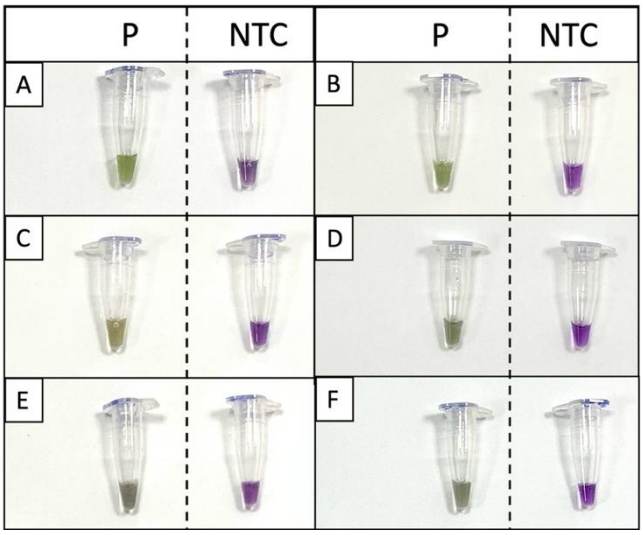

Figure 3.26 Visualization of LAMP results under white light during the shelf-life study at room temperature for animal internal control test. A: day zero, B: day three, C: day six, D: day nine, E: day twelve, and F: day fifteen. P: beef meat sample (positive sample), NTC: non-template control.

The overall evaluation of the storage stability results demonstrated that the optimized direct multi-LAMP platform maintained its amplification performance for up to 15 days when the reaction mixtures were stored at room temperature. Positive reactions remained distinguishable from the corresponding negative controls throughout the storage period, indicating that the Bst DNA polymerase and primer sets retained sufficient activity to support amplification after prolonged storage under non-refrigerated conditions. Although a gradual decrease in color intensity and a slight reduction in the contrast between positive and negative reactions were observed over time, the color changes remained visually detectable for all tests, allowing the amplification results to be interpreted reliably. The negative and non-template controls showed no evidence of non-specific amplification, supporting the stability of the reaction mixtures during storage. Overall, room-temperature storage for up to 15 days did not quite affect the functionality of the optimized reaction components. Therefore, the developed platform may offer practical advantages for applications where continuous cold-chain storage is difficult to maintain, while also simplifying the storage and transportation of pre-prepared LAMP reaction tubes. However, the

gradual decrease in color intensity suggests that longer storage times at room temperature may negatively affect visual discrimination. Thus, experimental testing is required before extending room temperature storage above 15 days.

CHAPTER 4

CONCLUSION

This study aimed to develop a multiplex LAMP system based on colorimetric identification that can simultaneously detect pork, chicken, and horse meat from meat samples. The assay conditions were systematically optimized, including the reaction buffer, $MgSO_4$ concentration, reaction temperature, and reaction time. Following the evaluation of the NBZ 10X Isothermal buffer at different pH values, a buffer pH of 8.75 and an $MgSO_4$ concentration of 6 mM were selected as the common reaction conditions. A reaction temperature of 65°C and a reaction time of 60 minutes were subsequently adopted to provide sufficient amplification for all target assays. The establishment of common reaction conditions for the porcine, equine, and chicken-specific assays provides a suitable foundation for the development of a multi-LAMP platform enabling the simultaneous detection of multiple meat species.

A dual-colorimetric detection system based on cresol red and hydroxy naphthol blue (HNB) was incorporated into the assay to enable visual interpretation of amplification results without specialized detection equipment. A final concentration of 250 μM cresol red and 50 μM HNB was selected as the optimal dye combination. The use of pH-responsive and metal-ion-responsive dyes together enabled amplification to be assessed directly through a visible color change, supporting the development of a simple and equipment-independent detection strategy (Zhang et al., 2022). This feature is particularly advantageous for direct LAMP, where rapid visual interpretation can further simplify the overall workflow.

The direct LAMP approach also eliminated the requirement for conventional DNA extraction prior to amplification. Instead, a small meat sample was directly treated, and the resulting supernatant was used as the amplification template. Direct

amplification strategies have previously been demonstrated to reduce sample-processing steps, analysis time, and assay cost in meat species identification (Lee et al., 2016). In the present study, combining direct sample preparation with colorimetric detection further reduced the complexity of the assay and supports its potential use as a rapid screening method.

The specificity experiments demonstrated that the porcine, equine and chicken-specific assays selectively amplified their corresponding target species without detectable amplification in the other tested ten meat species. The animal internal control primers were additionally used to confirm the suitability of the meat samples and the amplification process, providing an additional level of assay validation. The sensitivity experiments further demonstrated the potential of the system for detecting low-level species adulteration, which is particularly relevant to the detection of economically motivated substitution in meat products.

The applicability of the assay to processed meat samples was also investigated. Target-specific amplification was retained following heat treatment at 98°C for different durations and after autoclaving at 121°C for 15, 30, and 45 min. These findings demonstrate that the direct LAMP assay can retain its detection capability despite the DNA degradation associated with thermal processing and therefore has potential for application to processed meat products.

An important value of the developed system is its potential to provide a common LAMP-based framework for the detection of multiple meat species. Commercial meat authentication solutions currently include multiplex real-time PCR platforms capable of detecting several animal species within a single testing workflow, such as commercially available kits validated for the simultaneous detection of beef, horse, and pork, or pork, chicken, and turkey, each requiring a multi-channel real-time PCR instrument and an internal amplification control to confirm result validity (R-Biopharm AG, 2026a; R-Biopharm AG, 2026b). These platforms demonstrate that multi-species screening is already a recognized laboratory need, but their

dependence on fluorescence-based real-time instrumentation keeps them tied to well-equipped laboratories rather than field or point-of-need settings.

Dual-dye colorimetric strategies such as the HNB and cresol red combination have so far been developed and validated for single-target applications, including viral RNA detection and food allergen screening, rather than for a shared framework spanning multiple animal species (Zhang et al., 2022; Hongjaisee et al., 2023). To the extent that dual-colorimetric LAMP has not previously been established as a common, standardized platform across pork, horse, and chicken detection, the present approach combines species-specific LAMP detection with direct sample preparation and dual-colorimetric visual detection, providing a simpler alternative that does not require conventional DNA extraction or real-time fluorescence instrumentation. The combination of these features gives the developed platform particular potential for rapid and accessible meat species authentication and food fraud screening.

As a continuation of this work, the sensitivity of the complete multi-species system will be further evaluated using defined meat mixtures containing different percentages of target species. The internal control used in previous direct LAMP analyses for individual species, such as the 18S rRNA target, is designed to verify the presence of the template. However, to support the accuracy of the method in species-specific sensitivity measurements, a higher-sensitivity animal-universal internal control primer set will be designed. A more sensitive endogenous control will enable verification of templates with a low copy count or partially corrupted templates with higher confidence and will further reduce the risk of false negative results in all three target types (Lee et al., 2016). In addition, extended shelf-life studies will be performed under different storage conditions and for longer storage periods. Previous work from the same research group demonstrated the stability of the colorimetric direct LAMP system under selected storage conditions. The present study will therefore extend these investigations to evaluate the stability of the developed multi-species platform over longer periods. Finally, integration of the optimized assays into a microfluidic platform represents an important next step

toward simultaneous analysis of multiple meat species in a single device. Such integration could ultimately provide a compact, rapid, and visually interpretable platform for on-site meat authentication and food fraud detection.

REFERENCES

Abbas, O., Zadravec, M., Baeten, V., Mikuš, T., Lešić, T., Vulić, A., Prpić, J., Jemeršić, L., & Pleadin, J. (2018). Analytical methods used for the authentication of food of animal origin. *Food Chemistry*, *246*, 6–17. https://doi.org/10.1016/j.foodchem.2017.11.007

Ali, Md. E., Razzak, Md. A., Hamid, S. B., Rahman, Md. M., Amin, Md. A., Rashid, N. R., & Asing. (2015). Multiplex PCR assay for the detection of five meat species forbidden in Islamic Foods. *Food Chemistry*, *177*, 214–224. https://doi.org/10.1016/j.foodchem.2014.12.098

Ballin, N. Z. (2010). Authentication of meat and meat products. *Meat Science*, *86*(3), 577–587. https://doi.org/10.1016/j.meatsci.2010.06.001

Basanisi, M. G., La Bella, G., Nobili, G., Coppola, R., Damato, A. M., Cafiero, M. A., & La Salandra, G. (2020). Application of the novel droplet digital PCR technology for identification of meat species. *International Journal of Food Science and Technology*, *55*(3), 1145–1150. https://doi.org/10.1111/ijfs.14486

Becherer, L., Borst, N., Bakheit, M., Frischmann, S., Zengerle, R., & von Stetten, F. (2020). Loop-mediated isothermal amplification (LAMP) – review and classification of methods for sequence-specific detection. *Analytical Methods*, *12*(6), 717–746. https://doi.org/10.1039/c9ay02246e

Boyacı, İ. H., Temiz, H. T., Uysal, R. S., Velioğlu, H. M., Yadegari, R. J., & Rishkan, M. M. (2014). A novel method for discrimination of beef and horsemeat using Raman spectroscopy. *Food Chemistry*, *148*, 37–41. https://doi.org/10.1016/j.foodchem.2013.10.006

Brooks, S., Elliott, C. T., Spence, M., Walsh, C., & Dean, M. (2017). Four Years post-horsegate: An update of measures and actions put in place following the Horsemeat Incident of 2013. *Npj Science of Food*, *1*(1). https://doi.org/10.1038/s41538-017-0007-z

Calvo, J. H., Zaragoza, P., & Osta, R. (2001). Random amplified polymorphic DNA fingerprints for identification of species in poultry pâté. *Poultry Science*, *80*(4), 522–524. https://doi.org/10.1093/ps/80.4.522

Candoğan, K., Deniz, E., Güneş Altuntaş, E., İğci, N., & Özel Demiralp, D. (2020). Detection of pork, horse or donkey meat adulteration in beef-based formulations by Fourier transform infrared spectroscopy. *Gıda*, *45*(2), 369–379. https://doi.org/10.15237/gida.gd19146

Cavin, C., Cottenet, G., Cooper, K. M., & Zbinden, P. (2018). Meat vulnerabilities to economic food adulteration require new Analytical Solutions. *CHIMIA*, *72*(10), 697–703. https://doi.org/10.2533/chimia.2018.697

Chatterjee, R., & Sulabh, S. (2026). Naked eye meets nucleic acids: A review on dyes for isothermal amplification methods for detection of meat adulteration. *Journal of Fluorescence*, *36*(2), 1485–1497. https://doi.org/10.1007/s10895-025-04647-7

Chia, S., Teo, G., Tay, S. J., Loo, L. S., Wan, C., Sim, L. C., Yu, H., Walsh, I., & Pang, K. T. (2022). An integrative glycomic approach for quantitative meat species profiling. *Foods*, *11*(13), 1952. https://doi.org/10.3390/foods11131952

Cho, A.-R., Dong, H.-J., & Cho, S. (2014). Meat species identification using loop-mediated isothermal amplification assay targeting species-specific mitochondrial DNA. Korean Journal for Food Science of Animal Resources, 34(6), 799–807. https://doi.org/10.5851/kosfa.2014.34.6.799

Chou, C.-C., Lin, S.-P., Lee, K.-M., Hsu, C.-T., Vickroy, T. W., & Zen, J.-M. (2007). Fast differentiation of meats from fifteen animal species by liquid chromatography with electrochemical detection using copper nanoparticle plated electrodes. *Journal of Chromatography B*, *846*(1–2), 230–239. https://doi.org/10.1016/j.jchromb.2006.09.006

Cottenet, G., Sonnard, V., Blancpain, C., Ho, H. Z., Leong, H. L., & Chuah, P. F. (2016). A DNA macro-array to simultaneously identify 32 meat species in food samples. *Food Control*, *67*, 135–143. https://doi.org/10.1016/j.foodcont.2016.02.042

Ebbehøj, K. F., & Thomsen, P. D. (1991). Species differentiation of heated meat products by DNA Hybridization. *Meat Science*, *30*(3), 221–234. https://doi.org/10.1016/0309-1740(91)90068-2

European Parliament & Council of the European Union. (2011, October 25). Regulation (EU) No 1169/2011 of the European Parliament and of the Council on the provision of food information to consumers. https://eur-lex.europa.eu/eli/reg/2011/1169/oj/eng

Fischbach, J., Xander, N. C., Frohme, M., & Glökler, J. F. (2015). Shining a light on lamp assays — a comparison of lamp visualization methods including the novel use of Berberine. *BioTechniques*, *58*(4), 189–194. https://doi.org/10.2144/000114275

Gıda, Tarım ve Hayvancılık Bakanlığı. (2011, December 27). *Hayvansal gıdalar için özel hijyen kuralları yönetmeliği* (Official Gazette of the Republic of Turkiye, No. 28155). https://resmigazete.gov.tr/eskiler/2011/12/20111227-10.htm

Giovannacci, I., Guizard, C., Carlier, M., Duval, V., Martin, J.-L., & Demeulemester, C. (2004). Species identification of meat products by Elisa. *International*

Journal of Food Science and Technology, *39*(8), 863–867. https://doi.org/10.1111/j.1365-2621.2004.00859.x

Giusti, A., Malloggi, C., Magagna, G., Filipello, V., & Armani, A. (2023). Is the metabarcoding ripe enough to be applied to the authentication of Foodstuff of animal origin? A systematic review. *Comprehensive Reviews in Food Science and Food Safety*, *23*(1). https://doi.org/10.1111/1541-4337.13256

Goto, M., Honda, E., Ogura, A., Nomoto, A., & Hanaki, K.-I. (2009). Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *BioTechniques*, *46*(3), 167–172. https://doi.org/10.2144/000113072

Hartati, Y. W., Irkham, I., Sumiati, I., Wyantuti, S., Gaffar, S., Zakiyyah, S. N., Zein, M. I., & Ozsoz, M. (2023). The optimization of a label-free electrochemical DNA biosensor for detection of sus scrofa mtdna as food adulterations. *Biosensors*, *13*(6), 657. https://doi.org/10.3390/bios13060657

Hellberg, R. S., Hernandez, B. C., & Hernandez, E. L. (2017). Identification of meat and poultry species in food products using DNA barcoding. *Food Control*, *80*, 23–28. https://doi.org/10.1016/j.foodcont.2017.04.025

Hilger, C., Kohnen, M., Grigioni, F., Lehners, C., & Hentges, F. (1997). Allergic cross-reactions between cat and pig serum albumin. *Allergy*, *52*(2), 179–187. https://doi.org/10.1111/j.1398-9995.1997.tb00972.x

Hird, H., Chisholm, J., Sanchez, A., Hernandez, M., Goodier, R., Schneede, K., Boltz, C., & Popping, B. (2006). Effect of heat and pressure processing on DNA fragmentation and implications for the detection of meat using a real-time polymerase chain reaction. Food Additives and Contaminants, 23(7), 645–650. https://doi.org/10.1080/02652030600603041

Hitchcock, C. H. S., & Crimes, A. A. (1985). Methodology for meat species identification: A review. *Meat Science*, *15*(4), 215–224. https://doi.org/10.1016/0309-1740(85)90077-4

Hongjaisee, S., Kham-Kjing, N., Musikul, P., Daengkaokhew, W., Kongson, N., Guntala, R., Jaiyapan, N., Kline, E., Panpradist, N., Ngo-Giang-Huong, N., & Khamduang, W. (2023). A single-tube colorimetric loop-mediated isothermal amplification for rapid detection of SARS-COV-2 RNA. Diagnostics, 13(19), 3040. https://doi.org/10.3390/diagnostics13193040

Huang, T., Li, L., Liu, X., Chen, Q., Fang, X., Kong, J., Draz, M. S., & Cao, H. (2020). Loop-mediated isothermal amplification technique: Principle, development and wide application in Food Safety. Analytical Methods, 12(46), 5551–5561. https://doi.org/10.1039/d0ay01768j

Jawla, J., Kumar, R. R., Mendiratta, S. K., Agarwal, R. K., Singh, P., Saxena, V., Kumari, S., Boby, N., Kumar, D., & Rana, P. (2021). On-site paper-based loop-mediated isothermal amplification coupled lateral flow assay for pig tissue identification targeting mitochondrial CO I gene. *Journal of Food Composition and Analysis*, *102*, 104036. https://doi.org/10.1016/j.jfca.2021.104036

Kaneko, H., Kawana, T., Fukushima, E., & Suzutani, T. (2007). Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. *Journal of Biochemical and Biophysical Methods*, *70*(3), 499–501. https://doi.org/10.1016/j.jbbm.2006.08.008

Kim, M.-J., & Kim, H.-Y. (2019). A fast multiplex real-time PCR assay for simultaneous detection of pork, chicken, and beef in commercial processed meat products. LWT, 114, 108390. https://doi.org/10.1016/j.lwt.2019.108390

Kitpipit, T., Sittichan, K., & Thanakiatkrai, P. (2014). Direct-multiplex PCR assay for meat species identification in food products. *Food Chemistry*, *163*, 77–82. https://doi.org/10.1016/j.foodchem.2014.04.062

Koh, M. C., Lim, C. H., Chua, S. B., Chew, S. T., & Phang, S. T. W. (1998). Random amplified polymorphic DNA (RAPD) fingerprints for identification of red meat animal species. Meat Science, 48(3–4), 275–285. https://doi.org/10.1016/s0309-1740(97)00104-6

Kumar, Y., Bansal, S., & Jaiswal, P. (2017). Loop-mediated isothermal amplification (LAMP): A rapid and sensitive tool for quality assessment of Meat Products. Comprehensive Reviews in Food Science and Food Safety, 16(6), 1359–1378. https://doi.org/10.1111/1541-4337.12309

Lee, S. Y., Kim, M. J., Hong, Y., & Kim, H. Y. (2016). Development of a rapid on site detection method for pork in processed meat products using real-time loop-mediated isothermal amplification. Food Control, 66, 53–61. https://doi.org/10.1016/j.foodcont.2016.01.041

Manchester, K. L. (1996). Use of UV methods for measurement of protein and nucleic acid concentrations. *BioTechniques*, *20*(6), 968–970. https://doi.org/10.2144/96206bm05

Markoulatos, P., Siafakas, N., & Moncany, M. (2002). Multiplex polymerase chain reaction: A practical approach. Journal of Clinical Laboratory Analysis, 16(1), 47–51. https://doi.org/10.1002/jcla.2058

Martuscelli, M., Serio, A., Capezio, O., & Mastrocola, D. (2020). Safety, quality and analytical authentication of ḥalāl meat products, with particular emphasis on Salami: A review. *Foods*, *9*(8), 1111. https://doi.org/10.3390/foods9081111

Mee, P. T., Wong, S., O'Riley, K. J., da Conceição, F., Bendita da Costa Jong, J., Phillips, D. E., Rodoni, B. C., Rawlin, G. T., & Lynch, S. E. (2020). Field

verification of an African swine fever virus loop-mediated isothermal amplification (LAMP) assay during an outbreak in Timor-leste. *Viruses*, *12*(12), 1444. https://doi.org/10.3390/v12121444

Meyer, R., Höfelein, C., Lüthy, J., & Candrian, U. (1995). Polymerase chain reaction-restriction fragment length polymorphism analysis: A simple method for species identification in food. Journal of AOAC Internatıonal, 78(6), 1542-1551. https://doi.org/10.1093/jaoac/78.6.1542

Moehling, T. J., Choi, G., Dugan, L. C., Salit, M., & Meagher, R. J. (2021). Lamp diagnostics at the point-of-care: Emerging trends and perspectives for the developer Community. *Expert Review of Molecular Diagnostics*, *21*(1), 43–61. https://doi.org/10.1080/14737159.2021.1873769

Montowska, M., & Pospiech, E. (2007). Species identification of meat by electrophoretic methods. *Acta Scientiarum Polonorum:* Technologia Alimentaria, 06(1), 5-16.

Moon, Y.-J., Lee, S.-Y., & Oh, S.-W. (2022). A review of isothermal amplification methods and food-origin inhibitors against detecting food-borne pathogens. *Foods*, *11*(3), 322. https://doi.org/10.3390/foods11030322

Mori, Y., & Notomi, T. (2009). Loop-mediated isothermal amplification (LAMP): A rapid, accurate, and cost-effective diagnostic method for infectious diseases. *Journal of Infection and Chemotherapy*, *15*(2), 62–69. https://doi.org/10.1007/s10156-009-0669-9

Mori, Y., Kitao, M., Tomita, N., & Notomi, T. (2004). Real-time turbidimetry of lamp reaction for quantifying template DNA. *Journal of Biochemical and Biophysical Methods*, *59*(2), 145–157. https://doi.org/10.1016/j.jbbm.2003.12.005

Nagamine, K., Hase, T., & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. Molecular and Cellular Probes, 16(3), 223–229. https://doi.org/10.1006/mcpr.2002.0415

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, *28*(12). https://doi.org/10.1093/nar/28.12.e63

Nwe, M. K., Jangpromma, N., & Taemaitree, L. (2024). Evaluation of molecular inhibitors of loop-mediated isothermal amplification (LAMP). *Scientific Reports*, *14*(1). https://doi.org/10.1038/s41598-024-55241-z

Özden, D. (2024). *Development of a loop mediated isothermal amplification (LAMP) assay for identification of meat species* [Master's thesis, Middle East Technical University]. METU Open. https://hdl.handle.net/11511/108218

Pang, B., Yao, S., Xu, K., Wang, J., Song, X., Mu, Y., Zhao, C., & Li, J. (2019). A novel visual-mixed-dye for lamp and its application in the detection of foodborne pathogens. *Analytical Biochemistry*, *574*, 1–6. https://doi.org/10.1016/j.ab.2019.03.002

Park, J.-W. (2022). Principles and applications of loop-mediated isothermal amplification to point-of-care tests. *Biosensors*, *12*(10), 857. https://doi.org/10.3390/bios12100857

Rahmati, S., Julkapli, N. M., Yehye, W. A., & Basirun, W. J. (2016). Identification of meat origin in food products–A review. *Food Control*, *68*, 379–390. https://doi.org/10.1016/j.foodcont.2016.04.013

Ren, J., Deng, T., Huang, W., Chen, Y., & Ge, Y. (2017). A digital PCR method for identifying and quantifying adulteration of meat species in raw and processed food. PLoS One, 12(3). https://doi.org/10.1371/journal.pone.0173567

Roy, S., Rahman, I. A., Santos, J. H., & Ahmed, M. U. (2016). Meat species identification using DNA-redox electrostatic interactions and non-specific adsorption on graphene biochips. *Food Control*, *61*, 70–78. https://doi.org/10.1016/j.foodcont.2015.09.029

Şakalar, E., Abasiyanik, M. F., Bektik, E., & Tayyrov, A. (2012). Effect of heat processing on DNA quantification of meat species. Journal of Food Science, 77(9). https://doi.org/10.1111/j.1750-3841.2012.02853.x

Sul, S., Kim, M.-J., & Kim, H.-Y. (2019). Development of a direct loop-mediated isothermal amplification (LAMP) assay for rapid and simple on-site detection of chicken in processed meat products. *Food Control*, *98*, 194–199. https://doi.org/10.1016/j.foodcont.2018.11.025

Tanner, N. A., Zhang, Y., & Evans, T. C. (2015). Visual detection of isothermal nucleic acid amplification using ph-sensitive dyes. *BioTechniques*, *58*(2), 59–68. https://doi.org/10.2144/000114253

Tasrip, N. A., Mohd Desa, M. N., Khairil Mokhtar, N. F., Sajali, N., Mohd Hashim, A., Ali, Md. E., & Kqueen, C. Y. (2021). Rapid porcine detection in gelatin-based highly processed products using loop mediated isothermal amplification. *Journal of Food Science and Technology*, *58*(12), 4504–4513. https://doi.org/10.1007/s13197-020-04932-2

Thangsunan, P., Temisak, S., Jaimalai, T., Rios-Solis, L., & Suree, N. (2022). Sensitive detection of chicken meat in commercial processed food products based on one-step colourimetric loop-mediated isothermal amplification. *Food Analytical Methods*, *15*(5), 1341–1355. https://doi.org/10.1007/s12161-021-02210-1

Tomita, N., Mori, Y., Kanda, H., & Notomi, T. (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of

products. *Nature Protocols*, *3*(5), 877–882. https://doi.org/10.1038/nprot.2008.57

Tomlinson, J. A., Barker, I., & Boonham, N. (2007). Faster, simpler, more-specific methods for improved molecular detection of *phytophthora ramorum* in the field. *Applied and Environmental Microbiology*, *73*(12), 4040–4047. https://doi.org/10.1128/aem.00161-07

U.S. Food and Drug Administration. (2025, December 9). FDA Food Code. https://www.fda.gov/food/retail-food-protection/fda-food-code

Wang, J., Wan, Y., Chen, G., Liang, H., Ding, S., Shang, K., Li, M., Dong, J., Du, F., Cui, X., & Tang, Z. (2019). Colorimetric detection of horse meat based on loop-mediated isothermal amplification (LAMP). Food Analytical Methods, 12(11), 2535–2541. https://doi.org/10.1007/s12161-019-01590-9

Wang, S., Song, H., Wang, T., Xue, H., Fei, Y., & Xiong, X. (2025). Recent advancements with loop-mediated isothermal amplification (LAMP) in assessment of the species authenticity with meat and seafood products. *Critical Reviews in Food Science and Nutrition*, *65*(12), 2214–2235. https://doi.org/10.1080/10408398.2024.2329979

Wilfinger, W. W., Mackey, K., & Chomczynski, P. (1997). Effect of ph and ionic strength on the spectrophotometric assessment of Nucleic Acid Purity. *BioTechniques*, *22*(3), 474–481. https://doi.org/10.2144/97223st01

Wong, Y.-P., Othman, S., Lau, Y.-L., Radu, S., & Chee, H.-Y. (2018). Loop-mediated isothermal amplification (LAMP): A versatile technique for detection of micro-organisms. *Journal of Applied Microbiology*, *124*(3), 626–643. https://doi.org/10.1111/jam.13647

Xiao, B., Zhao, R., Wang, N., Zhang, J., Sun, X., Huang, F., & Chen, A. (2023). Integrating microneedle DNA extraction to hand-held microfluidic

colorimetric lamp chip system for meat adulteration detection. *Food Chemistry*, *411*, 135508. https://doi.org/10.1016/j.foodchem.2023.135508

Xing, R.-R., Wang, N., Hu, R.-R., Zhang, J.-K., Han, J.-X., & Chen, Y. (2019). Application of next generation sequencing for species identification in meat and poultry products: A DNA metabarcoding approach. *Food Control*, *101*, 173–179. https://doi.org/10.1016/j.foodcont.2019.02.034

Zahradnik, C., Martzy, R., Mach, R. L., Krska, R., Farnleitner, A. H., & Brunner, K. (2014). Loop-mediated isothermal amplification (LAMP) for the detection of horse meat in meat and processed meat products. *Food Analytical Methods*, *8*(6), 1576–1581. https://doi.org/10.1007/s12161-014-0072-8

Zhang, F., Gao, C., Bai, L., Chen, Y., Liang, S., Lv, X., Sun, J., & Wang, S. (2022). Dual-color blending based visual LAMP for food allergen detection: A strategy with enlarged color variation range and contrast. Food Chemistry: X, 13, 100201. https://doi.org/10.1016/J.FOCHX.2021.100201

Zhang, H., Cao, W., Zhang, Y., Chang, Y., Huang, H., Wei, T., Wu, J., Ye, L., & Shi, L. (2023). Identification for meat adulteration (pork, beef, sheep and duck) in foodstuff by microfluidic chip-based real-time fluorescent lamp. *Journal of Food Composition and Analysis*, *119*, 105223. https://doi.org/10.1016/j.jfca.2023.105223

Zhou, S., Zhong, G., Zhou, H., Zhang, X., Zeng, X., Wu, Z., Pan, D., He, J., Cai, Z., & Liu, Q. (2022). A heptaplex PCR assay for molecular traceability of species origin with high efficiency and practicality in both raw and heat processing meat materials. *Frontiers in Nutrition*, *9*. https://doi.org/10.3389/fnut.2022.890537

Zia, Q., Alawami, M., Mokhtar, N. F. K., Nhari, R. M. H. R., & Hanish, I. (2020). Current analytical methods for porcine identification in meat and meat

products. Food Chemistry, 324. https://doi.org/10.1016/j.foodchem.2020.126664

Zvereva, E. A., Popravko, D. S., Hendrickson, O. D., Vostrikova, N. L., Chernukha, I. M., Dzantiev, B. B., & Zherdev, A. V. (2020). Lateral flow immunoassay to detect the addition of beef, pork, lamb, and horse muscles in raw meat mixtures and finished meat products. Foods, 9(11), 1662. https://doi.org/10.3390/foods9111662

APPENDICES

A. Buffer and Solution Preparations

Tris-EDTA (TE) Buffer:

Table A.1 TE buffer preparation.

Chemicals	Final concentrations
pH 8.0 Tris	10 mM
EDTA	1 mM

The components were thoroughly mixed, followed by the addition of nuclease-free distilled water to a final volume of 100 ml.

Betaine:

Table A.2 Betaine preparation

Chemicals	Final concentrations
Betaine	5 M

The components were thoroughly mixed, followed by the addition of nuclease-free distilled water to a final volume of 10 ml. The resulting solution was subsequently filtered using a 0.2 μm membrane filter.

Lysis Buffer:

Table A.3 Lysis buffer preparation

Chemicals	Final concentrations
pH 8.0 Tris-HCl	10 mM
pH 8.0 EDTA	2 mM
NaCl	400 mM

The components were thoroughly mixed, followed by the addition of nuclease-free distilled water to a final volume of 100 ml.

PBS:

Table A.4 PBS preparation

Chemicals	Final concentrations
NaCl	137 mM
KCl	2.7 mM
Na2HPO4	10 mM
KH2PO4	1.8 mM

The components were thoroughly mixed, followed by the addition of nuclease-free distilled water to a final volume of 100 ml.

Cresol red:

Table A.5 Cresol red preparation

Chemicals	Final concentrations
Cresol red (CR)	5 mM

HNB:

Table A.6 HNB preparation

Chemicals	Final concentrations
Hydroxy naphthol blue	3 m M

The components were thoroughly mixed, followed by the addition of nuclease-free distilled water to a final volume of 10 ml.

B. *In Silico* Analysis of Primers

In silico analyses were performed using the MFE Primer 4.0 and MFE Primer 4.1.0 in silico evaluate the performance and specificity of the primer sets designed for porcine, equine, and chicken genomes. Initially, the binding capacities of these primers to their target genomes were verified to ensure efficiency. Subsequently, the cross-reactivity of these primer sets with non-target animal species was systematically examined.

This assessment was conducted based on Gibbs free energy (ΔG) values. ΔG value indicates the thermodynamic stability of a primer-target duplex, where more negative values signify stronger binding affinity. During the cross-reactivity analysis, interactions with ΔG values above -15 kcal/mol were considered negligible and disregarded. Potential cross-reactivity interactions identified below this threshold were further evaluated, and these *in silico* findings were subsequently supported and validated by experimental analysis.

In silico Binding Analysis of *Sus-Sucrofa* (pork)

The MFE Primer 4.1.0 tool was used to analyze the binding performance of the Sus scrofa-specific primer set. (Figure B.1) The MFE Primer 4.1.0 tool was used to analyze the binding performance of the Sus scrofa-specific primer set. The analysis showed that the porcine-specific F3 and B3 primers generated a 190 bp amplicon with 47.37% GC content on the mitochondrial reference sequence. The melting temperatures for the forward and reverse primers were 55.07 °C and 56.04 °C, with ΔG values of -19.42 kcal/mol and -19.78 kcal/mol, respectively.

Figure B.1 *In silico* binding analysis of the Sus scrofa specific forward-reverse primer set.

These results demonstrated complete sequence complementarity with 18/18 and 19/19 nucleotide matches**.** Consequently, it was observed that the primers bind to the target genome with high affinity and effectively amplify the *Sus scrofa* target region.

In silico **Binding Analysis of** ***Equus caballus*** **(horse)**

The binding performance of the *Equus caballus*-specific F3-B3 primer set was analyzed (Figure B.2). The analysis showed that the equine-specific F3 and B3 primers generated a 213 bp amplicon with 42.72% GC content on the mitochondrial reference sequence. The melting temperatures for the forward and reverse primers were 56.79 °C and 54.04 °C, with ΔG values of -20.78 kcal/mol and -19.28 kcal/mol, respectively.

Figure B.2 *In silico* binding analysis of the Equus caballus specific forward-reverse primer set.

These results demonstrated complete sequence complementarity with 20/20 and 20/20 nucleotide matches. Consequently, it was observed that the primers bind to the target genome with high affinity and effectively amplify the *Equus caballus* target region.

***In silico* Binding Analysis of *Gallus gallus* (chicken)**

The binding performance of the *Gallus gallus*-specific F3-B3 primer set was analyzed (Figure B.3). The analysis showed that the equine-specific F3 and B3 primers generated a 244 bp amplicon with 50.41 % GC content on the mitochondrial reference sequence. The melting temperatures for the forward and reverse primers were 58.38 °C and 55.79 °C, with ΔG values of -21.55 kcal/mol and -21.74 kcal/mol, respectively.

Figure B.3 *In silico* binding analysis of the Gallus gallus specific forward-reverse primer set.

These results demonstrated complete sequence complementarity with 20/20 and 23/23 nucleotide matches. Consequently, it was observed that the primers bind to the *Gallus gallus* genome with high affinity and effectively amplify the *Gallus gallus* target region.

In silico Binding Analysis of Internal Control Primers

The binding capacities of the Internal Control (IC) primers to *Sus scrofa*, *Equus caballus*, and *Gallus gallus* genomes were analyzed. The detailed findings regarding these evaluations are presented below.

In silico Binding Analysis of IC Primers with *Sus scrofa* Genome

The *in silico* analysis performed with the IC primers and the *Sus scrofa* genome is presented in Figure B.4. The analysis showed that the IC-F3 and IC-B3 primers generated a 210 bp amplicon with 61.43% GC content on the *Sus scrofa* reference sequence. The melting temperatures for the forward and reverse primers were 60.98 °C and 58.72 °C, with ΔG values of -20.98 kcal/mol and -19.63 kcal/mol, respectively.

Figure B.4 *In silico* binding analysis of the IC primers with the Sus scrofa genome.

These results demonstrated complete sequence complementarity with 16/16 and 15/15 nucleotide matches. Consequently, it was observed that the primers bind to the *Sus scrofa* genome with high affinity and effectively amplify the target region.

In silico Binding Analysis of IC Primers with *Equus caballus* Genome

The *in silico* analysis performed with the IC primers and the *Equus caballus* genome is presented in Figure B.5. The analysis showed that the IC-F3 and IC-B3 primers generated a 210 bp amplicon with 61.43% GC content on the *Equus callabus* reference sequence. The melting temperatures for the forward and reverse primers were 60.98 °C and 58.72 °C, with ΔG values of -20.98 kcal/mol and -19.63 kcal/mol, respectively.

Figure B.5 *In silico* binding analysis of the IC primers with the Equus caballus genome

These results demonstrated complete sequence complementarity with 15/15 and 16/16 nucleotide matches. Consequently, it was observed that the primers bind to the *Gallus gallus* genome with high affinity and effectively amplify the target region.

In silico Binding Analysis of IC Primers with *Gallus gallus* Genome

The *in silico* analysis performed with the IC primers and the *Gallus gallus* genome is presented in Figure B.6. The analysis showed that the IC-F3 and IC-B3 primers generated a 209 bp amplicon with 59.63% GC content on the *Sus scrofa* reference sequence. The melting temperatures for the forward and reverse primers were 59.67 °C and 60.08 °C, with ΔG values of -20.08 kcal/mol and -20.80 kcal/mol, respectively.

Figure B.6 In silico binding analysis of the IC primers with the Gallus gallus genome.

These results demonstrated complete sequence complementarity with 15/15 and 16/16 nucleotide matches. Consequently, it was observed that the primers bind to the *Gallus gallus* genome with high affinity and effectively amplify the target region.

In Silico Cross-Reactivity Analysis

The potential cross-reactivity of the porcine, equine, and chicken-specific primers was evaluated against genomic DNA sequences from goose, chicken, duck, horse, cattle, sheep, turkey, and fish with MFE-Primer 4.0 in silico PCR tool. A representative example of the resulting cross-reaction amplicon data is presented in Figure B.7.

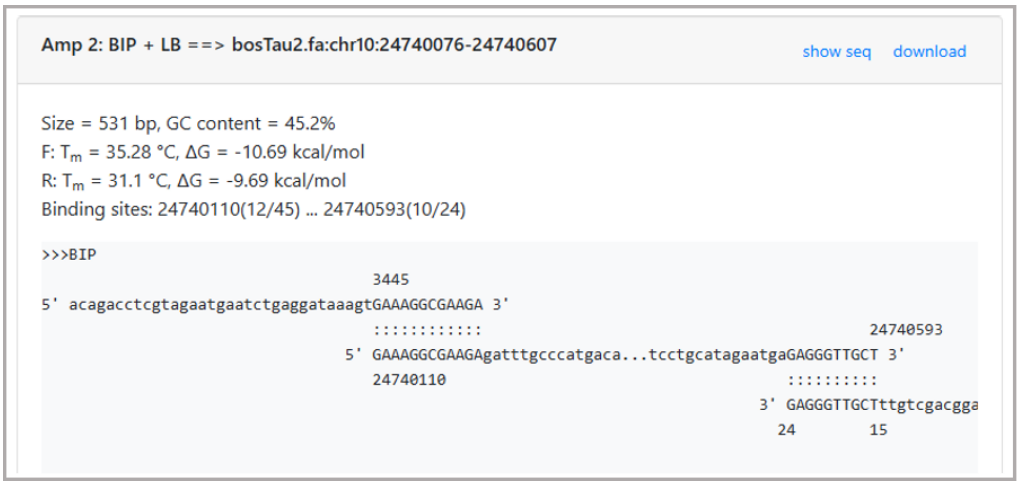

Figure B.7 Example of a potential amplicon formed between the porcine-specific primers and the cattle genome.

The scheme shows the mismatches identified at the primer-binding sites. None of the non-target sequences showed a complete match with the primers, and mismatches were observed in the primer-binding regions in all cases. Therefore, the detected partial matches were considered non-significant due to their low ΔG values. These results support the specificity of the primer set for porcine DNA. The complete results of the MFE-Primer 4.0 in silico PCR analysis are presented in Table B.1.

Table B.1 Summary of the *in silico* cross-reaction analysis for the porcine, equine, and chicken-specific primers.

Primer Set	Genome with Observed Cross Reaction	Product Size (bp)	ΔG (kcal/mol)
porcine	goose	121	-14.01
porcine	goose	127	-9.65
porcine	duck	537	-10.62
porcine	goose	244	-10.79
porcine	goose	276	-10.79
porcine	goose	284	-9.65
porcine	chicken	532	-9.75
porcine	goose	398	-10.18
porcine	chicken	250	-9.47
porcine	chicken	235	-9.35
porcine	chicken	583	-9.65
porcine	goose	297	-9.65
porcine	duck	581	-9.65
porcine	horse	115	-10.40
porcine	cattle	531	-10.69
porcine	horse	121	-9.61
porcine	horse	229	-9.65
porcine	horse	437	-9.65
porcine	horse	472	-9.65
porcine	cattle	176	-9.65
porcine	cattle	470	-9.65
chicken	horse	1141	-10.99
chicken	horse	1411	-9.46

In parallel with the *in silico* analysis, the specificity of the primer set was further evaluated experimentally using the LAMP analysis. The assay was performed using

genomic DNA obtained from the species listed in the table to assess the potential for cross-reactivity with non-target species. No amplification was observed in any of the tested non-target species. These results indicate that the primer set specifically detects the target DNA without producing detectable cross-reactions with the other species tested. Thus, the experimental findings were consistent with the *in silico* analysis and further support the specificity of the LAMP assay.

C. Commercial Products and News Reports on Meat Adulteration

Alternative Commercial Products

R-Biopharm AG – SureFood® ANIMAL ID 4plex Beef/Horse/Pork+IAAC
Commercial real-time PCR-based assay for the simultaneous identification of beef, horse, and pork DNA, including an internal amplification control (IAAC). Available at: https://food.r-biopharm.com/products/surefood-animal-id-4-plex-beef-pork-horse-iaac-new/

R-Biopharm AG – SureFood® ANIMAL ID 4plex Pork/Chicken/Turkey+IAAC
Commercial real-time PCR-based assay for the simultaneous identification of pork, chicken, and turkey DNA, including an internal amplification control (IAAC). Available at: https://food.r-biopharm.com/products/surefood-animal-id-4plex-porkchickenturkeyiaac/

News Reports on Meat Adulteration

GZT (March 12, 2026). *Mersin'de belediyeye ait aşevinde dağıtılan kavurmada at eti tespit edildi.* Available at: https://www.gzt.com/gundem/vatandasin-kavurma-tabagindan-yaris-ati-cipi-cikti-belediye-asevinde-skandal-4023299

Turkish Post (April 19, 2026). *İzmir'de pide harcının içinden at eti çıktı.* Available at: https://www.turkishpost.net/izmirde-pide-harcinin-icinden-at-eti-cikti/

GZT (July 30, 2026). *Tarım Bakanlığı taklit ve tağşiş listesi: 9 üründe at, eşek ve katır eti tespit edildi.* Available at: https://www.gzt.com/gundem/tarim-bakanligi-taklit-ve-tagsis-listesi-9-urunde-at-esek-ve-katir-eti-tespit-edildi-hangi-gidalarda-hile-var-tarim-bakanligi-ifsa-listesi-2026-pdf-4246993