ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

DEVELOPMENT OF FAST AND ECONOMIC QPCR-BASED METHOD FOR MEAT SPECIES DETECTION

M.Sc. THESIS

Eda ÇİFTÇİ

Department of Advanced Technologies

Molecular Biology-Genetics and Biotechnology Master Programme

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

HIZLI VE EKONOMİK ET TÜR TAYİNİ İÇİN EŞ ZAMANLI QPCR TABANLI BİR YÖNTEM GELİŞTİRİLMESİ

YÜKSEK LİSANS TEZİ

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HAZİRAN, 2013

Eda ÇİFTÇİ, a **M.Sc.** student of ITU **Graduate School of of science engineering and technology** student ID 521101121, successfully defended the **thesis** entitled "**DEVELOPMENT OF FAST AND ECONOMICAL QPCR-BASED METHOD FOR MEAT SPECIES DETECTION**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family,

FOREWORD

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ABBREVIATIONS

ANS : Anserine
ATP : Adenosine triphosphate
BAL : Balenine
BHQ : Black Hole Quencher
CAR : Carnosine
CE : Capillary Electrophoresis
CE-SDS : Sodium Dodecyl Sulfate Polymer-filled Capillary Electrophoresis
CTAB : Hexadecyltrimethylammonium Bromide
cyclic-GMP : Cyclic Guanosine Monophosphate
Cytb : Cytochrome b
DABCYL : 4-(dimethylamino) azobenzene-4'-carboxylic acid
ddNTP : Dideoxy Nucleotide triphosphates
DNA : Deoxyribonucleic Acid
dNTP : Deoxyribo Nucleotide triphosphate
dsDNA : Double-Stranded DNA
EDTA : Ethylene Diamine Tetraacetic Acid
ELISA : Enzyme-Linked Immunosorbent Assay
FRET : Fluorescence Resonance Energy Transfer
GC : Gas Chromatography
G-C : Guanin-Cytosin
HPLC : High-Performance Liquid Chromatography
HRM : High Resolution Melting
IEF : Isoelectric Focusing
MAbs : Monoclonal Antibodies
mtDNA : Mitochondrial DNA
PAbs : Polyclonal Antibodies
PAGE : Polyacrylamide Gel Electrophoresis
PCR : Polymerase Chain Reaction
PCR-RAPD : PCR-Random Amplified Polymorphic DNA
PCR-RFLP : PCR-Restriction Fragment Length Polymorphism
PCR-SSCP : PCR-Single-Strand Conformation Polymorphism
QPCR : Real-Time Quantitative PCR
RFU : Relative Fluorescence Units
RNA : Ribonucleic Acid
rRNA : Ribosomal Ribonucleic Acid
SDS : Sodium Dodecyl Sulfate
SDS-PAGE : Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SSP : Salt Soluble Protein
TAMRA : 6-carboxy-tetra-methyl-rhodamine
WSP : Water Soluble Protein

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DEVELOPMENT OF A FAST AND ECONOMIC QPCR-BASED METHOD FOR MEAT SPECIES DETECTION

SUMMARY

Meat contains amino acids, vitamins, fat and especially animal proteins, which are extremely important for human health. According to data from Turkish Statistical Institute (TUIK) meat consumption per capita in Turkey was 12 kg in 2012. The meat consumption per capita in United States of America (U.S.A.) and European Union (EU) are approximately 60 kg and 30 kg, respectively. These data show that; meat consumption in Turkey is lower than EU and U.S.A. Increasing human population and the cost of meat products have resulted in gradual decreases in meat consumption over the years. So that, the manufacturers started to mix different meat types (horse, donkey, pig, turkey and chicken) to reduce the costs. If the food is frozen and processed, it becomes impossible for consumer to differentiate the meat type which has similar pigmentation (beef- horse, chicken-pork, etc.). Therefore, forgery is commonly encountered within the production of meatball, sausage and salami.

According to the Turkish Food Regulations before 2013, mixed meat application is permitted as long as the producers state the mixed meat types on the label. On the other hand, the Ministry of Food, Agriculture and Livestock has determined undeclared mixed meat applications in the Turkish Food Market. This has led to the new regulations in 2013, which strictly prohibited the mixed meat application.

Protein and nucleic acid-based methods have been commonly used for meat species identification. The protein-based methods have been reported to be inadequate for the meat species identification since the protein structures deformed in thermally processed foods.

DNA based methods have been considered to be more advantageous than the protein based methods. DNA is thermo-stable, shows the same features in all cells and provides more information about the species. Polymerase chain reaction (PCR) based methods have a power of amplifying a specific DNA molecule that belongs to a certain animal species. On the other hand, the conventional PCR cannot provide quantitative results and the post PCR steps such as gel electrophoresis make it time consuming.

Quantitative Real Time PCR (qPCR) can provide both qualitative and quantitative results for meat type identification. In this technique, amplification of the target gene can be monitored online by the use of fluorescent reporters. The most commonly used reporters in meat type detection are Sybr Green dye and the oligonucleotide probes. Sybr Green can inhibit PCR reactions if used above a certain concentration and it cannot be used for detection of the multiple targets. This is why the oligonucleotide probes are the most frequently used reporters despite of their high costs. As an alternative, High Resolution Melting (HRM) dyes are preferred for use

with melting curve assays due to the more discrete signal change occurring upon DNA denaturation. HRM dyes only bind to double stranded DNA that prevents the dye molecule from redistribution during melting and provides superior melt curve resolution. Unlike SYBR Green dye, HRM dyes can be used at high concentrations because they do not inhibit DNA polymerases and PCR reaction. HRM dyes great ability to bind the hydrogen bond almost 4 times more than SYBR Green.

The aim of this study, develop a quick, reliable and low-cost qPCR based methodology to qualitatively detect different meat species (cattle, chicken, turkey, horse, donkey and pig) in food products. Firstly in this study, an enzyme free DNA extraction methodology which can be completed in less than 20 minutes was developed. The developed methodology was based on bead beating treatment and silica column method. In this methodology, hexadecyltrimethylammonium bromide (CTAB), Guanidinium thiocyanate and bead beating were used to disrupt the cells. Guanidinium thiocyanate also acted in PCR inhibitor removal and DNA binding.

The results showed that the purities and concentrations of the DNA extracts obtained using the developed DNA extraction methodology were in the desirable ranges: 1.6-2 and 50-1000 ng/µl, respectively. The obtained DNA qualities were also assessed by using 200 ng of the template DNAs in qPCR. The obtained threshold cycle numbers were less than 20, which implied that the obtained DNAs were suitable for PCR amplification. The current commercially available DNA extraction kits are based on time-consuming reactions that are completed in at least 1.5 hours. In this study, we have developed a DNA extraction protocol, which does not include enzymatic steps. The DNA extracts were obtained via only the physical and the chemical cell disruption. This has significantly decreased the total time (less than 20 minutes) and the cost of the DNA extraction.

Universal mitochondrial DNA sequences such as; 12S rRNA, cytochrome b and 16S rRNA genes have generally been chosen as the target for meat type specific probe design. This has led to specifity problems in the detections. Mitochondrial genes are highly conserved so that differentiation is difficult between the species that belongs to the same genus such as; horse and donkey. To obtain more specific results, we concentrated on the amplification of highly variable gene regions for the each animal type. This approach prevented the non-specific amplifications and led to easier workflow for the validation studies.

The qPCR methodology was designed to target both single and multiple DNA types. The multiple detection was based on melting temperature (T_m) differences of the different PCR amplification products with a single HRM dye (EvaGreen). The qPCR trials on the reference meat samples showed that the target specific melting peaks can be obtained at 82.02 ± 0.29˚C for horse, 84.3˚C ± 0.32˚C for pig, 78.80 ± 0.38˚C for donkey, 84.86 ± 0.29˚C for turkey, 81.91 ± 0.34˚C for chicken and 86.96 ± 0.31˚C for cattle. Q-PCR trials on the binary mixtures of turkey/cattle, chicken/cattle, turkey/chicken, pig/donkey, donkey/horse and horse/pig and triple mixtures of turkey/chicken/cattle and pig/donkey/horse resulted in multiple melting peaks that are specific to the intended targets.

To obtain the limit of detection (LOD), 10 g standard meat mixtures that contain 1-100 copies of the additive meat type DNA were prepared. The LODs were 4 chicken copies/gr cattle sample, 3 turkey gene copies/gr cattle sample, 1 horse gene copy/gr cattle sample, 1 donkey gene copy/gr cattle sample and 1 pig gene copy/gr cattle sample.

On the other hand, since the standard meat mixtures were not obtained from an acredited reference laboratory, the detected LODs were rough estimations of the real LODs.

Commercial samples which are intended to be introduced to the Turkish food market were screened. The commercial samples were obtained from acredited food laboratories. The sample types were sucuk, doner kebap, beef sausage, beef salami and the swab samples from meat production benches. 24 chicken, 9 turkey and 1 pig meat positive samples were detected among the 83 screened samples. The results were also confirmed via the DNA sequencing of PCR products.

The currently available qPCR based meat type identification methodologies are time and money consuming. The main reasons behind these are the long incubation times and high costs of the available DNA extraction and the multiplex qPCR methodologies. In this study, a new system was developed to overcome these problems. This was achieved via an enzyme free DNA extraction methodology and a multiplex qPCR using a single HRM dye. For the first time, this study introduced discrimination of three different qPCR amplicons from various animal specific gene products based on the differences in T_ms. The overall results proved that the developed method could give sensitive results in less than 75 minutes, which is at least two times faster than the currently available PCR-based methods for meat type detection.

The qPCR based methodology developed in this study is a potential molecular tool that can be used in rapid and routine detection of horse, donkey, pig, chicken and turkey meats present in heat treated meat mixtures. The use of species-specific primers makes the method very sensitive for determination in raw and processed meats. On the other hand, the methodology must be validated using the reference samples prepared by reference accredited food control laboratories.

HIZLI VE EKONOMİK ET TÜR TAYİNİ İÇİN QPCR TABANLI BİR YÖNTEM GELİŞTİRİLMESİ

ÖZET

Et, içerdiği amino asitler, vitaminler, yağ ve özellikle hayvansal protein ile insan sağlığı için vazgeçilmez bir besin kaynağıdır. Türkiye İstatistik Kurumu'nun (TUIK) 2012 verilerine göre; Türkiye'de yıllık kişi başına tüketilen et miktarı 12 kg'dır. Yine TUIK'in sonuçlarına göre, Avrupa ülkelerinde kişi başına tüketilen et miktarı 30 kg iken Amerika Birleşik Devletlerinde bu sayı 60 kg'a kadar çıkmaktadır. Bu veriler, Türkiye'de et tüketiminin son derece az olduğunu göstermektedir.Artan insan popülasyonu ve et ürünlerinin maliyetlerinin yüksek olması et tüketim oranını her yıl azaltmaktadır. Bu yüzden et üreticileri, fiyatları düşürmek için farklı et türlerini (at, esek, domuz, hindi, ve tavuk) karıştırmaya başlamıştır. Benzer pigmentasyona sahip et türleri (dana ve at, tavuk ve domuz gibi) dondurulduktan sonra veya işlenmiş et ürünlerinde kullanıldıklarında tüketici tarafından algılanması neredeyse imkansız hale gelir. Bu nedenle, köfte, salam, sosis, sucuk gibi ürünlerde sahteciliklerin yapılması oldukça kolaydır.

2013 yılından önceki Türk Gıda Kodeksi'ne göre, et üreticilerinin karıştırdığı hayvan türlerini, ürünlerin etiketlerinde bildirmesi koşuluyla karma et uygulamasına izin verilmekteydi. Ancak, Gıda, Tarım ve Hayvancılık Bakanlığı yaptığı çalışmalar sonucunda, piyasada bulunan bir çok ürünün etiketinde, içerdiği hayvan türünün belirtilmediğini tespit etmiştir. Bu durum, 2013 yılında revize edilen Türk Gıda Kodeksi'nde karma et uygulamasının tamamen yasaklanmasına neden olmuştur.

Et tür tayini analizlerinde en sık kullanılan yöntemler, protein ve nükleik asit tabanlıdır. Fakat, ısıl işleme maruz kalan ürünlerin protein yapıları bozulduğundan, protein tabanlı yöntemlerin et tür tayini için yetersiz kaldığı bildirilmiştir.

DNA tabanlı yöntemlerin, protein tabanlı yöntemlere göre daha avantajlı olduğu düşünülmektedir. DNA molekülü sıcaklığa dayanıklı bir moleküldür, tüm hücrelerde aynı özelligi gösterir ve ayrıca tür hakkında daha fazla bilgi sağlar. Polimeraz zincir reaksiyonu (PZR) tabanlı metotlar, belli bir hayvan türüne ait özgü DNA sekansını çoğaltma gücüne sahiptir. Diğer taraftan, konvensiyonel PZR ile kantitatif sonuçlar elde edilemez ve jel elektroforezi gibi PZR sonrası adımlar gerektirdiği için zaman alıcı bir yöntemdir.

Kantitatif eş zamanlı PZR (quantitative Real Time PCR- qPCR), hem kalitatif hem de kantitatif sonuçlar sağlar. Bu teknikte, hedef genin çoğalması, floresans işaretleyiciler kullanılarak eş zamanlı olarak görüntülenebilir. Et tür tayini çalışmalarında, Sybr Green ve oligonükleotit problar en çok kullanılan işaretleyicilerdir. Sybr Green belli bir konsantrasyonun üstünde kullanıldığında PZR reaksiyonunu inhibe edebilir ve ayrıca çoklu hedefleri tespit etmek için uygun değildir. Bu yüzden, yüksek maliyetli olmalarına rağmen oligonükleotit problar en çok tercih edilen işaretleyicilerdir. Alternatif olarak, Yüksek Çözünürlükte Erime

(HRM) boyaları, erime eğrisi analizlerinde, DNA denatürasyonu ile birlikte çok daha ayırt edilebilir sinyal değişimlerine neden oldukları için tercih edilmektedirler. HRM boyaları sadece çift zincirli DNAya bağlanır, bu da boya molekülünü erime sırasında tek zincirli DNAya yeniden bağlanmasını önler ve üstün erime eğrisi çözünürlüğü sağlar. SYBR Green boyalarının aksine, HRM boyaları yüksek konsantrasyonlarda kullanılabilir, çünkü HRM boyaları DNA polimerazı ve PZR reaksiyonunu inhibe etmezler. Ayrıca HRM boyaları Sybr Green ile karşılaştırıldığında hidrojen bağlarına 4 kat daha fazla bağlanır.

Bu tezin amacı; et ürünlerinin içerisine karıştırılan farklı et türlerinin (sığır, tavuk, hindi,at, eşek ve domuz) kalitatif olarak varlığını hızlı, güvenilir ve ekonomik bir biçimde tespit edilebilmesi için qPCR tabanlı bir sistem geliştirmektir. Bu çalışmada ilk olarak, 20 dakikadan az bir sürede tamamlanabilen, enzim içermeyen bir DNA izolasyon metodolojisi geliştirilmiştir. Geliştirilen metodoloji, boncuk ile parçalama ve silika kolon yöntemine dayalıdır. Bu metodolojide, hekzadesiltrimetilamonyum bromür (CTAB), guanidin tiyosiyanat ve boncuk ile parçalama uygulaması kullanılmıştır. Guanidin tiyosiyanat ve boncuk ile parçalama uygulaması hücreleri parçalamak için kullanılmıştır. Ayrıca guanidin tiyosiyanat DNA bağlanmasında ve PZR inhibitorlerinin uzaklaştırmasında rol oynar.

DNA izolasyon sonuçlarına göre, geliştirilen DNA izolasyon metodolojisi kullanılarak elde edilen DNAların saflıkları ve konsantrasyonları ulaşılmak istenen aralıklarda elde edilmiştir: sırasıyla 1.6-2 ve 50-1000 ng/µl. Elde edilen DNAların kalitesi, qPCR'da bu DNAların 200 nanogramının kalıp DNA olarak kullanılmasıyla sınanmıştır. Elde edilen eşik döngü sayılarının 20'nin altında elde edilmesi, elde edilen DNAların PZR çoğalması için uygun olduğunu kanıtlamıştır. Piyasada mevcut ticari DNA izolasyon kitleri, zaman alıcı reaksiyonlara dayalıdır ve DNA izolasyon işlemi en az 1.5 saat sürmektedir. Bu çalışmada, enzimatik adımlar içermeyen bir DNA izolasyon protokolü geliştirilmiştir. DNA izolatları, sadece fiziksel ve kimyasal hücre parçalamasıyla elde edilmiştir. Bu da, toplam analiz süresinin (20 dakikadan az) ve DNA izolasyonun maliyetini önemli ölçüde azaltmıştır.

Bu zamana kadar yapılan çalışmalarda, genellikle 12S rRNA, sitokrom b geni ve 16S rRNA gibi evrensel mitokondriyal genler, et türüne özgü prop dizaynı için hedef olarak seçilmişlerdir. Bu durum, özgüllük problemlerine neden olabilmektedir. Mitokondriyal genler son derece korunmuş genlerdir, bu yüzden at ve eşek gibi aynı cinse ait türler arasında ayrım yapmak zordur. Daha özgül sonuçlar elde etmek için, bu çalışmada her bir hayvan türü için yüksek derecede değişken gen bölgelerin çoğaltılmasına odaklanılmıştır. Bu yaklaşım sayesinde, özgül olmayan çoğalmalar önlenmiş ve validasyon çalışmaları için iş akışı kolaylaştırılmıştır.

Bu çalışmada geliştirilen qPCR metodolojisi, tekli ve çoklu DNA tiplerini hedef alacak şekilde dizayn edilmiştir. Bu metodoloji sayesinde, tek bir HRM boyası (EvaGreen) kullanılarak, farklı PZR ürünlerinin, erime sıcaklığı (T_m) farklılıklarına göre çoklu tespit yapılmıştır. Referans et örneklerinin qPCR sonuçlarına göre; hedefe özgü erime sıcaklıkları at için 82.02 ± 0.29˚C, domuz için 84.3˚C ± 0.32˚C, eşek için 78.80 ± 0.38˚C, hindi için 84.86 ± 0.29˚C, tavuk için 81.91 ± 0.34˚C ve sığır için 86.96 ± 0.31˚C olarak belirlenmiştir. Hindi/sığır, tavuk/sığır, tavuk/hindi, domuz/eşek, eşek/at, domuz/at ikili karışımlarının qPCR denemelerinde ve hindi/tavuk/sığır, domuz/eşek/at üçlü qPCR denemelerinde, istenilen hedeflere özgü olan birden fazla erime sıcaklığı tespit edilmiştir.

Tespit limitini (Limit of Detection –LOD) belirlemek için; 10 gramlık et karışımları hazırlanmıştır. Sırasıyla hedeflenenler hayvan eti, sığır eti ile karıştırılmıştır. Sığır etiyle karıştırılan her hayvan türü , karışımda 1 – 100 kopya gen sayısı bulunduracak şekilde karışımlar yapılmıştır. Sığır etinin 1 gramında tespit limiti; tavuk için 4 gen kopya sayısı; hindi için 3 gen kopya sayısı; at , eşek ve domuz için ise 1 kopya gen sayısı olarak belirlenmiştir. Bununla birlikte, standart et karışımları akredite referans laboratuvarlar tarafından hazırlanmadığı için, gerçek LOD'nin kabaca tahmini yapabilmek için bu çalışmalar yürütülmüştür.

Türkiye gıda piyasasına sunulması planlanan çiğ ve işlenmiş et ürünleri geliştirilen yöntemle başarıyla analiz edilmiştir. Numuneler akredite gıda kontrol laboratuvarları tarafından sağlanmıştır. Analiz edilen numune tipleri köfte, döner, sucuk, salam ve sosis gibi işlenmiş ürünler ve bir et üretim tesisinin üretim tezgahlarından alınan sürüntü numuneleridir. Analiz edilen toplam 83 örnekten; 24 tanesinin tavuk eti, 9 tanesinin hindi eti ve 1 tanesin domuz eti içerdiği tespit edilmiştir. Sonuçların doğruluğu, DNA sekanslama yöntemi kullanılarak onaylanmıştır. Geliştirilmiş olan bu yöntemin, mevcut PZR tabanlı yöntemlere göre en az iki kat daha hızlı olduğu ve 75 dakika içinde hassas sonuçlar verebildiği kanıtlanmıştır.

Et türü tayini için kullanılan mevcut qPCR tabanlı yöntemler yüksek zaman ve maliyet gerektirmektedir. Bunun temel nedeni DNA ekstraksiyonu ve qPCR adımlarındaki uzun inkübasyon süreleri ve yüksek sarf maliyetleridir. Bu çalışmada bu sorunlara çözüm getirmek için yeni bir sistem geliştirilmiştir. Bu sistemin başarısının altında enzim içermeyen DNA protokolü ve tek HRM boyası ile yapılan çoklu hedef tespiti yatmaktadır. Bu çalışmada ilk defa, farklı hayvan türlerinden çoğaltılmış üç farklı hedef DNA qPCR'da tek bir boya kullanılarak, T_m'lerindeki farktan faydalanılarak ayırt ve tespit edilebilmiştir. Elde edilen sonuçlar geliştirilen yöntemin 75 dakikadan kısa bir sürede hassas sonuçlar verebileceğini göstermiştir. Böylelikle mevcut PCR tabanlı et türü tayin yöntemlerine nazaran en az 2 kat daha hızlı sonuç elde edilebilmiştir.

Bu çalışmada geliştirilen qPCR'a dayalı metodoloji, ısıl işlem görmüş et karışımlarında at, eşek, domuz, tavuk ve hindi etlerinin hızlı ve rutin tespitleri için potansiyel bir moleküler araç olarak kullanılabilir. Türe özgü primerlerin kullanılması, bu metodu çiğ ve işlenmiş etlerin tespitinde son derece hassas kılmaktadır. Diğer taraftan, geliştirilen bu metodolojinin, akredite gıda kontrol labaratuvarları tarafından hazırlanan referans örnekler kullanılarak validasyonu yapılmalıdır.

1. INTRODUCTION

Meat contains animal protein, fat and essential amino acids which are extremely important for human health. Iron, zinc, phosphorus, magnesium, B6, B12, A, B1 vitamins are other important elements found in meat. Meats are good quality protein source. More consumption of protein is important especially in infancy and childhood therefore meat should be included in their diet. In our country, especially sausage, salami and sucuk (the traditional meat product in Turkey) are the indispensables for the breakfast. According to data from the Institute of Statistics of Turkey (TUIK) 2012, meat consumption per capita in Turkey was 12 kg (Beef meat: 10 kg/per person, Sheep/Goat meat: 2 kg/ per person). The meat consumption per capita in United States (U.S.) is approximately 60 kg and in Europe countries (EU), meat consumption per capita is approximately 30 kg. Meat consumption in Turkey is lower than EU countries and U.S. Increasing human population and the high cost of meat products cause sales of foods expensively. To remedy this situation, the manufacturers started to make tricks to reduce costs. The mixing meats of different species of animals are usually done to lower the cost of meat products.

According to the Turkish Food Codex regulations, the animal species, which present in the product, together with the name of the product should be indicated on the label. According to the revised new codex in 2013, mixed meat application is strictly prohibited. However, 100% beef meat-containing delicatessen products have not been identified on the market according to the surveys of Turkish Food, Agriculture and Livestock Ministry. Recently, horsemeat was determined in globally known food brands that have attracted worldwide attention. This deception causes consumer victimization, economic, religious, health problems and unfair market competition. In this context, to detect different meat types in food products reliable and precise analytical tools need to be developed to facilitate the routine control tests.

In meat species identification analysis, such as organoleptic analysis, the anatomical and histological distinctions based on a structure of the hair, electrophoretic analysis of proteins, chromatographic methods, imunoassays and DNA-based methods can be

used. Several studies have been performed with these methods such as electrophoretic method (Cota-Rivas & Vallejo-Cordoba, 1997), chromatographic (Aristoy and Toldrá 2004) and enzyme-linked immunosorbent assay (ELISA) (Chen & Hsieh, 2000) for the identification of meat product in meat and meat products. In protein analysis, protein structure are disrupted because of the products are exposed to heat treatment, and therefore the accurate results cannot be obtained.

Targeting DNA molecule, which is more stable to heat treatment, PCR based methods are highly sensitive and they are desirable than protein-based methods (Jason Sawyer 2002). In a mixed sample, conventional PCR is suitable to identify different meat types qualitatively, but it cannot provide quantitative results.

According to recent studies, the qPCR is a more appropriate technique to determine meat species due to the qualitative and quantitative results that it provides (Mendoza-Romero et al., 2004). In this technique, amplification of the target gene can be monitored as the fluorescence increases without using an additional detection method. In the recent studies conducted for the detection of meat species, hydrolysis and hybridization probes were used. However, costs of these probes are extremely high. DNA binding dyes such as Sybr Green-I have been commonly used instead of hydrolysis and hybridization probes for identification of meat species. However, at high concentrations, SYBR Green-I inhibits the DNA polymerase and PCR reaction. To allow reliable amplification, low concentrations of SYBR Green I should be used. To overcome this limitation a new class of dsDNA intercalating dyes; High Resolution Melting (HRM) dyes such as LC-Green, EvaGreen can be used. HRM dyes do not inhibit DNA polymerases and PCR reaction, these dyes can be used at high concentrations. Besides, HRM dyes great ability to bind the hydrogen bond almost 4 times more than SYBR Green. Therefore, there is a need for developing a quick and reliable system that can be produced locally to reduce meat species detection cost via qPCR using a single HRM dye.

1.1 Purpose of Thesis

In this thesis, it was aimed to develop a quick, reliable and low-cost qPCR based system to screen different meat species (cattle, horse, donkey, chicken, turkey and pig) in food samples. The methodology was designed to target both single and multiple DNA types. The multiple detection was based on melting temperature (T_m)

differences of the different PCR amplification products. A single high resolution melting (HRM) dye was used instead of the oligonucleotide probes to detect multiple targets, which was expected to decrease the consumable costs.

The total analysis time was intended to become shorter via developing a quick DNA extraction methodology that was mainly based on the physical and chemical cell disruption. This study can open a way through a wider application of qPCR in Turkey to screen meat types in foods.

2. METHODS TO DETECT MEAT SPECIES

2.1 Traditional Methods

The meat species identification has great importance in food quality control and safety. In identification of meat species, most commonly used methods are protein and nucleic acid-based analysis (Montowskaa and Pospiechab, 2012). Protein analysis is related with electrophoretic techniques; such as polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) techniques. In addition, chromatographic techniques and enzyme-linked immunosorbent assays (ELISA) are used for detection of meat species. DNA-based methods are DNA hybridization, PCR-based techniques and qPCR.

2.1.1 Electrophoretic techniques

Electrophoresis simply refers to the movement of charged particles or molecules in an electric field, wherein molecules with different mobilities migrate at different rates (Oelshlegel F. and Stahmann M., 1973). Protein electrophoresis is a well-known separation technique. The principle of this methods; in all animal species are assumed to have a homogeneous composition of a given protein. The Sarcoplasmic and Myofibrillar protein electrophoresis was evaluated as a reliable method for the determination of meat species. Conventional electrophoretical methods are PAGE, SDS-PAGE, and IEF techniques. These methods have some advantages which are cheaper, faster, needs less complicated equipment and fewer personnel compared with the other techniques. On the other hand, they require extreme care and the results can be affected by many influences. These are the most important disadvantages of these methods.

2.1.1.1 PAGE and SDS-PAGE

In PAGE and SDS-PAGE techniques, proteins are separated according to their electrophoretic mobility. In PAGE method, agents which may distort to the natural structure of proteins are not used. It is not possible to obtain precise information about the molecular weight of the protein because, besides the molecular size, molecular shape and charge affects the separation. SDS is a detergent which separates subunits from oligomeric proteins. With SDS binding, denature proteins will have the same shape and charge / mass ratio. Thus, in the SDS-PAGE technique, in an electric field, negatively charged denatured proteins running through in a polyacrylamide gel are separated on the basis of molecular weight. Owing to provide high resolution, reproducibility and molecular weight based discrimination; SDS-PAGE can be acceptable method to determination different meat species in protein mixture. For instance, SDS-PAGE method has been evaluated to identify meats of: cattle, sheep, lambs, goats, red deer and rabbits (Parisi and Aguiari 1985). Recently, Ekici and Akyüz (2003) used SDS-PAGE technique to identify the animal species in raw meat species adulteration in binary mixture. Characteristic banding patterns of proteins for each species (beef, pork, sheep and horse) were used in identifying the existence of other species in a meat mixture. For detect of meat species successfully, the protein structures of different species must be sufficiently different from each other. This method is not very convenient because the obtained results can be influenced by many factors, among others, by: age, nutritional stage of animals, stress, meat quality deviations.

2.1.1.2 Isoelectric focusing

Isoelectric Focusing is an electrophoretic method for the separation of proteins based on their isoelectric point (pl), in a stabilized pH gradient. Separation is carried out in a slab of polyacrylamide or agarose gel that contains a mixture of amphoteric electrolytes (ampholytes) (European Pharmacopoeia 2005). Instead of buffer system like in electrophoresis, a strong acid at the anode and strong base at the cathode are used. When subjected to an electrical current, ampholytes are arranged according to isoelectric points in the gel. The most acidic ampholyte moves to the anode, the most basic ampholyte moves to the cathode. As a result, a decreasing pH gradient from anode to the cathode occurs in the gel. Proteins which are applied into gel, running

through the cathode and the anode based on their charges. Proteins migrate until the pH values of the net charges are zero on the gel and stop stationary at this point. In the final stage, the obtained protein profiles can be visualized by following an appropriate staining step. The most commonly used dyes for the species identification include Coomassie Blue, silver salts, or enzymatic staining (Hofmann 1997).

For instance, the silver-staining technique has been proved to be a useful method for the visualization of small amounts of protein in the electrophoretic gels (Rabilloud, 1992). Polyacrylamide gel isoelectric focusing (PAGIF) has been extensively applied in meat speciation studies because it's higher resolution capability than that of conventional electrophoresis. For example; Protein isoelectric focusing and the analysis of restriction fragments of amplified DNA were used to identify raw pork, beef, chicken and turkey meats or their presence in cooked mixes (Barbieri and Forni, 2000). In another study, Skarpeid and others (1998) developed an assay that based on intensity profiles from isoelectric focusing of water-soluble proteins in mixtures of ground meat. Samples containing various amounts of beef, pork and turkey meat were analyzed by isoelectric focusing in immobilized pH-gradients. PAGIF has been extensively utilized in meat identification. However, the results of PAGIF are influenced by many factors, such as age, sex, gender of the animals, or different metabolic state of the muscles in the same animal (Kesmen and Yetim, 2012).

2.1.1.3 Capillariy electrophoresis

In capillary electrophoresis (CE), analytes moves along the capillary tube under the influence of an applied electrical field and they are separated based on their different electrophoretic mobilties. CE provides high-resolution separation of extremely small amount (5-10 nL) of the sample (Temizkan and Arda, 2008). Therefore, CE is a widely used technique for analysis of amino acids, peptides, proteins, nucleic acids. CE is combined with various detectors to detect proteins such as; fluorescence, refractive index, UV absorbance and mass spectrometers.

Cota-Rivas and Vallejo-Cordoba (1997) developed and optimized a sodium dodecyl sulfate (SDS) polymer-filled capillary gel electrophoresis (CE-SDS) method for the determination of meat proteins for species differentiation. They employed CE-SDS

method to separate both sarcoplasmic and myofibrillar meat proteins. According to the CE-SDS sarcoplasmic protein profiles, sarcoplasmic protein was more specific for each species both qualitatively and quantitatively and could be employed for differentiation and identification purposes. In another study, Vallejo-Cordoba and others (2010) used CE-SDS method to characterize, compare and quantify the water soluble protein (WSP) and salt soluble protein (SSP) fractions from bovine and ostrich muscle. The WSP profiles showed differences for bovine and ostrich meat, both qualitatively and quantitatively and could be employed for species differentiation. CE separation has been utilized as a powerful analytical method for the species identification in the mixtures. On the other hand, there are some disadvantages of CE, such as low sensitivity and reproducibility.

2.1.2 Chomatographic methods

Chromatographic methods are high-performance liquid chromatography (HPLC) and gas chromatography (GC) which have been commonly used in the analysis of food samples to detect food components and contaminants. Gas chromatography is a simple, versatile, fast and very sensitive technique which provides separation of very small molecules. However, the most important limitation of the technique is analyzed samples need to be volatile and resistant to higher temperatures (200-250 ºC) (Temizkan and Arda 2008). Therefore, only volatile or derivative of volatile molecules can be used in gas chromatography. HPLC technique is basically a modern liquid chromatography which automatically optimized. In HPLC technique; analysis and separation rates are higher than the traditional liquid chromatography. The technique also has superiorities such as; continuous availability, reproducibility and the automation of data easily.

The minor and specific compounds or groups of meats have been utilized for the identification of meat species in chromatographic studies. The histidine-containing dipeptides (the imidazole dipeptide carnosine (CAR), its methylated analogs anserine (ANS) and balenine (BAL)) are present in high concentrations in the skeletal muscle of many mammals. The relative concentrations of the three dipeptides are characteristic for each species (Carnegie et al., 1983) and can be used for the identification of meat species (Kesmen and Yetim 2012).

For instance, Tinbergen and Slump (1976) found a distinctive difference between the ANS/CAR ratio in beef or pork and of that in chicken/meat. According the study, the high ANS/CAR ratio of chicken meat should be considered to be a suitable parameter for the presence of chicken meat in meat products. Similarly, Carnegie and others (1985) used HPLC method to monitor the adulteration of cooked beef products with meat from other species. They used the ANS/CAR ratio to distinguish differences between sheep, cattle, horse and kangaroo. Recently, a simple, rapid and reliable method based on HPLC with electrochemical detection was developed to routinely differentiate among meat products from fifteen food animal species. They used using copper nanoparticle-plated electrodes for the rapid differentiation (Chou et al., 2007).

The chromatographic methods are not most suitable method to use in meat authentication analysis, because of the difficulties in understanding the complex chromatographic data sets observed from meat mixtures including target adulterants and more time is usually required for sample preparation and derivatization steps (Kesmen and Yetim 2012).

2.1.3 Immunoassays

Immunoassays are the biochemical tests that based on antigen-antibody interaction in order to measure the presence or concentration of a macromolecule in a sample. Enzyme-linked immunosorbent assay (ELISA) is a method that uses antibodies and color change to detect a target substance. The ELISA is the most common used technique for meat identification. Many commercial ELISA kits are available for widely used in food identification. Eurofins, EuroProxima, ELISA Technologies Inc., Neogen Corporation, Strategic Diagnostics Inc., Tepnel are the commercial companies have developed a variety of ELISA test kits for meat identification.

Numerous ELISA methods have been applied with using both polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) to detect the species of origin of the meat products.

In early studies PAbs has been used, for instance, ELISA has been developed to differentiate between unprocessed beef, sheep, horse, kangaroo, pig and camel meats with using species-specific rabbit antisera (Whittaker et al., 1983). In another study, a double-antibody sandwich ELISA has been successfully developed by using horse-

specific antibodies for the detection of defined amounts of horse meat (1-50%) in unheated meat mixtures (Martin et al., 1988). Compared with MAbs, PAbs are more preferred for the detection of denatured proteins because PAbs provide more robust detection and tolerance to small changes in the nature of the antigen. However, PAbs have reproducibility problems and extensive purification procedures. Unlike PAbs, MAbs usually have very high specificity and reproducibility.

On the other hand, the MAbs development requires high-level technology, besides it is costly and time consuming than the development of PAbs.

MAbs have been applied in many studies for authentication meat species (Billett et al., 1996; Djurdjevic et al., 2005; Liu et al., 2006). Chen and Hsieh (2000) developed ELISA using a monoclonal antibody to a porcine thermo-stable muscle protein for detection of pork in cooked meat products. Djurdjevic and others (2005) developed a monoclonal antibody (Mab)-based ELISA for the quantitative detection of chicken and turkey meat adulterated in cooked (100 °C, 15 min) mammalian meat.

The ELISA is preferred because of its specificity, simplicity, sensitivity, and suitability for routine controls of the foods (Hsieh 2005). On the other hand, detection limit in processed products depend on various parameters, such as the fat content, the severity of heat processing, the origin of muscles, and the maturation state of the meat (Giovannacci et al., 2004). Besides, producing a specific antibody to a target is difficult and antibodies may be unstable at extreme pH or high salt or solvent concentrations. These are main advantages of ELISA methodology.

2.2 DNA-Based Methods

DNA is more thermo-stable and resistant to pressure and chemical compounds than many proteins, it shows the same features in all cells and tissues. That facilitates for extraction the DNA from various types of samples: blood, liver tissue, bones, muscle or from hair. DNA has the potential to provide a greater amount of information. Due to all these features, in the past three decades DNA-based technologies are preferred rather than protein-based technologies for authenticating meat species.

2.2.1 DNA hybridization

Nucleic acid hybridization techniques are based on ability to create double-stranded hybrid molecules by itself from a single-stranded nucleic acid molecule under appropriate conditions and with complementary sequences. These original reactions are used to determine a specific nucleotide sequences on both RNA and DNA molecules. The target nuclear material can be detected and quantified by using labeled probes. Nucleic acid Hybridization techniques are Southern Blotting (for DNA), Northern Blotting (for RNA) and In Situ Hybridization (both DNA and RNA in cell or tissue).

In early studies, DNA hybridization techniques were utilized for the detection of meat species. Ebbehøj and Thomsen (1991a) was developed a method for quantitation of pork by using a 32P-labeled probe made from genomic porcine DNA in heat-treated meat products. However, this technique was unsuccessful in discrimination of closely related species because of cross-hybridization. The same researchers reduced the cross hybridization between probe and DNA sequences from closely related species by addition of unlabeled DNA from the cross hybridizing species (Ebbehøj and Thomsen, 1991b). In another study, Chikuni and others (1990) utilized dot-blots hybridization technique to the detection of species-specific DNA fragments by using biotin-labeled chromosomal DNA fragments in the cooked meats of chicken, pig, goat, sheep, and beef. The oligonucleotide probes which are highly specific for species are developed for the identification of meat from cattle, sheep/goat, horse, deer, pig, chicken and turkey. It was reported that the differentiation between closely related species like chicken and turkey was possible (Buntjer et al., 1995).

The quantitative hybridization signal is influenced by factors such as tissue origin and sample processing (Buntjer et al., 1999). Also, DNA hybridization is expensive and time-consuming methodology. Therefore DNA hybridization is not suitable for the routine species determination in food and food products.

2.3 PCR- Based Techniques

The Polymerase Chain Reaction (PCR) is used to obtain multiple copies of a desired gene or specific DNA sequences from 1980s with development of thermo-stable

Thermus aquaticus (Taq) DNA polymerase by Kary Mullis. The best description of PCR is "The process comprises treating separate complementary strands of the (target) nucleic acid with a molar excess of two oligonucleotide primers to form complementary primer extension products which act as templates for synthesizing the desired nucleic acid sequence." by US patent number 4,683,202. A PCR cycle comprises of denaturation (at ~95°C), primer binding (annealing, at 50-65°C depends on GC% content) and extension (at 72°C) steps.

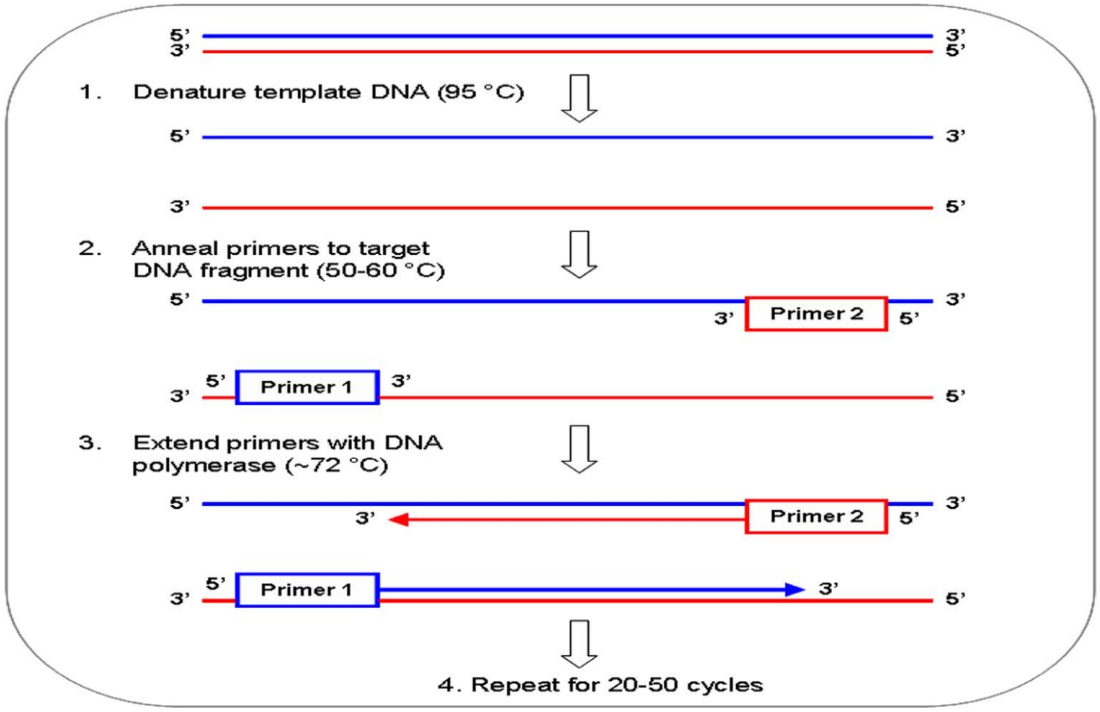


Figure 2.1 : Main steps in the amplification of a target DNA fragment with the polymerase chain reaction (Rasmussen and Morrissey 2008).

PCR based methods have been used in basic molecular biological research (cloning, sequencing, DNA mapping etc.) and for the diagnosis based on DNA of many diseases (Leukemia, cystic fibrosis, AIDS etc.) in clinical medicine. PCR-based methods provide a potential for the detection of the animal species, even for the products that have been exposed to heat processing (Kesmen, Sahin and Yetim, 2010). A number of PCR-based methods have been developed for species detection in meat products. These studies are summarized as follows.

2.3.1 Sequencing of PCR products

DNA sequencing is the most straightforward way of acquiring information of a DNA molecule sequence. In the mid-1970's two methods were developed for directly sequencing DNA. These were the Maxam-Gilbert chemical cleavage method and the Sanger chain-termination method. In the Maxam-Gilbert method; DNA is labeled and then chemically cleaved in a sequence-dependent manner. However chemical reactions of most protocols are slow and the use of hazardous chemical requires special handling care and automation of this method is difficult. In Sanger sequencing, the DNA to be sequenced serves as a template for DNA synthesis and is based on the use of dideoxynucleotides (ddNTP's) in addition to the normal nucleotides (dNTP's) found in DNA. The chain-termination is most popular protocol for sequencing and it is adaptable, scalable to large sequencing projects, it uses fewer toxic chemicals and lower amounts of radioactivity than the Maxam method.

Sequencing is used for acquiring information from PCR products in authentication meat species studies. For example, the 18S ribosomal RNA gene is targeted for the detection of kangaroo, cattle, crocodile, turkey, frog, and Alaska Pollack species (Matsunaga et.al., 1998). In other study; cattle, pig, sheep, chicken and turkey were detected with the sequence analysis of cyt b gene amplification products (Bartlett and Davidson, 1991). Although sequencing is accurate and precise method, it cannot be used to detect adulterants in admixed meats because the evaluation of the sequence data from a mixture is not possible. Therefore, it is generally used to confirm the results that are obtained from species-specific PCR method and qPCR.

2.3.2 Species-specific PCR and species-specific multiplex PCR

Species-specific PCR assay was found to be rapid and cost effective for identification of meat species due to specific detection of target sequence without the need of further sequencing or digestion of the PCR products with restriction enzymes (Rodriguez et al., 2004) and successfully used for identification of various species of meat (Frezza et al., 2008). Under optimized amplification conditions, species-specific primers can produce a specific amplicon as a complementer only to the DNA of the target species within a heterogeneous DNA pool obtained from a food product. If the complete sequence information of an amplified fragment is present, identification can be verified according to the amplicon size determined

electrophoretically (Lockley and Bardsley 2000).Recently in many studies, specific primers for many animal species were designed on mitochondrial genes; such as cyt b gene (Pascoal et al., 2005), and 12S ribosomal DNA (Che Man et al., 2007) and actin genes (Rodríguez et al., 2003); these genes have been successfully used in species detection in meat products. For instance, Ilhak and other (2006) determined the origin of horse, dog, cat, bovine, sheep, porcine, and goat meat by PCR technique, using species-specific primers that designed on mitochondrial DNA. Recently, a highly specific single step PCR was employed for the detection of pig meat by using designed species-specific primer pairs based on mitochondrial D-loop and 12S ribosomal ribonucleic acid (rRNA) gene (Kumar et al., 2012).

Although species-specific PCR methods are the most appropriate method for the detection of different meat species in meat mixtures; false-positives because of cross-homology and the semi-quantitative results are the major drawbacks of these methods.

Multiplex PCR is the process of amplification of many target regions at the same time with using more than one primer pair in a single reaction. In species-specific Multiplex PCR, primer design is critically important in this methodology. The length of the amplicons that are produced by these primers is the key point to analyze different species. The length of each fragment can be predicted if the complete sequence is known, and a given species can be identified by gel-based visualization of an amplicon of appropriate size (Lockley and Bardsley 2000).

Matsunaga and others (1999) developed a quick and simple multiplex PCR method for the identification of six different meat species (cattle, pork, chicken, sheep, goat, and horse) in raw and cooked meats. Similarly, a duplex PCR-based assay was described for the detection of pork meat in fresh horse sausages and it was also used to evaluate the presence of fraudulently added pork meat (Di Pinto et al., 2005). Even though these two PCR based methods are extremely useful and appropriate for identification meat species, on the other hand they are time consuming and impractical when compared to the qPCR.

2.3.3 PCR-restriction fragment length polymorphism

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis is based on the generation of a species-specific pattern of the restriction fragments by the

digestion of PCR amplicons with one or more appropriate restriction enzyme that recognizes specific DNA sequences (Kesmen and Yetim, 2012). Both nuclear and mitochondrial genes have been targeted for the identification of meat species in several PCR-RFLP studies. Among the widely used mitochondrial genes, the cytochrome b gene (Murugaiah et al., 2009; Erwanto et al., 2012), 12S rRNA gene (Gupta et al., 2008), and the 16S rRNA gene (Borgo et al., 1996) have been used for species identification in raw and heat-treated meat samples. Advantage of this methodology is closely related species can be separated without the need for a sequence analysis.

In addition, although this technique is suitable for the identification of raw and heat-treated pure species, the analysis of meat mixtures is difficult since the results may not be representative of the target species present in the mixture (Partis et al., 2000).

2.3.4 PCR-random amplified polymorphic DNA

Unlike traditional PCR analysis, PCR-Random Amplified Polymorphic DNA (PCR-RAPD) does not require any specific knowledge of the DNA sequence of the target organism; it is possible to detect the meat species using short PCR primers of ~10 bases which are designed randomly. Arbitrary primers generate species-specific "fingerprints" whose visualization occurs after performing electrophoresis (Spychaj and Mozdziak, 2009). This technique has been applied successfully in many meat identification studies. For example, meats of 8 poultry (chicken, turkey, gull, ostrich, duck, goose, quail, and partridge) were identified by RAPD method using two different primers of 10 nucleotides each (Arslan et al., 2004).

Saez and others (2004) used the PCR-RAPD for the simultaneous identification of five animal species (pork, beef, lamb, chicken, and turkey) in meat products, such as; hamburgers, raw sausages, dry fermented sausage, and cooked meat products.

PCR-RADP was also used to identify raw meats of: a wild boar, a pig, a horse, a bi-son, a cow, a dog, a cat, a rabbit and a kangaroo. In this study, they used a commercially available set of primers to obtain characteristic electrophoretic patterns (Koh et al., 1998). The main advantages of the PCR-RADP method are relatively cheap and simple to perform.

However, this method has also its drawbacks: the interpretation of gel results is generally difficult, the results of the analysis vary depending on intraspecific polymorphisms and PCR conditions, and it is not suitable for the species identification of meat mixtures (Kesmen and Yetim, 2012).

2.3.5 PCR-single-strand conformation polymorphism

The PCR-single-strand conformation polymorphism (PCR-SSCP) technique allows detection of mutations as well as polymorphisms occurring in DNA (Spychaj and Mozdziak, 2009). PCR-SSCP is a simple and reliable method containing sequentially PCR amplification, denaturation of PCR product, and the analysis of denatured fragments by electrophoresis.

Under proper conditions, denatured products with different secondary structures move at different speeds and produce species-specific profiles (Lockley and Bardsley, 2000). SSCP has been applied successfully to distinguish domestic and wild porcine species (Rea et al., 1996) and to identify many fish species (Weder et al., 2004).

2.3.6 QPCR

The most recent reports showed that meat species identification studies have focused on the use of real-time PCR. In the real-time quantative PCR (qPCR) technique, amplification of the target gene is monitored and measured after each cycle by an increased fluorescent signal. This system enables direct assessment of the results after PCR application without additional detection steps. Thus, qPCR obviates the need for gel electrophoresis to detect amplification products.

The fluorescent signal increases directly proportional to the amount of PCR product in a reaction. Meanwhile, the fluorescent signal is monitored in the qPCR system. Computer data analysis software recorded and displayed the amount of fluorescence emission at each cycle in relative fluorescence units (RFU). This analysis system enables real-time calculation and plotting.

In real-time assays, quantification of target sequences is determined by identifying the cycle number at which the reporter dye emission intensity rises above background noise. That cycle number is referred to as the threshold cycle (C_t). Thus, the C_t value is a quantitative measurement of the copies of the target present in any

sample and is inversely proportional to the copy number of the target. Primer design is the most critical step in qPCR. Generally, primers lengths should be 18-24 nucleotides and primers pairs should have compatible melting temperature with each other. The temperature differences between primer pairs should be within 5°C. Additionally, primer pairs should contain approximately 50% Guanin-Cytosin (G-C) content.

A number of fluorescence-based approaches have been employed to obtain a fluorescent signal from PCR products and each has specific assay design requirements. These are DNA-binding dyes, hybridization probes, hydrolysis probes. The most commonly utilized detection chemicals in meat identification are briefly reviewed below.

2.3.6.1 Probe-based detection systems

Target-specific probes use fluorogenic probes to detect the PCR products of interest that accumulates during PCR. Thus, fluorogenic probes allow the specific detection of target sequences. Fluorescence is the property of emitting electromagnetic radiation in the form of light as the result of (and only during) the absorption of light from another source (Lakowicz, 2006). Probe-based detection systems, including hybridization and hydrolysis probes, use the fluorescence resonance energy transfer (FRET) principle. FRET is a mechanism that based on distance-dependent energy transfer between two chromophore/dye molecules that can interact with each other. FRET is the transmission of energy from a donor molecule to an acceptor molecule. The donor molecule is the dye and is usually called the reporter that initially absorbs the energy. The other one is acceptor or quencher molecule, can be fluorescent dye or a non-fluorescent molecule that absorbs any fluorescence emitted by the reporter when in close vicinity. When probe structure disrupted during PCR cycle, reporter dye gives off its energy and the emitted fluorescent signal from the reporter dye is monitored during the reaction. The most widely used reporter dye is 6-FAM, the other common fluorescent dyes are ROX™, VIC™, HEX™, JOE™, TET™, Yakima Yellow™, Cy3™, and Cy5™ (Kesmen and Yetim, 2012). Several commonly used quenchers are 6-carboxy-tetra-methyl-rhodamine (TAMRA), 4-(dimethylamino) azobenzene-4'-carboxylic acid (DABCYL), and black hole quencher (BHQ) (Kesmen and Yetim, 2012).

TaqMan is the most commonly used fluorogenic probe system among the hydrolysis probe-based chemistries. TaqMan probe is designed to bind to the amplified sequence by the primers. TaqMan probes are designed with the fluorescent reporter dye at the 5' end and a quencher dye that inhibits fluorescence at the 3' end. In annealing phase of the PCR cycle, the hydrolysis probe has bound to target sequence on the template DNA after denaturation step. During the extension phase, the probe is cleaved by the 5'- 3' nuclease activity of the Taq DNA polymerase; this separates the quencher from reporter dye, released reporter dye generates a fluorescent signal that increases with each cycle (Figure 2.2). The accumulation of probe-specific PCR product is monitored and quantified by a real-time PCR instrument.

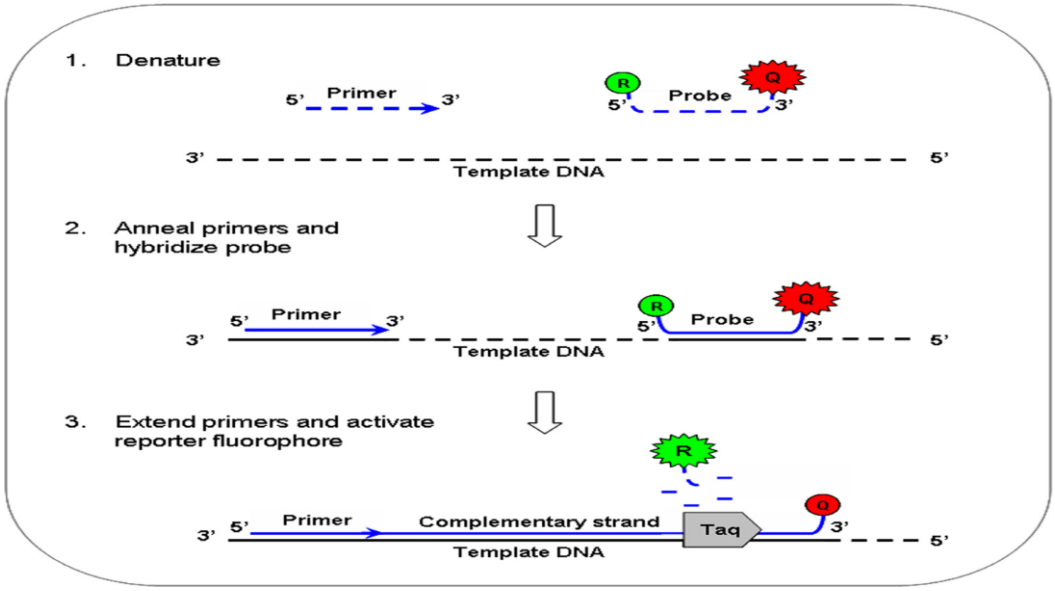


Figure 2.2 : QPCR using TaqMan probes. (Rasmussen and Michael T. Morrissey 2008)

Taqman probes have been commonly used in meat species identification. Numerous species-specific qPCR (TaqMan) assays have been developed for the species identification studies. For example, Dooley and others (2004) developed a qPCR assay based on the amplification of a fragment mitochondrial cytochrome b (cytb) with using two different TaqMan probes (mammalian, poultry) for detection of beef, pork, lamb, chicken and turkey. In the other study, specific primers and TaqMan probes were designed on the mitochondrial ND2, ND5 and ATP 6-8 genes for donkey, pork and horse, respectively (Kesmen et al., 2009). Similarly, Rodríguez and others (2004) developed a highly specific qPCR, based on the amplification of a fragment of the mitochondrial 12S ribosomal RNA gene (rRNA) for the quantitation

of pork in binary pork/beef muscle mixtures. Laube and others (2007) developed species-specific system that able to amplify DNA regions with located on the single-copy genes cyclic guanosine monophosphate (cyclic GMP) phosphodiesterase, ryanodine receptor and interleukin-2 precursor for detection of beef, pork, lamb, goat, chicken, turkey and duck in processed foods.

Alternative fluorescence detection system is Scorpion containing two primers; one of which serves as a probe and contains a hairpin-loop structure at the 5’ end, the other one is primer sequence at the 3’ end. The hairpin structure of Scorpion brings the reporter and quencher into close proximity, so that the quencher absorbs the emitted fluorescence by the reporter. During the first amplification cycle, target-specific primer of the Scorpion anneals to the target sequence and then the DNA polymerase synthesizes the complementary strand. During next cycle, the hairpin loop unfolds and the loop-region of the probe hybridizes intra-molecularly to newly synthesized target sequence. After the conformational reorganization, reporter is no longer in close proximity to quencher and emitted fluorescence from reporter dye can be observed. The Scorpions probe contains a PCR blocker, just 3' of the quencher, to prevent read-through during the extension of the opposite strand (Figure 2.3).

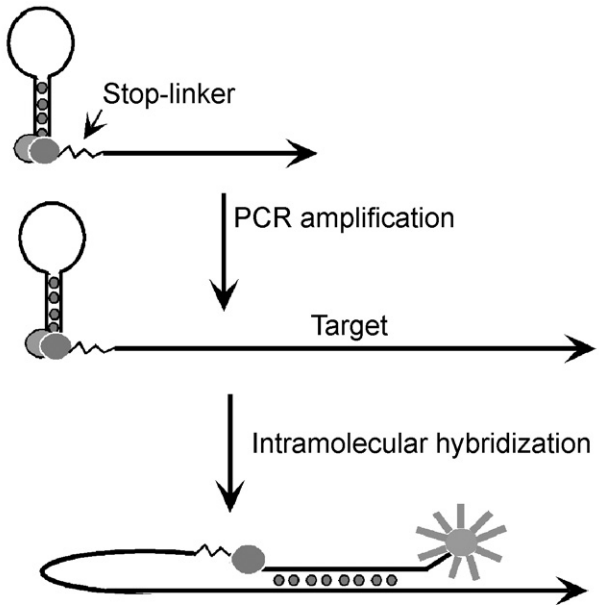


Figure 2.3 : Schematics of the Scorpion probe (Broude, 2004).

Sawyer and others (2003) utilized the Scorpion primer to measure of beef in mixed sample.Hybridization probe (HybProbes) is another fluorescence-based detection

system. HybProbes consists of two sequence-specific hybridization probes that are designed bind adjacent to sequences in the target. One probe has a donor dye and is labeled at the 3' end, and the other probe has an acceptor dye and is labeled at the 5' end. The free 3' hydroxyl group of second probe blocked to prevent extension during the annealing step. During the annealing step of qPCR, both probes hybridize to their target sequence in a head-to-tail arrangement. This brings the donor and acceptor dyes into close proximity and the reporter is excited and passes its energy to the acceptor dye by FRET. The emitted fluorescence wavelength from the acceptor dye is detected by the real-time instrument and recorded. The increasing amount of fluorescence signal is directly proportional to the amount PCR product present. HybProbes has been utilized in many meat species identification studies. Frezza and others (2008) designed four species-specific primers and probes for the detection and quantification of bovine, ovine, swine and chicken mitochondrial DNA (16S rRNA, cyt b, ATPase 8) in feeds. In another study, Rensen and others (2006) used a single set of primers and two sets of FRET probes targeting the ruminant-specific mitochondrial cytochrome b gene for detecting and discriminating between bovine, ovine, and caprine contaminates in cattle feed.

Probe color and melting temperature can be used for the simultaneous amplification and detection of two or more DNA targets in a single reaction. The multiplex PCR provides powerful real-time analysis. Differentiate the target genes is possible with sequence-specific oligonucleotide TaqMan or Hyb probes that are labeled with fluorophores that emit light at different wavelengths. Köppel and others (2008) developed a quantitative multiplex PCR for the quantification of beef, pork, chicken and turkey. In another study, this time; to measure the fractional proportion of each pork, beef, chicken, turkey, horse meat, sheep (mutton) and goat meat types simultaneously, a quantitative multiplex PCR has been developed (Köppel et al., 2009).Probe based systems are highly sensitive and specific detection of DNA and allows quantification of multiple meat species simultaneously. However, costs of these probes are extremely high.

2.3.6.2 Intercalating dyes-based detection systems

The most commonly-used intercalating fluorescent dye is SYBR Green I in qPCR studies. SYBR Green I binds to double-stranded DNA (dsDNA) in the reaction,

including nonspecific PCR products or primer dimers. SYBR Green I dye forms three different interactions with DNA: intercalation between base pairs, electrostatic interaction and extended contact with the groove of DNA (Dragan et al., 2012). Sybr Green dye has a slight preference for AT-rich sequence in the minor groove of DNA (Mao et al., 2007). The intensity of the fluorescent emissions of DNA-binding dyes increases when bound to dsDNA. As dsDNA accumulates, the intensity of the fluorescent signal that is proportional to the PCR product and can be detected using real-time PCR instruments. The major drawback of intercalating dyes is their lack of specificity; PCR artifacts such as primer-dimers and non-specific products can be detected by real-time PCR instruments. This overestimate to overall fluorescent signal and affect the accuracy of quantification. So, false positives can arise. This drawback can easily be overcome by using melting curve analysis to determine the melting temperatures. The melting curve analysis can help discrimination between the desired PCR products and any nonspecific products, or between different amplicons in a multiplexed reaction; based on the G + C% content and length of the amplicon.

SYBR Green I-based PCR methods have been employed for the identification and quantification of meat species in food and feed products. For example, uniplex and duplex qPCR assays with a SYBR Green I post-PCR melting curve analysis were evaluated for the identification and quantification of bovine, porcine, horse, and wallaroo DNA in food products (López-Andreo et al., 2006).

In another study, three species-specific intra-SINE-based PCR assays have been developed for the identification and quantitation of bovine, porcine, and chicken DNA and a multispecies ruminant-specific intra-SINE-based PCR assay for the sensitive detection of common ruminant species (Walker et al., 2003). The same researchers designed series of class-specific (Aves), order-specific (Rodentia), and species-specific (equine, canine, feline, rat, hamster, guinea pig, and rabbit) quantitative PCR assays based on the amplification of genome-specific short and long interspersed elements with using SYBR Green-based detection (Walker et al., 2004). Recently, Martín and others 2009 developed a qPCR SYBR Green method using primers targeting the porcine-specific mitochondrial 12S rRNA gene for the detection and quantification of porcine DNA in mixtures containing <0.1% porcine tissue in a heat-treated material. According to studies published to date; when

compared SYBR Green and TaqMan-based detection system, no significant difference sensitivity was observed. In SYBR Green based qPCR system, only two sequence-specific primers are needed and probe design is not necessary. Also, it has the ability to test multiple genes quickly without designing multiple probes and cheaper than probe-based systems. However, SYBR Green can inhibit PCR reactions if used above a certain (non-saturating) concentration. At high concentrations, SYBR Green-I does not only intercalate between base pairs, it also binds to single stranded DNA as a result of electrostatic interactions and inhibits the DNA polymerase. High Resolution Melting (HRM) dyes are preferred for use with high resolution melt assays due to the more discrete signal change occurring upon DNA denaturation. HRM dyes only bind to double stranded DNA that prevents the dye molecule from redistribution during melting and provides superior melt curve resolution. Unlike SYBR Green dye, HRM dyes can be used at high concentrations because they do not inhibit DNA polymerases and PCR reaction. HRM dyes great ability to bind the hydrogen bond almost 4 times more than SYBR Green. HRM dyes such as EvaGreen, LC Green and LC Green Plus, ResoLight, Chromofy and SYTO 9 are available on the market. Especially, EvaGreen (also known "release-on-demand" dye) has emission spectra very close to those of fluorescein (FAM) or SYBR dye Green I. Also, it is non-mutagenic and extremely stable both thermally and hydrolytically. This is novel method of fluorescence emission, where the fluorescent signal is quenched when the dye is free in solution. Upon binding to duplex DNA, the quenching factor is released and the dye emits high fluorescent signal. This allows non-saturating concentrations of the dye to be used, ensuring that there is no PCR inhibition, whilst the unique dye chemistry provides highly sensitive HRM analysis (Figure 2.4).

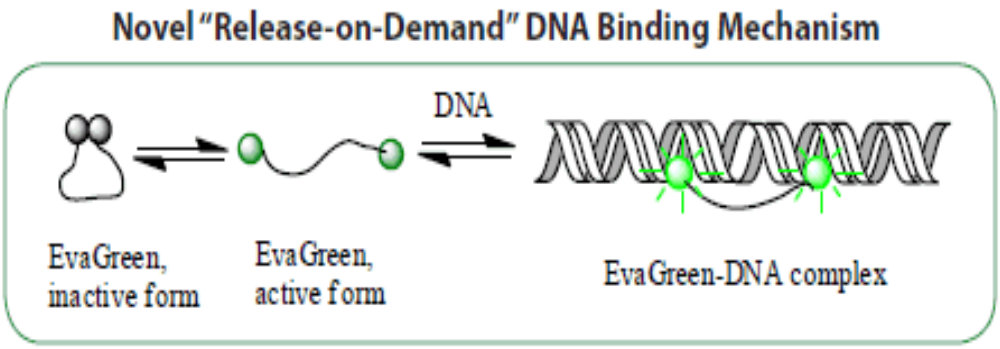


Figure 2.4 : EvaGreen dye mechanism.

3. MATERIALS AND METHODS

3.1 Oligonucleotide Primer Design

PCR-based species detection in meat products mostly focused on the amplification of mitochondrial DNA (mtDNA). Recently, some researchers have been used for the identification of animal derived material in meat mixture mtDNA genes as 12 S rRNA (Rodriguez et al., 2005), cytochrome b gene (Dooley et al., 2004), and 16S rRNA (Sawyer et al., 2003). However, possibility of non-specific amplification is very high due to highly conserved nature of these genes. This is why we selected target gene regions which are highly variable and not conserved between species for the primer design. Selected target gene regions are listed in Table 3.1. Primers were designed for the selected regions by using *Primer 3 (http://frodo.wi.mit.edu)* program. Specificity of the primer pairs were tested by using *Primer Blast* program. For each species, non-specific products were not observed in *Primer Blast* program. Thereby, selected primer pairs were determined whether they amplify only the intended regions. All primers were synthesized by Oligo Macrogen, Korea.

Table 3.1 : Selected target gene regions and primer sets.

Target Species	The Primer sets	Sequence (5'-3')	T_m °C	Target Gene	Product Size
Cattle	Forward	gttccagttccccaaaacaa	59.2	Mucin-like glycoprotein (GLYCAM1) gene, exon 1	257
	Reverse	taaggatggcgagagaggtg	61.2		
Turkey	Forward	tgaatggggagacacatgaa	59.5	MYBP-H gene, 3'UTR Sequence	224
	Reverse	tgctggtcaaaggtgagatg	60.4		
Chicken	Forward	agtaggacgccacctcagaa	62.9	Phosphoenolpyruvate carboxykinase (GTP) gene	102
	Reverse	actgttgagtcgcatggtgt	62.4		
Donkey	Forward	tgcctggttttccactgact	60.6	Isolate F6 BAT1 gene, partial sequence	133
	Reverse	tttgggtatctggcttaggg	59.5		
Pig	Forward	ctgggacatcatccttctgg	60	Ryr1 gene for ryanodine receptor	132
	Reverse	acacacacagggaacacagg	62.6		
Horse	Forward	aaggggcttccaaagttgat	60.4	Apolipoprotein B (ApoB) gene,exon 26 and partial cds	370
	Reverse	actttttggccattggaaag	57.8		

3.2 DNA Extraction

5 different DNA extraction protocols that were different in cell disruption method (Table 3.2) were tried for DNA isolation. The first method was standart hexa decyltrimethylammonium bromide (CTAB) methodology for DNA isolation (Yang et al., 1998). CTAB is a cationic detergent that disrupts protein and lipid molecules, and precipitates carbohydrate molecules. The second one was modification of first CTAB methodology that includes bead beating for physical cell disruption. The third methodology was based on NaCl- HCl treatment (Özsenşoy et al., 2008). In this method high base and high acid concentration were used to destroy the cells and tissues rapidly. The fourth one was modification of the third methodology that includes proteinase K and CTAB treatment. In all of the methodologies, guanidium thiocyanate was used for PCR inhibitor inactivation and as a catiotrophic agent for DNA binding. The best results were obtained using the Protocol 5. Details of the Protocol 5 were given below. The screening of the commercial samples was carried out using this protocol.

1- 400 mg beat and 400 mg homogenized sample and 800 lysis solution (%2 CTAB (100 mM TrisHCl [pH=8], 20 mM EDTA, 1.4 M NaCl) was added into 2 ml eppendorf tube, respectively.
2- In order to homogenization of sample, the mixture was centrifuged at 4500-6000 rpm for 1 minute.
3- The mixture was incubated at 95°C for 10 minutes.
4- The mixture was centrifuged at 14000 rpm for 2 minutes and 400 µl supernatant was transferred into new 2 ml microfuge tubes.
5- 800 µl binding solution (6.75M Guanidinium thiocyanate, 15mM Tris-Cl pH 8.0) and 400 µl isopropanol were added and the sample was vortexed.
6- 800 µl mixtures was added into DNA colon and centrifuged at 14000 rpm for 1 minute and the precipitate was discarded. This step was repeated for the centrifugation of whole sample.
7- 500 µl inhibitor solution (% 60 (5 M thiocyanate, 20 mM Tris-HCl, pH 6.6), %40 EtOH) was added into DNA colon and was centrifuged at 14000 rpm for 1 minute and the precipitate was discarded.

8- 500 µl wash solution (20 mM NaCl, 2 mM Tris-HCl, pH 7.5; 80% v/v Ethanol) was added into DNA colon and was centrifuged at 14000 rpm for 1 minute and the precipitate was discarded.
9- 500 µl wash solution (20 mM NaCl, 2 mM Tris-HCl, pH 7.5; 80% v/v Ethanol) was added into DNA colon and was centrifuged at 14000 rpm for 1 minute and the precipitate was discarded.
10- The empty colon was centrifuged at 14000 rpm for 1 minute and transferred into new clean microfuge tube.
11- Finally, 100 µl elution solution (10 mM Tris-HCl pH 8) was added and incubated for 1 minute. The column was centrifuged at 14000 rpm for 1 min. The eluted DNA was stored at -20 °C.

Table 3.2 : DNA extraction methods.

Protocol Number	Bead Beating	Proteinase K Treatment	NaOH- HCL Treatment	CTAB	Guanidine thiocyanate
1	-	+	-	+	+
2	+	+	-	+	+
3	-	-	+	-	-
4	-	+	+	+	+
5	+	-	-	+	+

3.3 Sampling and the Production of the Reference Material

Pig, cattle, turkey and chicken raw meats were obtained from randomly selected retail butcher shops. Horse and donkey hair were collected from Ankara University, Faculty of Veterinary Medicine. After the genomic DNA extraction, the target DNAs were amplified by qPCR. PCR products from the reference samples were purified by using a commercial PCR product purification kit (GF-1 CLEAN-UP Kit, Vivantis, Malaysia). The purified DNAs were sequenced using the Sanger method. It was determined from sequence analysis that the amplified PCR products were the targeted gene regions. Positive samples are used as a reference DNA.

The commercial processed meat products were obtained from Environmental Industrial Analysis Laboratory, Control Laboratory and Quality System Laboratory. Samples that were collected from different sources were analyzed: 1- the swab samples from different production stages of a meat producer who intended to replace

beef meat production with chicken and turkey meat production and; 2- some meat and delicatessen products which are intended to be introduced to Turkish Food Market. The analyzed samples are listed in Table 3.3.

Table 3.3 : Type and amount of the analysed samples.

Sample Numbers	Sample Type	Target Species
1 – 24	Swab sample	Turkey and Chicken
25 – 32	Sucuk	Horse, Donkey, Pig, Cattle, Turkey, Chicken
33 – 60	Doner Kebap	Turkey and Chicken
61 – 75	Beef sausage	Horse, Donkey, Pig, Cattle, Turkey, Chicken
76 – 83	Beef salami	Horse, Donkey, Pig, Cattle, Turkey, Chicken

3.4 Concentration Determination of Isolated DNA

DNAs extracted from 200 mg samples were diluted in 100 µl and concentrations of DNA extracts were measured by NanoDrop 2000 spectrophotometer (Thermo scientific, USA). The absorbance values were measured at 260 nm and 280 nm for each sample. DNA absorbs UV light at 260 nm, but it is also required to know the absorbance values of proteins at 280 nm in order to evaluate the purity of DNA samples. The ratio of A260/ A280 represents the purity of the samples. Pure DNA should have a A260/ A280 ratio of approximately 1.6-2. If there is contamination with protein and aromatic substances, the A260/ A280 value will be below 1.6 and the A260/ A280 value above 2 indicates possible contamination with RNA (Clark and Christopher 2001). Alternatively, phenolate ion, thiocyanates, and other organic compounds contamination is indicated by 230/260 ratios greater than 0.5 (Clark and Christopher, 2001).

3.5 QPCR

The primer sets and their targets were given in Table3.1. SsoFast™ EvaGreen® Supermix (dNTPs, Sso7d fusion polymerase, MgCl2, EvaGreen dye) and Roche LightCycler® 480 system was utilized for all reactions. Reaction mixes contained 50 ng template DNA, 0.25 µM of each primer and 2.5 µM MgCl2. The following thermo-cycling program was applied: 95°C for 10 min, 30 cycles of 10 s at 95°C, 20s at 65 °C and 25s at 72°C.

Melt-curve analysis was performed from 65°C to 95°C at 0.02 °C/sec ramp rate and the continuous fluorescence acquisition mode to determine T_m of the amplified products.QPCR runs were analyzed using Roche LightCycler® 480 Real Time PCR Software.

3.6 DNA Sequencing

QPCR products amplified from the reference samples were purified by ethanol precipitation and sequenced using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA). The obtained sequences were analyzed in Chromas software package version 1.45 (http://www.technelysium.com/au/chromas.html) and manually checked for reading errors. Homology searches of the sequences in DNA databases were performed with FASTA provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/nucleotide.html). Gene sequences showing 97% similarity or higher was considered to belong to the same gene.

4. RESULTS

4.1 DNA Isolation

The current methodologies for DNA extraction from the meat samples generally results in DNA purities between 1.6-2 and DNA concentrations between 25-1000 ng/µl (Clark and Christopher, 2001; Lahiff et al., 2001; Pinto et al., 2007). The purities and concentrations of the DNA extracts obtained in this study were in the desirable ranges: 1.6-2 and 50-1000 ng/µl, respectively. All DNAs were extracted from 200 mg sample of each target animal. All DNA isolation protocols were performed for 3 times. The spectrophotometer results and standard deviations of each extracted DNA for each isolation protocol were given in Table 4.1. $A_{260/280}$ ratios of DNA extracts from all of the methods were quite similar. On the other hand, $A_{260/280}$ index changed when the DNA source (animal type) changed. This showed that all of the methods results in DNAs with similar purities and the obtained DNA purity depends on the sample type. The best results in terms of DNA concentration were obtained from Protocols 2 and 5 which include beads and CTAB. In these two methods approximately one and a half fold more DNA concentration were obtained compared to the other methods. To comparatively evaluate the DNA quality obtained by different protocols, the same amount of template DNAs (200 ng) were used in qPCR. Since the DNA concentrations and purities were the same for all templates obtained from different protocols, the obtained C_t values indicated the presence of PCR inhibitors. The amplification charts, melting curves and melting peaks onbtained from 5 different protocols were shown in Figure A.1. The obtained C_t values were also given in Table 4.2. All of the templates were resulted in animal species specific T_m values. C_t values obtained using Protocols 2 and 5 were slightly lower than the other protocols. This showed that these protocols were more successful in eliminating the PCR inhibitors. Since Protocol 5 does not include enzymatic digestion steps, Protocol 5 was selected in this thesis. Inclusion of enzymatic steps makes Protocol 2 morre expensive and time consuming than

Protocol 5. DNAs from the commercial samples were isolated using Protocol 5. The spectrophotometer results and standard deviations of the commercial samples were given in Table 4.3. The results showed that the obtained DNAs were in the desired ranges in terms of DNA purity and concentration (Clark and Christopher 2001; Lahiff et al., 2001; Pinto et al., 2007).

Table 4.1 : DNA concentration and purities obtained using 5 different protocols.

	1st Protocol		**2nd Protocol**	
Sample	**Concentration**	**A260/280**	**Concentration**	**A260/280**
Cattle	524.7 ± 20.2	1.86 ± 0.04	701.2 ± 22.3	1.9 ± 0.08
Chicken	604.6 ± 24.6	1.75 ± 0.1	772.2 ± 24.6	1.66 ± 0.05
Turkey	481.3 ± 15.2	1.88 ± 0.08	614.3 ± 23.2	1.87 ± 0.1
Pig	545.1 ± 22.8	1.67 ± 0.05	640.2 ± 26.8	1.68 ± 0.05
Horse	301.9 ± 23	1.83 ± 0.06	479.6 ± 23.4	1.83 ± 0.06
Donkey	454.7 ± 23.18	1.95 ± 0.05	500.4 ± 20.5	1.87 ± 0.1

	3rd Protocol		**4th Protocol**	
Sample	**Concentration**	**A260/280**	**Concentration**	**A260/280**
Cattle	514.9 ± 24.6	1.9 ± 0.1	471.6 ± 25.6	1.91 ± 0.08
Chicken	528.1 ± 20.5	1.72 ± 0.08	543.1 ± 27.4	1.69 ± 0.06
Turkey	382.8 ± 23.6	1.86 ± 0.06	378.6 ± 22.9	1.88 ± 0.09
Pig	443.7 ± 21.2	1.68 ± 0.06	416.9 ± 25.9	1.68 ± 0.05
Horse	323.7 ± 18.9	1.83 ± 0.06	355.3 ± 21.1	1.83 ± 0.06
Donkey	331.3 ± 25.2	1.88 ± 0.09	388.4 ± 19.6	1.87 ±0.09

	5th Protocol	
Sample	**Concentration**	**A260/280**
Cattle	762.2 ± 27.1	1.87 ± 0.05
Chicken	827.4 ± 26.9	1.7 ± 0.07
Turkey	615.2 ± 27.2	1.86 ± 0.06
Pig	672.9 ± 25.3	1.84 ± 0.05
Horse	493.05 ± 23.7	1.87 ± 0.04
Donkey	502.8 ± 18.2	1.87 ± 0.1

Table 4.2 : C_t values obtained using 5 different protocols.

	1. Protocol	**2. Protocol**	**3. Protocol**	**4. Protocol**	**5. Protocol**
Sample	C_t	C_t	C_t	C_t	C_t
Cattle	17.12 ± 0.19	15.45 ± 0.28	18.45 ± 0.56	17.48 ± 0.43	15.37 ± 0.32
Chicken	20.21 ± 0.31	18.52 ± 0.48	21.33 ± 0.45	19.34 ± 0.57	17.12 ± 0.25
Turkey	18.71 ± 0.13	16.62 ± 0.53	19.12 ± 0.18	17.84 ± 0.32	15.25 ± 0.18
Pig	20.13 ± 0.22	18.17 ± 0.43	20.78 ± 0.13	19.38 ± 0.61	17.38 ± 0.46
Horse	17.77 ± 0.43	16.27 ± 0.23	18.25 ± 0.37	17.15 ± 0.52	14.98 ± 0.28
Donkey	18.16 ± 0.23	16.53 ± 0.17	18.76 ± 0.41	17.27 ± 0.53	14.88 ± 0.58

Table 4.3 : DNA concentration and purities of DNAs obtained from the commercial samples.

Sample	Concentration	A260/280	Sample	Concentration	A260/280
1	317.8 ± 18	1.90 ± 0.03	**43**	517.7 ± 20.7	1.89 ± 0.07
2	383.7 ± 20.2	1.93 ± 0.04	**44**	528.5 ± 22.1	1.90 ± 0.07
3	311.4 ± 11.7	1.83 ± 0.05	**45**	660 ± 24.4	1.90 ± 0.02
4	490.0 ± 18.4	1.90 ± 0.04	**46**	546.2 ± 26	1.86 ± 0.07
5	343.4 ± 28.1	1.86 ± 0.05	**47**	596.5 ± 27.8	1.90 ± 0.07
6	594.8 ± 25.4	1.90 ± 0.07	**48**	546.6 ± 21.4	1.86 ± 0.07
7	286.2 ± 18.2	1.86 ± 0.07	**49**	664.8 ± 24.2	1.90 ± 0.06
8	323.6 ± 24.4	1.86 ± 0.09	**50**	548.6 ± 27.0	1.85 ± 0.05
9	484.9 ± 21.3	1.90 ± 0.06	**51**	638.4 ± 19.9	1.89 ± 0.07
10	309 ± 22.2	1.86 ± 0.09	**52**	618.4 ± 19.0	1.90 ± 0.07
11	408.9 ± 17.1	1.87 ± 0.04	**53**	657.6 ± 28.4	1.90 ± 0.02
12	291.3 ± 13.4	1.88 ± 0.06	**54**	543.9 ± 26.6	1.86 ± 0.07
13	313 ± 19	1.86 ± 0.05	**55**	571.9 ± 24.5	1.90 ± 0.07
14	458.8 ± 20.4	1.93 ± 0.04	**56**	313 ± 19	1.86 ± 0.05
15	417.5 ± 25.9	1.83 ± 0.05	**57**	458.8 ± 20.4	1.89 ± 0.02
16	478.4 ± 17.5	1.82 ± 0.03	**58**	417.5 ± 25.9	1.83 ± 0.05
17	387.9 ± 22.8	1.86 ± 0.05	**59**	478.4 ± 17.5	1.82 ± 0.03
18	445.9 ± 23.2	1.88 ± 0.06	**60**	484.9 ± 21.3	1.86 ± 0.06
19	405.1 ± 17.9	1.91 ± 0.08	**61**	647.9 ± 27.2	1.81 ± 0.09
20	572.3 ± 21.1	1.86 ± 0.09	**62**	668.9 ± 24.5	1.82 ± 0.02
21	484.9 ± 21.3	1.86 ± 0.06	**63**	666.5 ± 15.1	1.86 ± 0.05
22	647.9 ± 27.2	1.81 ± 0.09	**64**	672.8 ± 23.1	1.87 ± 0.03
23	668.9 ± 24.5	1.83 ± 0.06	**65**	446.5 ± 63.6	1.89 ± 0.07
24	680.9 ± 22.1	1.86 ± 0.05	**66**	447.8 ± 25.9	1.83 ± 0.05
25	676.3 ± 22.1	1.87 ± 0.03	**67**	477.1 ± 18.8	1.90 ± 0.04
26	677.7 ± 19.4	1.89 ± 0.07	**68**	633.1 ± 20.5	1.86 ± 0.05
27	618.4 ± 19.0	1.90 ± 0.07	**69**	449.9 ± 22.8	1.90 ± 0.07
28	657.6 ± 28.4	1.90 ± 0.02	**70**	423.9 ± 21.1	1.86 ± 0.07
29	543.9 ± 26.6	1.86 ± 0.07	**71**	642.9 ± 20.9	1.86 ± 0.05
30	571.9 ± 24.5	1.90 ± 0.07	**72**	547.9 ± 20.2	1.90 ± 0.07
31	463.7 ± 26.1	1.86 ± 0.07	**73**	568.2 ± 17.1	1.86 ± 0.07
32	517.8 ± 26.7	1.86 ± 0.09	**74**	561.5 ± 24.5	1.86 ± 0.09
33	677.4 ± 17.2	1.90 ± 0.06	**75**	543.3 ± 42.1	1.86 ± 0.06
34	537.9 ± 23.7	1.85 ± 0.05	**76**	466.7 ± 13.1	1.86 ± 0.07
35	414.9 ± 22.8	1.87 ± 0.04	**77**	422.6 ± 20.4	1.86 ± 0.07
36	545.8 ± 27.2	1.86 ± 0.06	**78**	445.0 ± 24.4	1.85 ± 0.05
37	641.4 ± 20.4	1.93 ± 0.04	**79**	525.1 ± 24.7	1.89 ± 0.07
38	447.8 ± 25.9	1.83 ± 0.05	**80**	440.0 ± 27.1	1.90 ± 0.07
39	497 ± 27.3	1.90 ± 0.04	**81**	520.9 ± 18.4	1.90 ± 0.02
40	635.5 ± 15.3	1.86 ± 0.05	**82**	533.7 ± 26.2	1.85 ± 0.05
41	538.2 ± 26.1	1.90 ± 0.07	**83**	530.3 ± 23.9	1.90 ± 0.07
42	523.3 ± 21.8	1.86 ± 0.07			

4.2 QPCR Trials on References Materials

DNA extracts from pork, cattle, turkey, chicken raw meats and horse, donkey hair were amplified via qPCR using the species-specific primer pairs (Table 3.1). DNA amplification curves were analyzed via the second derivative maximum method and C_t value was calculated based on the start of exponential DNA amplification. There was an inverse relationship between identified C_t value and the amount of target DNA present in the analyzed sample.

After the amplification cycles, melting curve analysis was performed and the T_m of PCR products were calculated. T_m is the temperature at which one-half of a particular DNA duplex will dissociate. Each dsDNA has sequence-specific T_m degree. As the PCR product melts and the Eva Green is released into the solution, its fluorescence intensity decreases. A negative first derivation curve of the fluorescence intensity curve over temperature produced by the instrument's software clearly indicates the T_m of the PCR product (peak of the –dF/dT curve). T_m degrees of the each PCR product were shown in Table 4.4. The amplification charts, melting curves and melting peaks were shown in Figure 4.1.

The target specific melting peaks were obtained at as seen in 82.02 ± 0.29˚C for horse, 84.3˚C ± 0.32˚C for pig, 78.80 ± 0.38˚C for donkey, 84.86 ± 0.29˚C for turkey, 81.91 ± 0.34˚C for chicken and 86.96 ± 0.31˚C for cattle. In cattle specific qPCR, a primer dimer was observed around 73˚C. This primer dimer is not important because the second and the much higher cattle specific T_m peaks were obtained at 86.96 ± 0.31˚C.

Table 4.4 : Tm and standard deviations of each target.

Target	T_m (˚C)	Target	T_m (˚C)
Horse	82.02 ± 0.29	**Turkey**	84.86 ± 0.29
Donkey	78.80 ± 0.38	**Chicken**	81.91 ± 0.34
Pig	84.33 ± 0.32	**Cattle**	86.96 ± 0.31

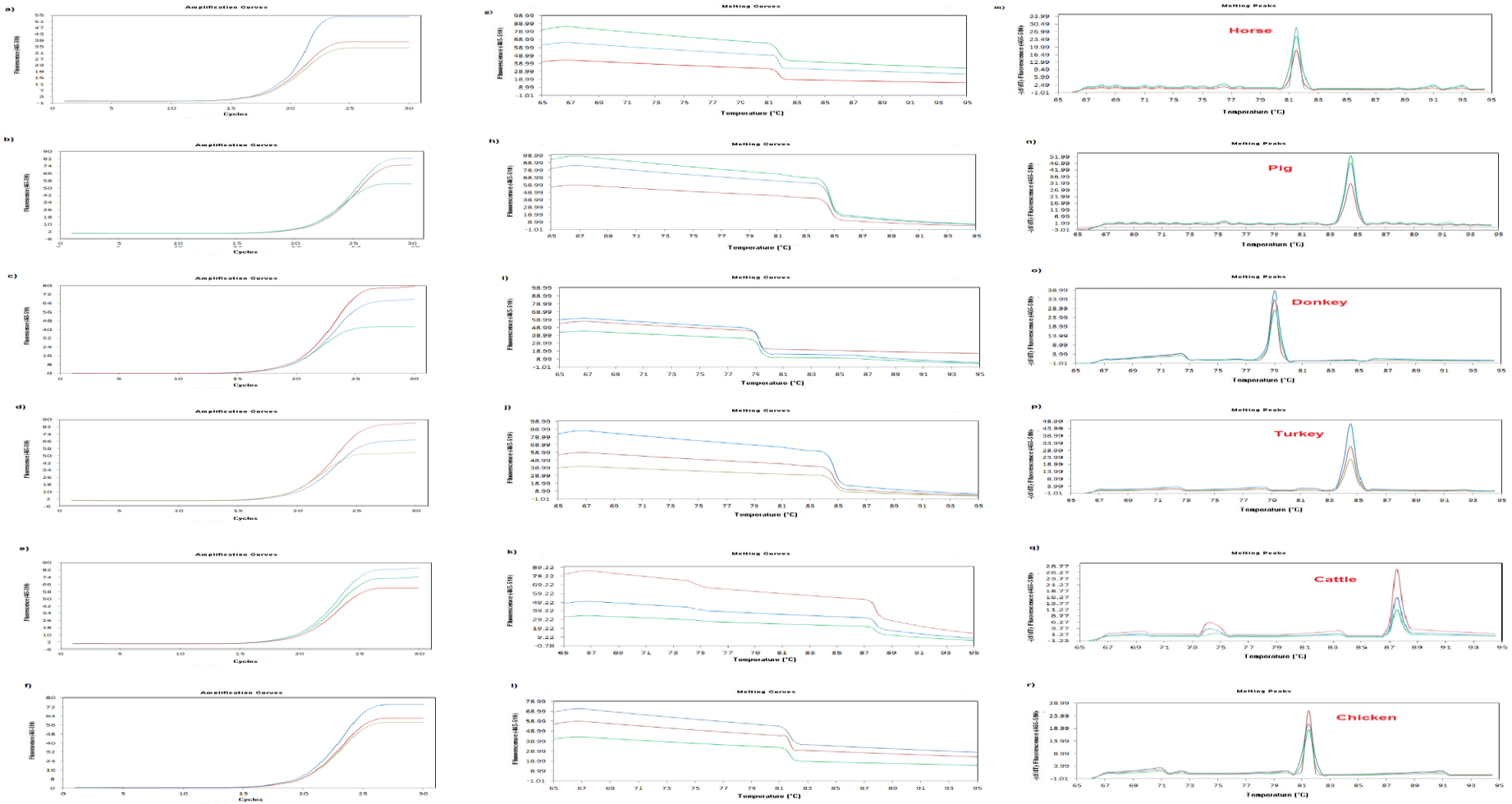


Figure 4.1 : The amplification charts (a, b, c, d, e, f), the melting curves (g, h, I, j, k, l) and the melting peaks (m, n, o, p, q, r) of horse, pig, donkey, turkey, cattle, chicken, respectively. First, second and third runs were shown in blue, red and green, respectively.

4.3 Multiplex QPCR Trials on Reference Materials

The each reference sample was diluted to 50 ng/µl and subjected to qPCR. Binary DNA mixture combinations of reference samples were prepared. The binary combinations were named and represented in Table 4.5. Each prepared mixture was amplified by qPCR. The level of product specific T_m peaks in binary qPCRs negatively correlated to the C_t values of the single template qPCRs. In some of the binary reactions, the qPCR template with a low single qPCR C_t value over-dominated and inhibited the template with a high single qPCR C_t value. DNAs obtained from the different reference samples were mixed at different ratios (1/1, 1/10, 1/100 and 1/1000) and amplified by qPCR to detect the relative copy number detection limit of the primer pairs which result in lower T_m peaks. The amplification charts, melting curve and melt peak charts of these trials were shown in Figures A.2 and Figure A.3. The amplification charts, melting curve and melt peak charts of 1/1 relative template concentrations were given in Figure 4.2. The results showed that; two different T_m peaks were not obtained under 1/100 relative template concentrations but two different T_m peaks were obtained for each target above 1/100 relative template concentrations.

Table 4.5 : The binary combinations.

Mixture Name	Target DNA 1	Target DNA2	Mixture Name	Target DNA 1	Target DNA2
1	Cattle	Chicken	4	Horse	Pig
2	Cattle	Turkey	5	Horse	Donkey
3	Chicken	Turkey	6	Donkey	Pig

After successful binary mixtures, triple mixtures were prepared using the selected dilution of the reference samples. The triple combinations were named and represented in Table 4.6. QPCR results of the 1/1/1 triple mixtures were given in Figure 4.3. Since the existence of three different meat types in a commercial sample is impossible, the detected relative copy number effects on T_m peaks for binary combinations can be applied for the triple combinations. On the other hand, in order to show that three different primer pairs can specifically binds to their targets and does not from additional primer dimers, triple combinations were applied to 1/1/1 relative copy number ratios.

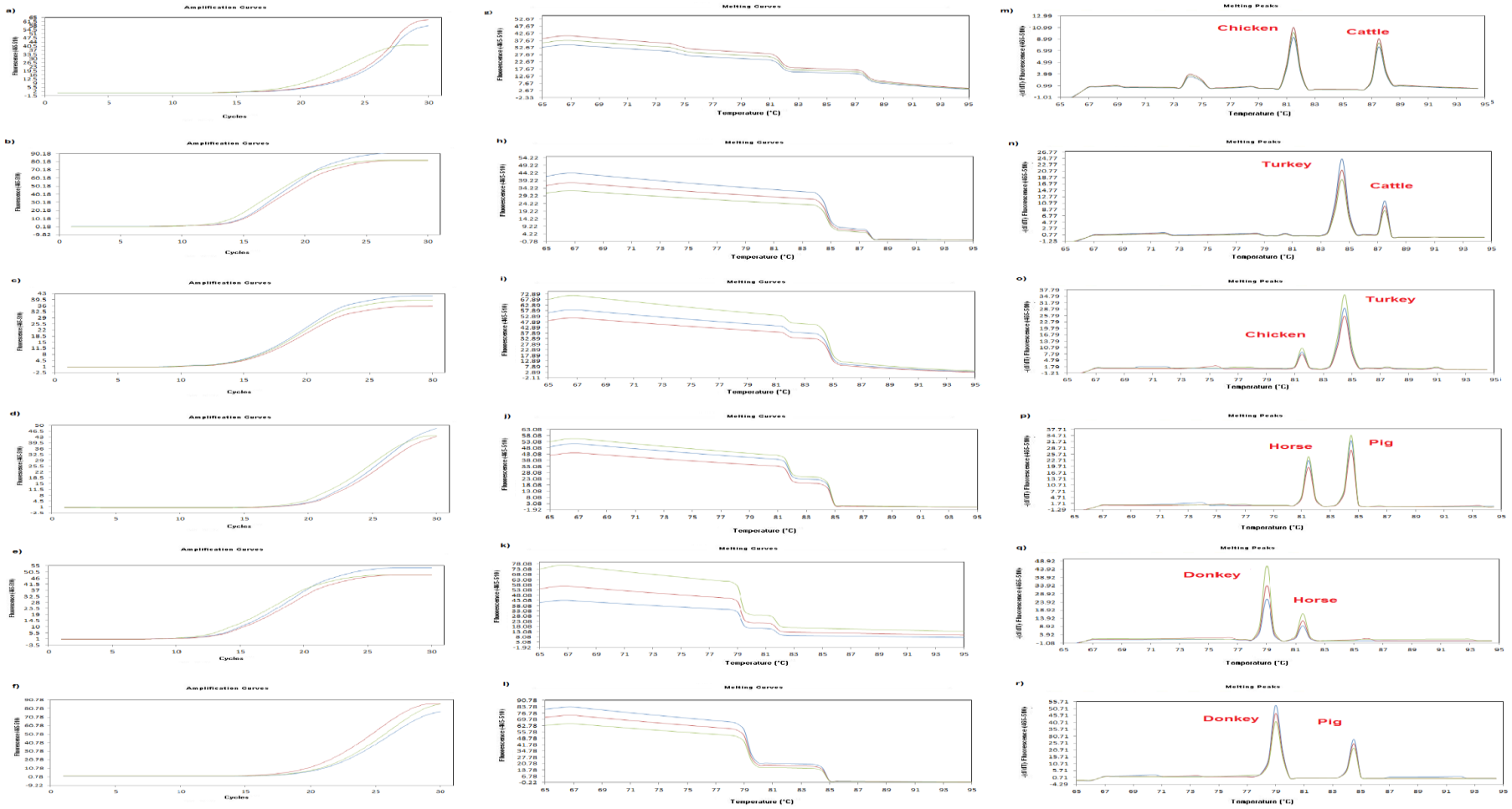


Figure 4.2 : The amplification charts, melting curve and melt peak charts of binary mixtures obtained from Roche LightCycler® 480 Real Time PCR Software. First, second and third runs were shown in blue, red and green, respectively.

Table 4.6 : The triple combinations.

Mixture Name	Target DNA 1	Target DNA 2	Target DNA 3
1a	Cattle	Chicken	Turkey
2a	Horse	Donkey	Pig

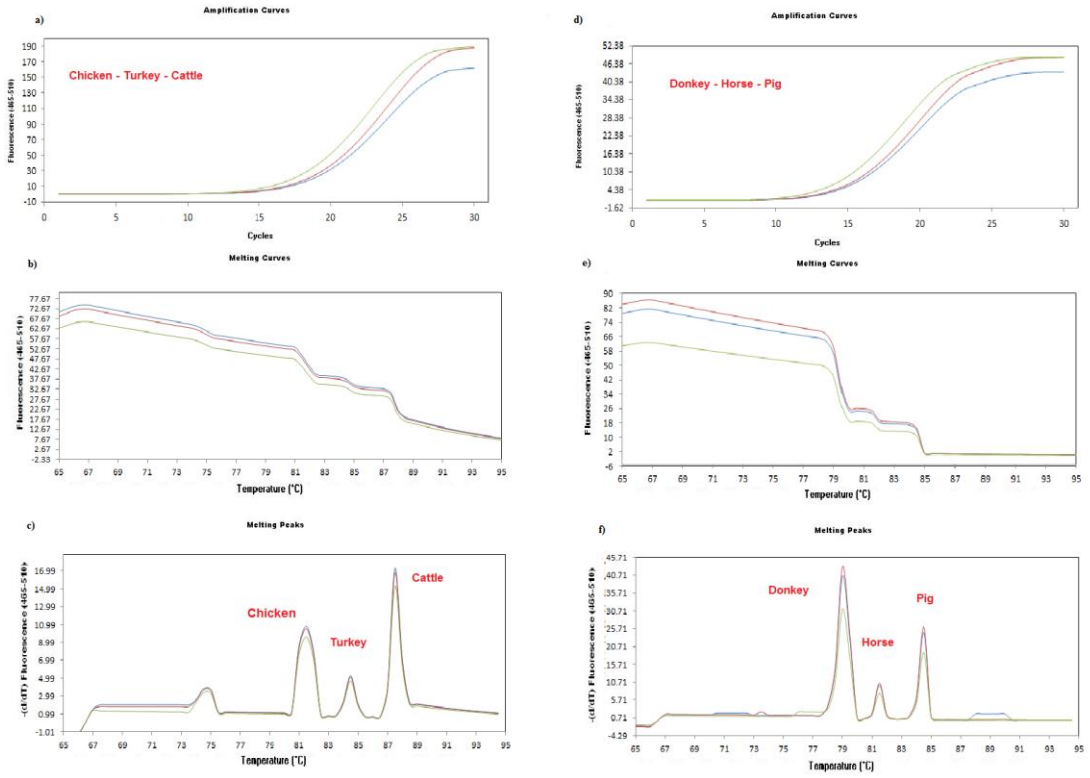


Figure 4.3 : The amplification chart (a), melting curve (b) and melt peak charts (c) of 1a triple mixtures. The amplification chart (d), melting curve (e) and melt peak charts (f) of 2a triple mixtures. First, second and third runs were shown in blue, red and green, respectively.

T_m degrees and standard deviations of the each PCR products for the binary qPCRs were given in Table 4.7. As seen in Figure 4.2, for chicken-cattle specific multiplex qPCR reaction, two different melting peaks were obtained. The melting peak corresponding to chicken species were observed at 81.81 ± 0.12˚C and , the melting peak corresponding to cattle species were observed at 86.86 ± 0.15˚C. In Turkey-cattle specific multiplex qPCR reaction, the melting peak corresponding to turkey species were observed at 84.82 ± 0.10˚C. Both chicken-cattle and turkey–cattle specific qPCRs resulted in the primer dimer peaks around 74˚C. This primer dimer peaks was belong to the cattle specific primer pair. This primer dimer is not important because the cattle, turkey and chicken specific T_m peaks were obtained along with the primer dimer peaks.

In horse-pig specific multiplex qPCR, the melting peak corresponding to horse and donkey species were observed at 82.1 ± 0.36˚C and 84.46 ± 0.15˚C respectively. In horse-donkey specific multiplex qPCR, the melting peak corresponding to donkey were observed at 78.73 ± 0.15˚C.

T_m degrees and standard deviations of each qPCR product of the triple combinations were shown in Table 4.8. As seen in Figure 4.3, in cattle-turkey-chicken specific multiplex qPCR results, the melting peak corresponding to cattle, turkey and chicken species were observed at 87.61 ± 0.18˚C, 84.63 ± 0.15˚C and 81.44 ± 0.19˚C respectively along with the primer dimer at 74˚C.

For horse-donkey-pig specific multiplex qPCR results, the melting peaks corresponding to horse, donkey and pig species were observed at 81.76 ± 0.21˚C, 79.46 ± 0.31˚C and 84.53 ± 0.21˚C respectively.

Table 4.7 : T_m values for each binary combinations.

Target	**T_m (˚C)**	**Target**	**T_m (˚C)**
Horse	82.1 ± 0.36	**Turkey**	84.82 ± 0.10
Donkey	78.73 ± 0.15	**Chicken**	81.81 ± 0.12
Pig	84.46 ± 0.15	**Cattle**	86.86 ± 0.15

Table 4.8 : T_m values for each triple combinations.

Target	**T_m (˚C)**	**Target**	**T_m (˚C)**
Horse	81.76 ± 0.21	**Turkey**	84.63 ± 0.15
Donkey	79.46 ± 0.31	**Chicken**	81.44 ± 0.19
Pig	84.53 ± 0.21	**Cattle**	87.61 ± 0.18

4.4 Specifity and Sensitivity of the Detection Method

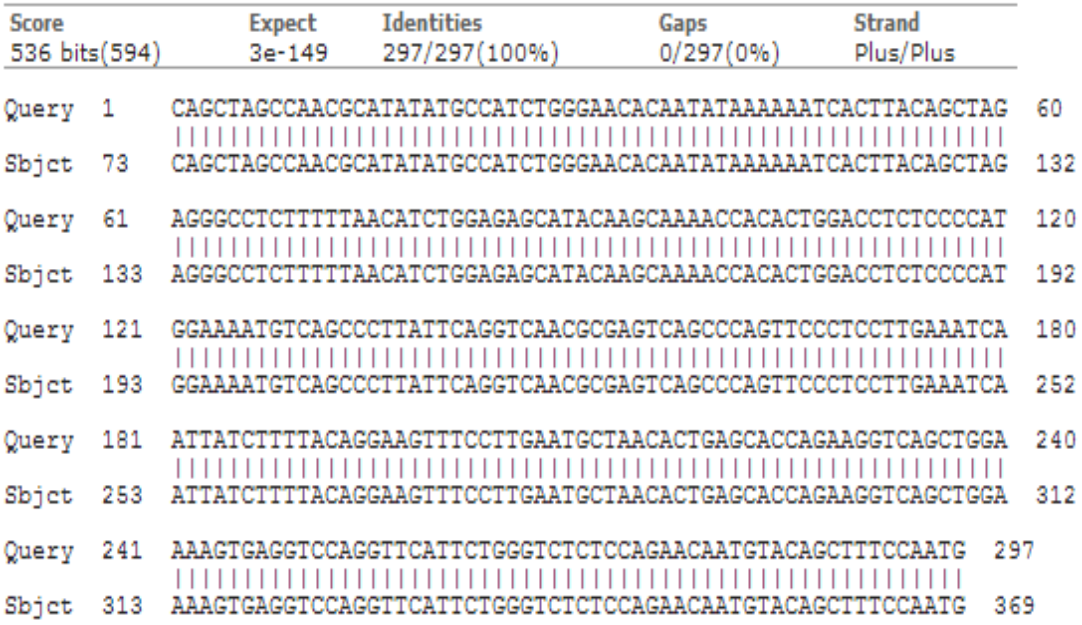
QPCR quantification standards were prepared using the purified PCR products from the reference samples. Molecular weights of the PCR products were calculated based on their DNA sequences.

The gene copy numbers were calculated via dividing DNA concentrations by the molecular weights. Serial dilutions were done to obtain standard samples containing 10^0-10^{10} copies of the targeted gene.

To obtain the limit of detection (LOD), 10 g standard meat mixtures that contain 1-100 copies of the additive meat type were prepared. The limit of detections were 3 cattle gene copies/gr chicken sample, 4 chicken copies/gr cattle sample, 3 turkey gene copies/gr cattle sample, 1 horse gene copy/gr cattle sample, 1 donkey gene copy/gr cattle sample and 1 pig gene copy/gr cattle sample. On the other hand, since the standard meat mixtures were not obtained from an accredited reference laboratory, the detected LODs were rough estimations of the real LODs.

A DNA mixture that contained 50 ng/µl of the each cattle, chicken, turkey, donkey, pig, horse DNAs was prepared to test specificity of the primers. The final mixture contained 300 ng/µl DNA and 0.5 µl of this mixture was used as a template in the trials. The DNA mixture was amplified by qPCR by using species-specific primers. The specifity of the qPCR reactions was examined via sequencing of each amplified PCR products. Homology searches of the obtained sequences were done using blast-n tool of National Center for Biotechnology Information and ClustalW2 of The European Bioinformatics Institute (http://www.ebi.ac.uk/). The homology search results were given in Table 4.9.

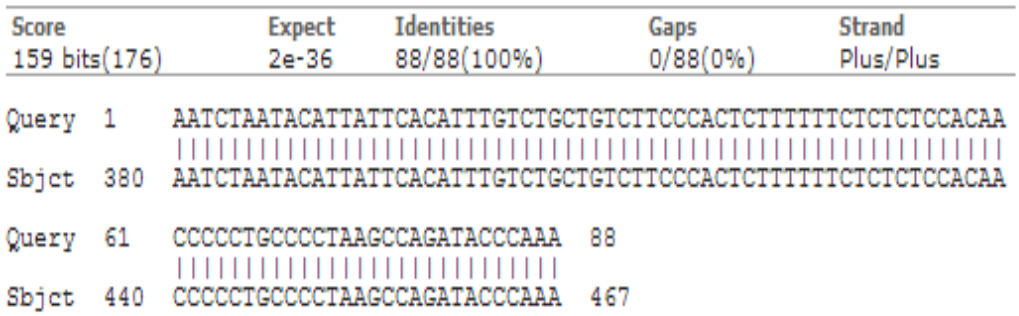
The blast analyses of sequences were explained at Figures 4.4-4.9. The ClustalW2 results of sequences were given Figure 4.10, Figure 4.11, Figure 4.12, Figure 4.13, Figure 4.14, Figure 4.15. Sequence chromatograms of target sequences were given at Figure A.4. The results showed that all of the PCR amplicons were the targeted DNA sequences.



Score	Expect	Identities	Gaps	Strand
536 bits(594)	3e-149	297/297(100%)	0/297(0%)	Plus/Plus

```
Query  1    CAGCTAGCCAACGCATATATGCCATCTGGGAACACAATATAAAAAATCACTTACAGCTAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73   CAGCTAGCCAACGCATATATGCCATCTGGGAACACAATATAAAAAATCACTTACAGCTAG  132

Query  61   AGGGCCTCTTTTTAACATCTGGAGAGCATACAAGCAAAACCACACTGGACCTCTCCCCAT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  133  AGGGCCTCTTTTTAACATCTGGAGAGCATACAAGCAAAACCACACTGGACCTCTCCCCAT  192

Query  121  GGAAAATGTCAGCCCTTATTCAGGTCAACGCGAGTCAGCCCAGTTCCCTCCTTGAAATCA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  193  GGAAAATGTCAGCCCTTATTCAGGTCAACGCGAGTCAGCCCAGTTCCCTCCTTGAAATCA  252

Query  181  ATTATCTTTTACAGGAAGTTTCCTTGAATGCTAACACTGAGCACCAGAAGGTCAGCTGGA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  253  ATTATCTTTTACAGGAAGTTTCCTTGAATGCTAACACTGAGCACCAGAAGGTCAGCTGGA  312

Query  241  AAAGTGAGGTCCAGGTTCATTCTGGGTCTCTCCAGAACAATGTACAGCTTTCCAATG  297
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  313  AAAGTGAGGTCCAGGTTCATTCTGGGTCTCTCCAGAACAATGTACAGCTTTCCAATG  369
```

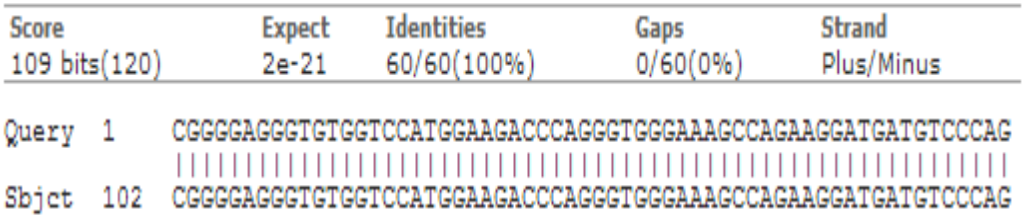
Figure 4.4 : Blast hit analysis of horse sequencing results and targeted Equus caballus apolipoprotein B (ApoB) gene, exon 26 and partial cds (|, indicates the homologous base pairs).



Score	Expect	Identities	Gaps	Strand
159 bits(176)	2e-36	88/88(100%)	0/88(0%)	Plus/Plus

```
Query  1    AATCTAATACATTATTCACATTTGTCTGCTGTCTTCCCACTCTTTTTTCTCTCTCCACAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  380  AATCTAATACATTATTCACATTTGTCTGCTGTCTTCCCACTCTTTTTTCTCTCTCCACAA  439

Query  61   CCCCCTGCCCCTAAGCCAGATACCCAAA  88
            ||||||||||||||||||||||||||||
Sbjct  440  CCCCCTGCCCCTAAGCCAGATACCCAAA  467
```

Figure 4.5 : Blast hit analysis of donkey sequencing results and targeted Equus asinus isolate F6 BAT1 gene, partial sequence (|, indicates the homologous base pairs).



Score	Expect	Identities	Gaps	Strand
109 bits(120)	2e-21	60/60(100%)	0/60(0%)	Plus/Minus

```
Query  1    CGGGGAGGGTGTGGTCCATGGAAGACCCAGGGTGGGAAAGCCAGAAGGATGATGTCCCAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  102  CGGGGAGGGTGTGGTCCATGGAAGACCCAGGGTGGGAAAGCCAGAAGGATGATGTCCCAG  43
```

Figure 4.6 : Blast hit analysis of pig sequencing results and targeted S.scrofa gene for skeletal muscle ryanodine receptor (|, indicates the homologous base pairs).

Score	Expect	Identities	Gaps	Strand
111 bits(60)	5e-22	60/60(100%)	0/60(0%)	Plus/Plus

```
Query  17   GTGAAACACCATCAGCTGAAAGGGAGCCAAATCCCCACTGACACCATGCGACTCAACAGT  76
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  371  GTGAAACACCATCAGCTGAAAGGGAGCCAAATCCCCACTGACACCATGCGACTCAACAGT  430
```

Figure 4.7 : Blast hit analysis of chicken sequencing results and targeted Chicken phosphoenolpyruvate carboxykinase (GTP) gene, 5' end (|, indicates the homologous base pairs).

Score	Expect	Identities	Gaps	Strand
223 bits(246)	3e-55	127/128(99%)	1/128(0%)	Plus/Plus

```
Query  4    GCCCCTGCCAGGCAGCAGCCTCCTCACCAGCACCAAGCAGCCTGCCCGGGGAAAACGGAT  63
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  209  GCCCCTGCCAG-CAGCAGCCTCCTCACCAGCACCAAGCAGCCTGCCCGGGGAAAACGGAT  267

Query  64   GCTGCTACAGCCCCACCATGAAATTCCTCTGCGTCCTGCTTCTGGCCAGCTTGGCCGCCA  123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  268  GCTGCTACAGCCCCACCATGAAATTCCTCTGCGTCCTGCTTCTGGCCAGCTTGGCCGCCA  327

Query  124  CCTCTCTC  131
            ||||||||
Sbjct  328  CCTCTCTC  335
```

Figure 4.8 : Blast hit analysis of cattle sequencing results and targeted Bos taurus mucin-like glycoprotein (GLYCAM1) gene, exon 1 (|, indicates the homologous base pairs).

Score	Expect	Identities	Gaps	Strand
219 bits(242)	4e-54	125/128(98%)	0/128(0%)	Plus/Minus

```
Query  1    GTTCAGCTGCAGACCTTCAGCTCTGCTCCTCTGTCTCACACACCCTTATGCCTGCCCCCA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357  GTTCAGCTGCAGACCTTCAGCTCTGCTCCTCTGTCTCACACACCCTTATGCCTGCCCCCA  298

Query  61   GCCCCCTGTATGTGTATTCAGCTCCATATCTCCAAGCCCTGGCTGTTGTTCATGCGCCTC  120
            |||||||||||| |||||||||||||||||||||||||||||||||| | |||
Sbjct  297  GCCCCCTGTATGTRTATTCAGCTCCATATCTCCAAGCCCTGGCTGTTGTTCATGTGTCTC  238

Query  121  CCCATTCA  128
            ||||||||
Sbjct  237  CCCATTCA  230
```

Figure 4.9 : Blast hit analysis of turkey sequencing results and targeted Meleagris gallopavo MYBP-H gene, 3'UTR sequence (|, indicates the homologous base pairs).

Table 4.9 : The homology search results.

TARGET	BLAST HIT	ACCESSION NUMBER	SIMILARITY
Horse	*Equus caballus* apolipoprotein B (ApoB) gene, exon 26 and partial cds	JN414029.1	100%
Donkey	*Equus asinus* isolate F6 BAT1 gene, partial sequence	HM195470.1	100%
Pig	*Sus scrofa* gene for skeletal muscle ryanodine receptor	X65504.1	100%
Turkey	*Meleagris gallopavo* MYBP-H gene, 3'UTR sequence	AY577442.1	98%
Chicken	*Gallus gallus* phosphoenolpyruvate carboxykinase (GTP) gene, 5' end	K03270.1	100%
Cattle	*Bos taurus* mucin-like glycoprotein (GLYCAM1) gene, exon 1	L36852.1	99%

```
HorseTemp       TGAAGCTGCAAGGGGCTTCCAAAGTTGATGATATCTGGAACCTTGAAGTAAAAGGAAATT 60
HorseSeq        ------------------------------------------------------------

HorseTemp       TTTCTGGAGAAACAGCTAGCCAACGCATATATGCCATCTGGGAACACAATATAAAAAATC 120
HorseSeq        ------------CAGCTAGCCAACGCATATATGCCATCTGGGAACACAATATAAAAAATC 48
                            **********************************************

HorseTemp       ACTTACAGCTAGAGGGCCTCTTTTTAACATCTGGAGAGCATACAAGCAAAACCACACTGG 180
HorseSeq        ACTTACAGCTAGAGGGCCTCTTTTTAACATCTGGAGAGCATACAAGCAAAACCACACTGG 108
                ************************************************************

HorseTemp       ACCTCTCCCCATGGAAAATGTCAGCCCTTATTCAGGTCAACGCGAGTCAGCCCAGTTCCC 240
HorseSeq        ACCTCTCCCCATGGAAAATGTCAGCCCTTATTCAGGTCAACGCGAGTCAGCCCAGTTCCC 168
                ************************************************************

HorseTemp       TCCTTGAAATCAATTATCTTTTACAGGAAGTTTCCTTGAATGCTAACACTGAGCACCAGA 300
HorseSeq        TCCTTGAAATCAATTATCTTTTACAGGAAGTTTCCTTGAATGCTAACACTGAGCACCAGA 228
                ************************************************************

HorseTemp       AGGTCAGCTGGAAAAGTGAGGTCCAGGTTCATTCTGGGTCTCTCCAGAACAATGTACAGC 360
HorseSeq        AGGTCAGCTGGAAAAGTGAGGTCCAGGTTCATTCTGGGTCTCTCCAGAACAATGTACAGC 288
                ************************************************************

HorseTemp       TTTCCAATGGCCAAAAAGAGGCACGCCTTGACGTTGCAGGTTCCCTAGAAGGATACCTAC 420
HorseSeq        TTTCCAATG------------------------------------------------- 297
                *********

HorseTemp       GGTTCTTCAAAGATATTGTCCTACCAGTTTATGACAAGAGCTTATGGGACCTCCTTAAGT 480
HorseSeq        ------------------------------------------------------------

HorseTemp       TGGATGTAACCACCAGCATTGATAGGAAACAGTATCTTCGTGCCTCAACTGCCCTTGTGT 540
HorseSeq        ------------------------------------------------------------

HorseTemp       ACACCAAAAACCCCAATAAGTATTCTTTCTCTATCCCTGTGCAAGAATTGGCTGATAAAT 600
HorseSeq        ------------------------------------------------------------

HorseTemp       TTATTATTCCTGGACTGAAACTAAATAATCTGAATTCAGTTCTTGTCACACCTGCGTTCC 660
HorseSeq        ------------------------------------------------------------

HorseTemp       AAGTCCCATTTACTGGTCACGAGGTTCCATCCTACACACTAGACTTARGTGAAATAATAA 720
HorseSeq        ------------------------------------------------------------

HorseTemp       TCTACAAGAAGCTAAGTACTTCGCCGTTTGTACTCAGCATACCAACACTACCCAAAGTGA 780
HorseSeq        ------------------------------------------------------------
```

```
HorseTemp       AATTCCCCRAAGTTGATGTGTTAACAAAATATTCTGGACCAAAAGACTCCTCAGATCCCT 840
HorseSeq        ------------------------------------------------------------

HorseTemp       TTTTTGAGATAACTGTGCCTAAATCTCAGTTAACTGTGTCCCAGTACACTCTTCCAAAAA 900
HorseSeq        ------------------------------------------------------------

HorseTemp       ATATTTCAATTGGCAATACTGTTTTGGATCTAAATGAGGTGGCCAGCAAGATTGCAGACT 960
HorseSeq        ------------------------------------------------------------

HorseTemp       TCGAGCTGCCAACCATCACTGTGCGTGAGCAGACTATTGAGATTCCTTCCATTACATTCT 1020
HorseSeq        ------------------------------------------------------------

HorseTemp       CTGTACCTGCTGAAATTTCCATTCCTTCCTTTGGAGCACTGACGGCACGTTTCRGGGTGG 1080
HorseSeq        ------------------------------------------------------------

HorseTemp       CCTCACCCCTGTATAATGCCACTTGGAGCACTGGTTGGAAAAACAAAAAAGATCGCATTG 1140
HorseSeq        ------------------------------------------------------------

HorseTemp       AAACATTCCTGAGTTCCACG 1160
HorseSeq        --------------------
```

Figure 4.10 : Similarity between the amplified horse sequence and the target horse sequence via ClustalW2 (*;indicates the homologous base pairs, ⟶ ; indicates forward primer, ⟵; indicates reverse primer).

```
DonkeyTemp      AGGGGGATGTNNNNTNATGGNTGATTTCAAGCTACTGTCATGAGGCAATTGAACATGGAG 60
DonkeySeq       ------------------------------------------------------------

DonkeyTemp      TTAGGAAATAGGTACCCAGTTTTCATGGGCTGGGAAGAGCTGGCTCTATTCTGCTAAATT 120
DonkeySeq       ------------------------------------------------------------

DonkeyTemp      CACTTACATTATCTCATTTAATCTTATCAAAAATCCTAGGAGTTAGGCATTATTATTTCC 180
DonkeySeq       ------------------------------------------------------------

DonkeyTemp      ATTTTATAGAACAGGAAATCGAGGCATAGAGAAGGAAAGTAACCTGGTAAGGTTACAGAG 240
DonkeySeq       ------------------------------------------------------------

DonkeyTemp      GTCTTAAGCGGAGCTGGGATTTGAAGCCAGGCCTGTTTGATCCCAGTGGCATCCCTATCA 300
DonkeySeq       ------------------------------------------------------------

DonkeyTemp      AACACTACACTATATCCAGTCTCCTTGTTTACATTGCCTGGTTTTCCACTGACTCCCCAC 360
DonkeySeq       ------------------------------------------------------------

DonkeyTemp      CCCCAATCAAATCAGAAAGAATCTAATACATTATTCACATTTGTCTGCTGTCTTCCCACT 420
DonkeySeq       -------------------AATCTAATACATTATTCACATTTGTCTGCTGTCTTCCCACT 41
                                   ****************************************

DonkeyTemp      CTTTTTTCTCTCTCCACAACCCCCTGCCCCTAAGCCAGATACCCAAACTTTAATCTGTCT 480
DonkeySeq       CTTTTTTCTCTCTCCACAACCCCCTGCCCCTAAGCCAGATACCCAAAA------------ 89
                ********************************************

DonkeyTemp      TCATCCCATCTCAGAATTTCATGTGCATGTTTTCAGTTTGCTGGTGTTAATGAGTCTTTG 540
DonkeySeq       ------------------------------------------------------------
```

```
DonkeyTemp      TTACCATGAACATACATCCCCCTA 564
DonkeySeq       -------------------------
```

Figure 4.11 : Similarity between the amplified donkey sequence and the target donkey sequence via ClustalW2 (*, indicates the homologous base pairs, ⟶; indicates forward primer, ⟵; indicates reverse primer).

```
PigTemp         GGCTTTCACCACCTCTTCTCAGTCACATCCCCACCTCCCACCCTGGGACATCATCCTTCT 60
PigSeq          ----------------------------------------CTGGGACATCATCCTTCT 18
                                                         ******************

PigTemp         GGCTTTCCCACCCTGGGTCTTCCATGGACCACACCCTCCCCGGCAAGTGCCCTCACACCT 120
PigSeq          GGCTTTCCCACCCTGGGTCTTCCATGGACCACACCCTCCCCG------------------ 60
                ****************************************

PigTemp         TGACCTCTGACCTTGACCCCTAGGTGCTGGATGTCCTGTGTTCCCTGTGTGTGTGCAATG 180
PigSeq          ------------------------------------------------------------

PigTemp         GTGTGGCCGTGCGCTCCAACCAAGATCTCATTACTGAGAACTTGCTCCCTGGCCGCGAGC 240
PigSeq          ------------------------------------------------------------

PigTemp         TTCTGCTGCAGACAAACCTCATCAACTATGTCACCAGGTCTGGCCCCCCAACCTTTGACC 300
PigSeq          ------------------------------------------------------------

PigTemp         CCAGAGCTTAGAACCCTCCACCACCCCGCCCCGACTCAGAGACTCCACTCCGGTGAATGG 360
PigSeq          ------------------------------------------------------------

PigTemp         CCCTTCCTCCGTCCCCCACCCCCGGACTTAATGCCAGTCCCCACCCCTGTGGTGCTTGTC 420
PigSeq          ------------------------------------------------------------

PigTemp         CCAGCTTGTCCCTGGCTTCTTACTTCTCTTACCCTTCTTCCCCAAACTCTTTCTCCCCTC 480
PigSeq          ------------------------------------------------------------

PigTemp         TGTCCTCTT 489
PigSeq          ---------
```

Figure 4.12 : Similarity between the amplified pig sequence and the target pig sequence via ClustalW2 (*, indicates the homologous base pairs, ⟶; indicates forward primer, ⟵; indicates reverse primer).

```
ChickenSeq      ------------------------------------------------------------
ChickenTemp     CTGCTGGGTTGCGCCAGCTCCCTGTTCAGGTTGTACCCAACCTTTTCAAAAAGAGTTGTG 60

ChickenSeq      ------------------------------------------------------------
ChickenTemp     ACGCTTTTGGTTTAAAGCTAAATGCATAAAAGTGTGGTTAAACCTTCATCGAACGTTTTG 120

ChickenSeq      ------------------------------------------------------------
ChickenTemp     TAACTGCTTAAACAGCAAACCCGGGGACTAAGAACCACGCTTTACGCCATTCATTAATCA 180

ChickenSeq      ------------------------------------------------------------
ChickenTemp     GAGCTGTTAAATGATTACTGCAGGGCTGTTGACACTCGCAGATGAAGTGTGTCAGTGGCA 240

ChickenSeq      ------------------------------------------------------------
ChickenTemp     GGTCCCAAAACACAACCATGGTGGTGTAAAGGAGGAAGCCTCCACCACCTCACCCGGTGG 300
```

Figure 4.13 : Similarity between the amplified chicken sequence and the target chicken sequence via ClustalW2 (*, indicates the homologous base pairs, ⟶ ; indicates forward primer, ⟵ ; indicates reverse primer).

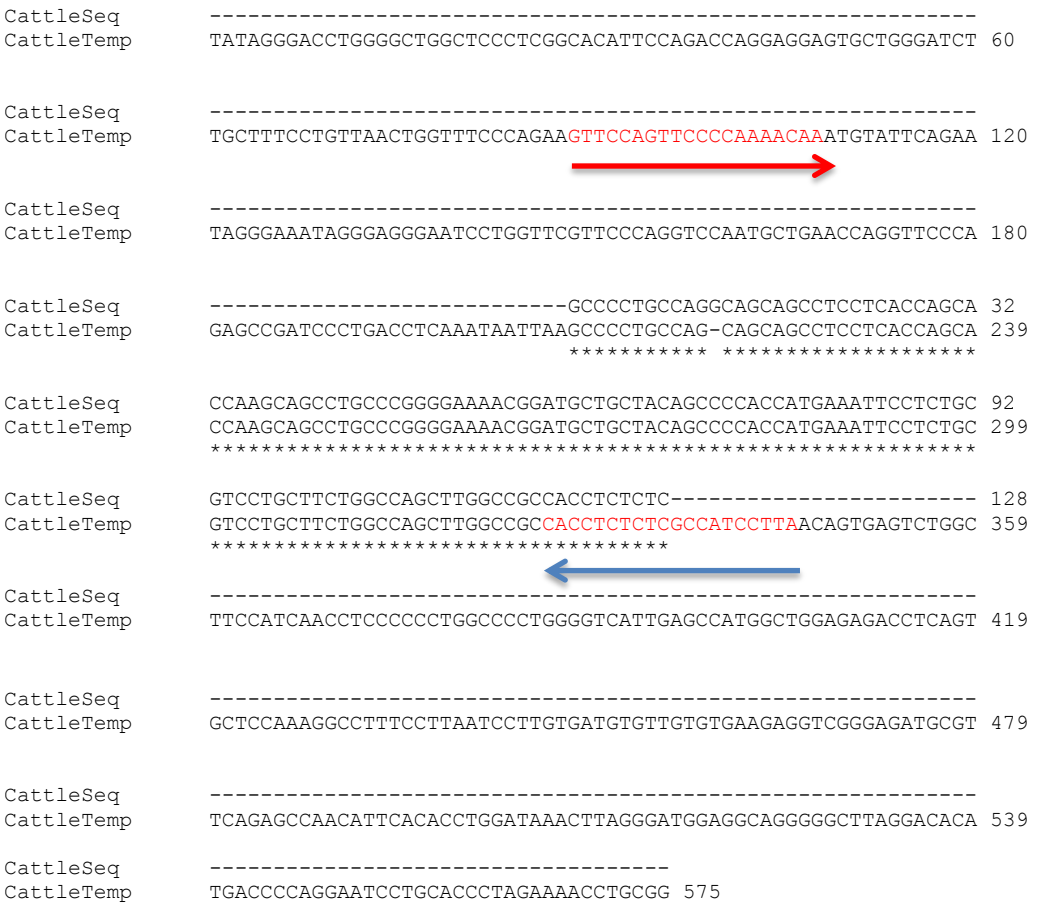


Figure 4.14 : Similarity between the amplified cattle sequence and the target cattle sequence via ClustalW2 (*, indicates the homologous base pairs, ⟶ ; indicates forward primer, ⟵ ; indicates reverse primer).

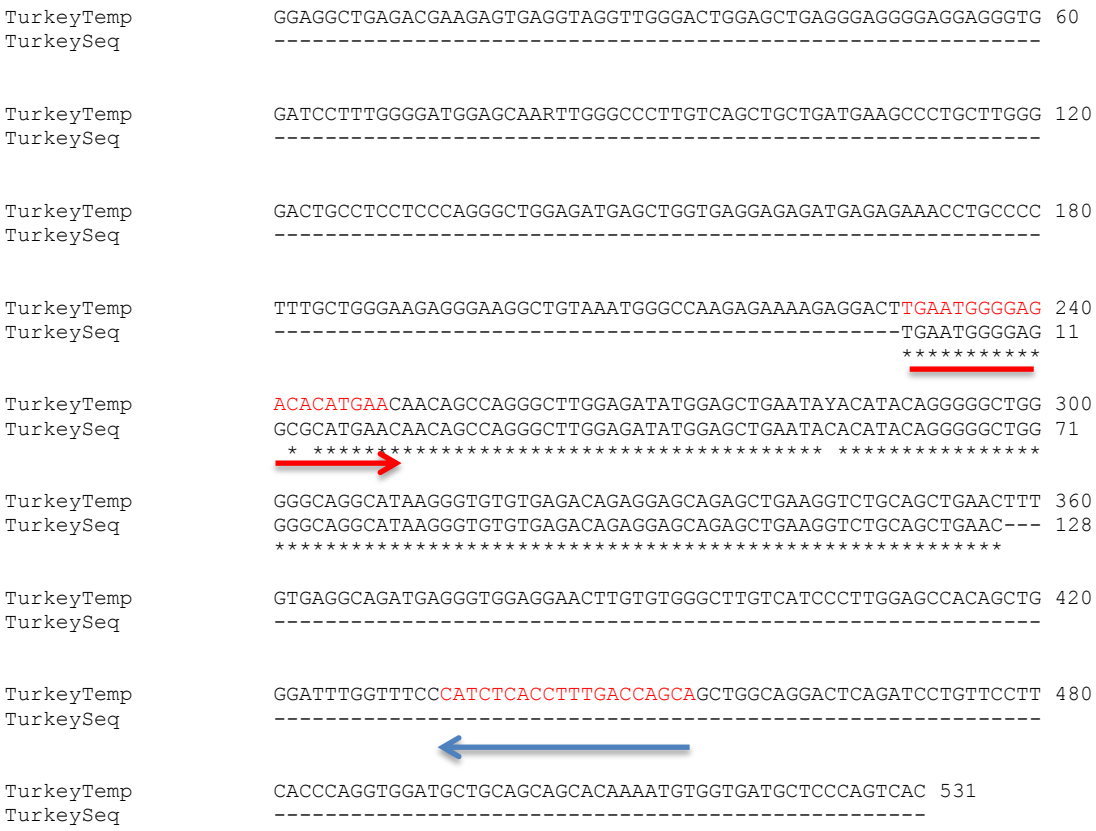


Figure 4.15 : Similarity between the amplified turkey sequence and the target turkey sequence via ClustalW2 (*, indicates the homologous base pairs, ⟶; indicates forward primer, ⟵ ; indicates reverse primer).

4.5 Commercial Food Screening Using the Developed Methodology

To test the practical application of the developed methodology, total of 83 commercial processed beef meat products and the swab samples from different production stages of a meat producer were tested using the developed method. The results were given in Table 4.10. Among the 83 screened samples, 24 gave positive amplification signal in chicken specific PCR, 9 gave positive amplification signal in turkey specific PCR, 1 gave positive amplification signal in pig specific PCR. The amplification curves, melting curves and melt peak charts of some of the chicken, turkey and pig positive commercial samples were shown in Figure 4.16. The results were shown for the cattle-chicken-turkey positive swap sample number 6, cattle positive sucuk sample number 30, pig positive sucuk sample number 30, chicken-cattle positive doner kebap sample number 39, turkey-cattle positive doner kebap sample number 45, chicken-cattle positive sausage sample number 61, turkey-cattle positive sausage sample number 74 and chicken-cattle positive salami sample number 81.

Cattle-turkey-chicken multiplex qPCR were performed on the swab samples that were obtained from a meat producer. Three different meat types were found in some of these samples. Figure 4.16 shows qPCR results of one (sample number 6) of the positive samples. This result was expected, because this sample was collected from a meat production bench where three different meat types can be treated based on the production demand. Melting peaks were obtained at 81.45˚C, 84.98˚C, 87.55˚C for chicken, turkey, cattle, respectively.

Both cattle-turkey-chicken and horse-pig-donkey multiplex qPCR were performed for sucuk sample (sample number 30). Only the melting peak that corresponding to cattle species was observed at 87.25˚C in cattle-turkey-chicken multiplex qPCR results as expected. The melting peak corresponding to pig species was observed at 84.98˚C in horse-pig-donkey multiplex qPCR (Figure 4.16).

Cattle-turkey-chicken multiplex qPCR were performed for doner kebap, salami and sausage samples. As seen in Figure 4.16, in chicken-cattle positive doner kebap sample (sample number 39); the melting peak that corresponding to chicken species was observed at 81.22˚C and the melting peak that corresponding to cattle species was observed at 87.18˚C.

In turkey-cattle positive doner kebap sample (sample number 45); the melting peak that corresponding to turkey species was observed at 84.88˚C and the melting peak that corresponding to cattle species was observed at 87.21˚C (Figure 4.16). In chicken-cattle positive sausage samples (sample number 61); the melting peak that corresponding to chicken species was observed at 81.18˚C and the melting peak that corresponding to cattle species was observed at 87.21˚C for (Figure 4.16).

The melting peaks were obtained at 84.88˚C, 87.34˚C for turkey and cattle, respectively in turkey-cattle positive sausage sample (sample number 74). In chicken-cattle positive salami sample (sample number 81); melting peaks were observed at 81.55˚C, 87.74˚C for chicken and cattle, respectively (Figure 4.16).

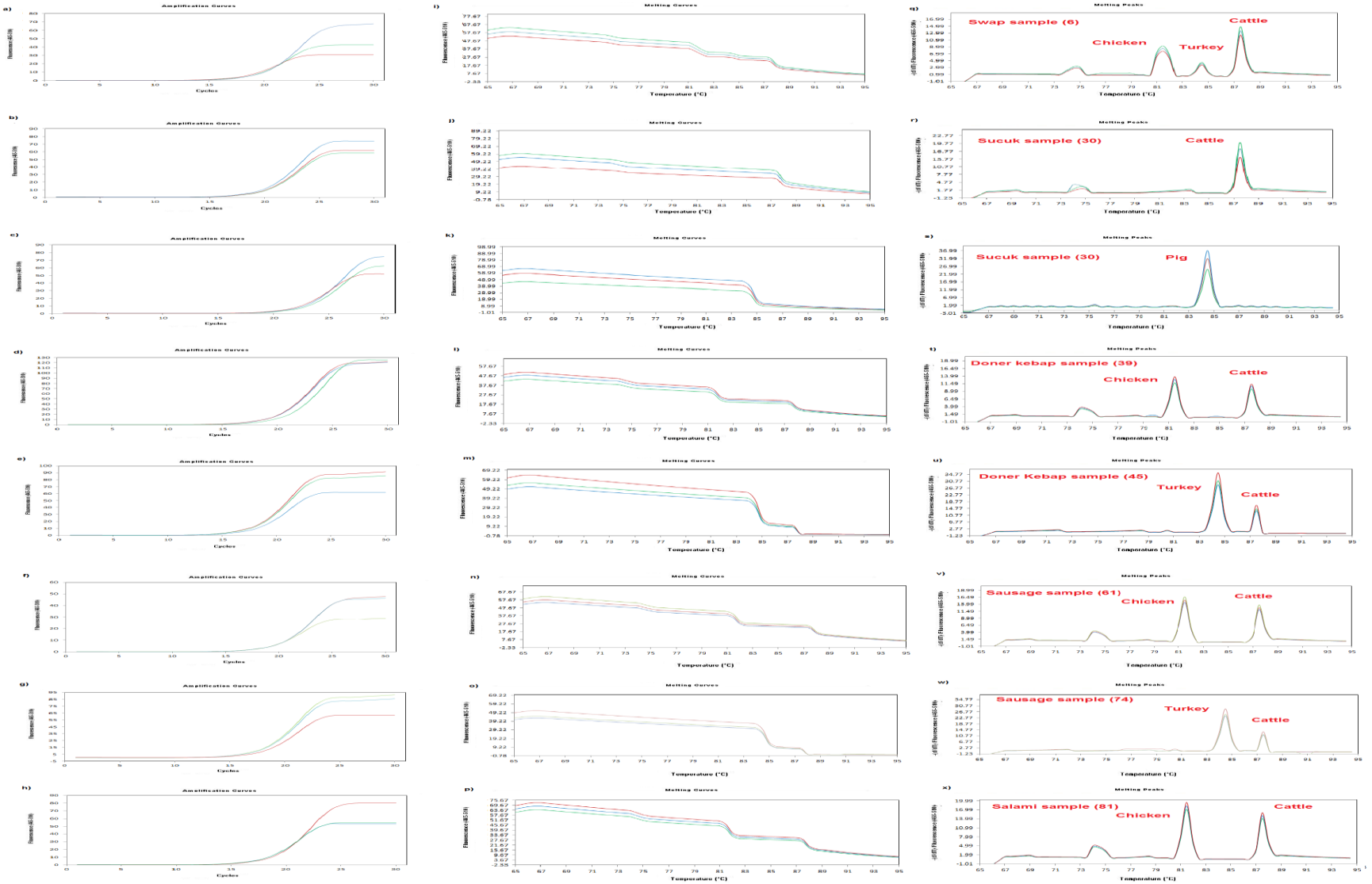


Figure 4.16 : The amplification curves, melting curves and melt peak charts of one of the types of the analyzed commercial samples. First, second and third runs were shown in blue, red and green, respectively.

Table 4.10 : The positive and negative results of commercial food products and swab sample (+; positive sample, -; negative samples, U; unanalyzed.)

					Cattle-Turkey-Chicken specific multiplex PCR						**Horse-Donkey-Pig specific multiplex PCR**		
Sample No	**Sample Name**	**Cattle**	**Chicken**	**Turkey**	**Cattle**	**Chicken**	**Turkey**	**Horse**	**Donkey**	**Pig**	**Horse**	**Donkey**	**Pig**
1	**Swap Sample**	+	-	-	+			U	U	U	U	U	U
2		+	+	-	+	+		U	U	U	U	U	U
3		+	+	-	+	+		U	U	U	U	U	U
4		+	-	+	+		+	U	U	U	U	U	U
5		+	+		+	+		U	U	U	U	U	U
6		+	+	+	+	+	+	U	U	U	U	U	U
7		+	-	-	+	-	-	U	U	U	U	U	U
8		+	-	-	+	-	-	U	U	U	U	U	U
9		+	+	-	+	+	-	U	U	U	U	U	U
10		+	+	-	+	+	-	U	U	U	U	U	U
11		+	+	+	+	+	+	U	U	U	U	U	U
12		+	-	-	+	-	-	U	U	U	U	U	U
13		+	+	-	+	+	-	U	U	U	U	U	U
14		+	-	-	+	-	-	U	U	U	U	U	U
15		+	-	+	+	-	+	U	U	U	U	U	U
16		+	+	-	+	+	-	U	U	U	U	U	U
17		+	-	-	+	-	-	U	U	U	U	U	U
18		+	-	+	+	-	+	U	U	U	U	U	U
19		+	-	-	+	-	-	U	U	U	U	U	U
20		+	+	-	+	+	-	U	U	U	U	U	U
21		+	+	-	+	+	-	U	U	U	U	U	U
22		+	+	-	+	+	-	U	U	U	U	U	U

Table 4.10 (continued) : The positive and negative results of commercial food products and swab sample (+; positive sample, -; negative samples, U; unanalyzed.)

					Cattle-Turkey-Chicken specific multiplex PCR						Horse-Donkey-Pig specific multiplex PCR		
Sample No		**Cattle**	**Chicken**	**Turkey**	**Cattle**	**Chicken**	**Turkey**	**Horse**	**Donkey**	**Pig**	**Horse**	**Donkey**	**Pig**
23		+	-	-	+	-	-	U	U	U	U	U	U
24		+	-	-	+	-	-	U	U	U	U	U	U
25	**Sucuk**	+	+	-	+	+	-	-	-	-	-	-	-
26		+	-	-	+	-	-	-	-	-	-	-	-
27		+	-	-	+	-	-	-	-	-	-	-	-
28		+	+	-	+	+	-	-	-	-	-	-	-
29		+	-	-	+	-	+	-	-	-	-	-	-
30		+	-	-	+	-	-	-	-	+	-	-	+
31		+	-	-	+	-	-	-	-	-	-	-	-
32		+	-	-	+	-	-	-	-	-	-	-	-
33	**Doner Kebap**	+	-	-	+	-	-	U	U	U	U	U	U
34		+	-	-	+	-	-	U	U	U	U	U	U
35		+	+	-	+	+	-	U	U	U	U	U	U
36		+	-	-	+	-	-	U	U	U	U	U	U
37		+	-	-	+	-	-	U	U	U	U	U	U
38		+	-	-	+	-	-	U	U	U	U	U	U
39		+	+	-	+	+	-	U	U	U	U	U	U
40		+	+	-	+	+	-	U	U	U	U	U	U
41		+	-	-	+	-	-	U	U	U	U	U	U
42		+	-	-	+	-	-	U	U	U	U	U	U
43		+	-	-	+	-	-	U	U	U	U	U	U
44		+	-	-	+	-		U	U	U	U	U	U

Table 4.10 (continued) : The positive and negative results of commercial food products and swab sample (+; positive sample, -; negative samples, U; unanalyzed.)

		Cattle-Turkey-Chicken specific multiplex PCR									Horse-Donkey-Pig specific multiplex PCR		
Sample No		**Cattle**	**Chicken**	**Turkey**	**Cattle**	**Chicken**	**Turkey**	**Horse**	**Donkey**	**Pig**	**Horse**	**Donkey**	**Pig**
45		+	-	+	+	-	+	U	U	U	U	U	U
46		+	-	-	+	-	-	U	U	U	U	U	U
47		+	-	-	+	-	-	U	U	U	U	U	U
48		+	-	-	+	-	-	U	U	U	U	U	U
49		+	-	-	+	-	-	U	U	U	U	U	U
50		+	+	-	+	+	-	U	U	U	U	U	U
51		+	-	-	+	-	-	U	U	U	U	U	U
52		+	-	-	+	-	-	U	U	U	U	U	U
53		+	-	-	+	-	-	U	U	U	U	U	U
54		+	-	-	+	-	-	U	U	U	U	U	U
55		+	+	-	+	+	-	U	U	U	U	U	U
56		+	-	-	+	-	-	U	U	U	U	U	U
57		+	-	-	+	-	-	U	U	U	U	U	U
58		+	-	-	+	-	-	U	U	U	U	U	U
59		+	-	-	+	-	-	U	U	U	U	U	U
60		+	-	-	+	-	-	U	U	U	U	U	U
61	**Beef sausage**	+	+	-	+	+	-	-	-	-	-	-	-
62		+	-	-	+	-	-	-	-	-	-	-	-
63		+	-	-	+	-	-	-	-	-	-	-	-
64		+	-	-	+	-	-	-	-	-	-	-	-
65		+	+	-	+	+	-	-	-	-	-	-	-
66		+	-	+	+	-	+	-	-	-	-	-	-

Table 4.10 (continued) : The positive and negative results of commercial food products and swab sample (+; positive sample, -; negative samples, U; unanalyzed.)

					Cattle-Turkey-Chicken specific multiplex PCR						Horse-Donkey-Pig specific multiplex PCR		
Sample No		**Cattle**	**Chicken**	**Turkey**	**Cattle**	**Chicken**	**Turkey**	**Horse**	**Donkey**	**Pig**	**Horse**	**Donkey**	**Pig**
67		+	-	-	+	-	-	-	-	-	-	-	-
68		+	-	+	+	-	+	-	-	-	-	-	-
69		+	-	-	+	-	-	-	-	-	-	-	-
70		+	+	-	+	+	-	-	-	-	-	-	-
71		+	-	-	+	-	-	-	-	-	-	-	-
72		+	-	-	+	-	-	-	-	-	-	-	-
73		+	-	-	+	-	-	-	-	-	-	-	-
74		+	-	+	+	-	+	-	-	-	-	-	-
75		+	-	-	+	-	-	-	-	-	-	-	-
76	**Beef salami**	+	-	-	+	-	-	-	-	-	-	-	-
77		+	+	-	+	+	-	-	-	-	-	-	-
78		+	-	-	+	-	-	-	-	-	-	-	-
79		+	-	-	+	-	-	-	-	-	-	-	-
80		+	-	-	+	-	-	-	-	-	-	-	-
81		+	+	-	+	+	-	-	-	-	-	-	-
82		+	-	-	+	-	-	-	-	-	-	-	-
83		+	-	-	+	-	-	-	-	-	-	-	-

5. DISCUSSION

Universal mitochondrial DNA sequences such as; 12S rRNA (Rodriguez et al., 2005), cytochrome b gene (Dooley et al., 2004), and 16S rRNA (Sawyer et al., 2003) have generally been chosen as the target for meat type specific probe design. This has led to specifity problems in the detections. Mitochondrial genes are highly conserved so that differentiation is difficult between the species that belongs to the same genus such as; horse and donkey. To obtain more specific results, we concentrated on the amplification of highly variable gene regions for the each animal type. This approach prevented the non-specific amplifications and led to easier workflow for the validation studies.

QPCR technique is a very sensitive and robust technique on species identification. Recently, hydrolysis (Chisholm et al., 2005) and hybridization (Whitcombe et al., 1999) probes were used for the detection of meat species in contaminated food and feedstuffs. However, the most important disadvantage of the probe-based techniques is the high costs of the probes. As an alternative to probe based detection, SYBR Green I is the most frequently used intercalating dye in qPCR studies. Several SYBR Green I-based PCR methods have been proposed for the identification and quantification of animal species in food and feed products. For example, Walker and others (2003) targeted the short interspersed elements(SINE) gene for the detection of cattle, chicken and pig species in food products. They selected SINE gene as target, because each of the SINE families within the different genomes was derived independently,every mammalian order has a significant number (in excess of 100,000) of characteristic mobile elements (Deininger et al., 1993). These large dispersed gene families serve as novel markers that identify the DNA from the species within that order. In the other study, cytochrome b gene was selected as target gene to detect cattle horse and pig species in a meat mixture.

Also, multiplex qPCR was performed for multiple detection of cattle-horse, cattle-pig combinations (Lopez-andreo et.al. 2006). Martin and others (2009) targeted 12S rRNA to detect pig species in food products.

Although the SYBR Green qPCR system is a more convenient and cheaper alternative to approaches where specific amplicons are detected by fluorogenic probes, Sybr Green has some disadvantages such as; it can inhibit PCR reactions if used above a certain concentration. As an alternative, HRM dyes are preferred for use with high resolution melt assays due to the more discrete signal change occurring upon DNA denaturation. HRM dyes only bind to double stranded DNA that prevents the dye molecule from redistribution during melting and provides superior melt curve resolution. Unlike SYBR Green dye, HRM dyes can be used at high concentrations because they do not inhibit DNA polymerases and PCR reaction. HRM dyes great ability to bind the hydrogen bond almost 4 times more than SYBR Green. In this study, we used a single HRM dye instead of using fluoregenic probes or SYBR Green I to identify triple targets. Since melting curve analysis combined with HRM dyes gives very sensitive sequence specific profiles, the same specifity of the probe based methods was obtained using a single HRM dye in this study.

It was previously reported that a DNA extraction methodology must be resulted in DNA purities and concentrations between 1.6-2 and 25-1000 ng/µl, respectively (Lahiff et al., 2001; Pinto et al., 2007). In our study, the purities and concentrations of the DNA extracts obtained using five different methodologies were in the desirable ranges. The current commercially available DNA extraction kits are based on time consuming reactions that are completed in at least 1.5 hours. In this study, we have developed a DNA extraction protocol which does not include enzymatic steps. The DNA extracts were obtained via only the physical and the chemical cell disruption. This has significantly decreased the total time (less than 20 min.) and the cost of the extraction.

This study has shown that it is possible to develop a quick, reliable and cost effective system based on qPCR for meat authentication. It was proved that the developed method can give sensitive results in less than 75 minutes which is at least two times faster than the currently available PCR based methods for the meat type detection.

6. CONCLUSION

Mixing meats of different animal species causes consumer victimization, religious and health problems and unfair market competition. Thus, the meat species identification has great importance in food quality control and safety.

The current qPCR based methodologies for meat species identification are time and money consuming. The main reasons behind these are the long incubation times and high costs of the available DNA extraction and the multiplex qPCR methodologies. In this study, a new system was developed to overcome the analysis time and cost related problems in the meat type detection. This was achieved via an enzyme free DNA extraction methodology and a multiplex qPCR using a single HRM dye. For the first time, this study introduced discrimination of three different qPCR amplicons from various animal specific gene products based on the differences in T_ms. The results also showed that all of the PCR amplicons were specific. The overall results proved that the developed method could give sensitive results in less than 75 min., which is at least two times faster than the currently available PCR-based methods for meat type detection.

The qPCR using a single HRM dye assays evaluated in this study have a high potential as a molecular tools that can be used in rapid and routine detection of horse, donkey, pig, chicken and turkey meats present in heat treated ground meat mixtures. The use of species-specific primers makes the method very sensitive for determination in raw and processed meats. Consequently, qPCR based assay described in this study is a practical method that can be used by the food control laboratories to quickly detect technically inevitable contamination and/or intentional admixtures in meat products. On the other hand, the methodolgy must be validated using the reference samples prepared by reference accredited food control laboratories.

6.1 Future Aspects

The developed method is now being further validated by Turkey's oldest food control laboratory, Environmental Industrial Analysis Laboratory. Our group are going to developed an automated DNA isolation, PCR set-up and qPCR system. Our methodology will be adapted to this automated system.

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APPENDICES

APPENDIX A: QPCR Results and Sequence Chromatograms of cattle, chicken, turkey, horse, donkey and pig species.

APPENDIX A

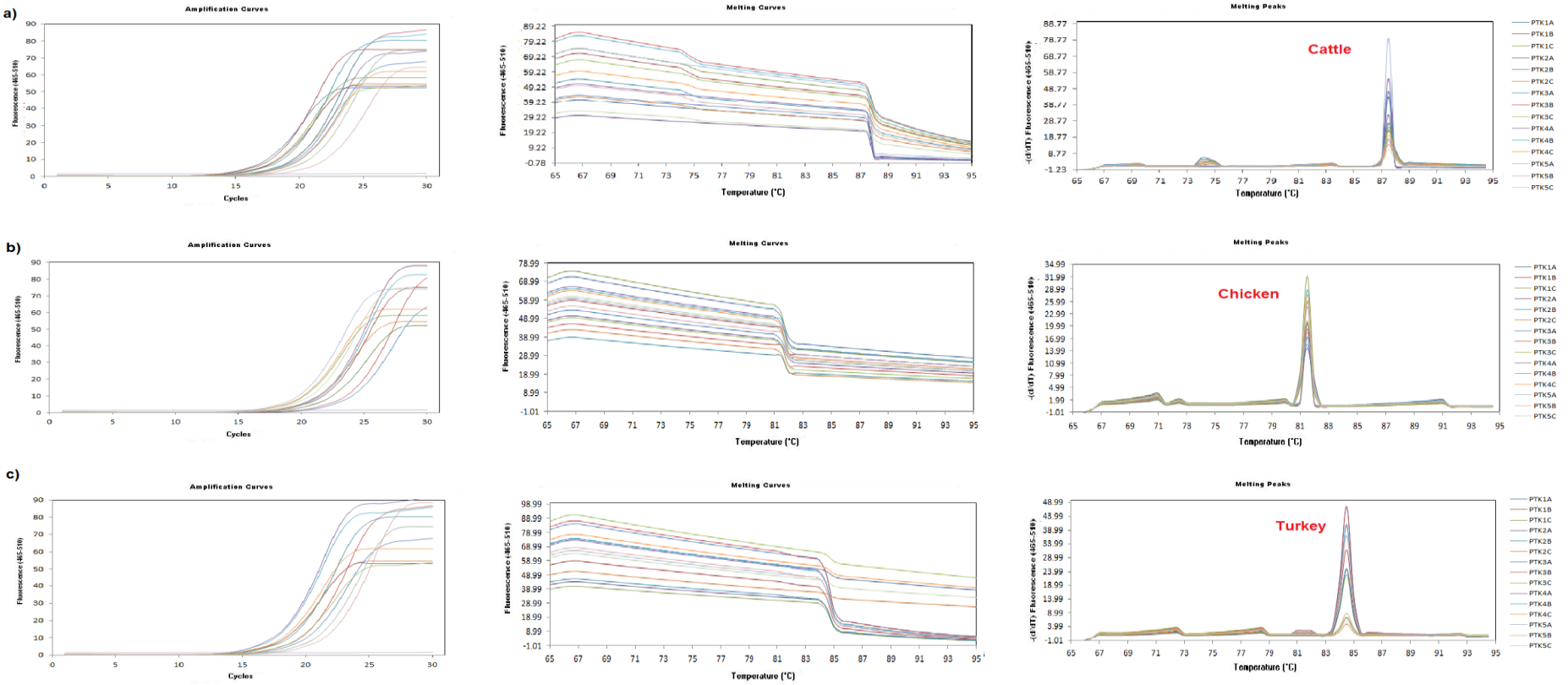


Figure A.1 : QPCR results obtained from the 5 different protocols of cattle, chicken and turkey sample. The color of the each data series was shown on the top right corner of the melting peak charts.

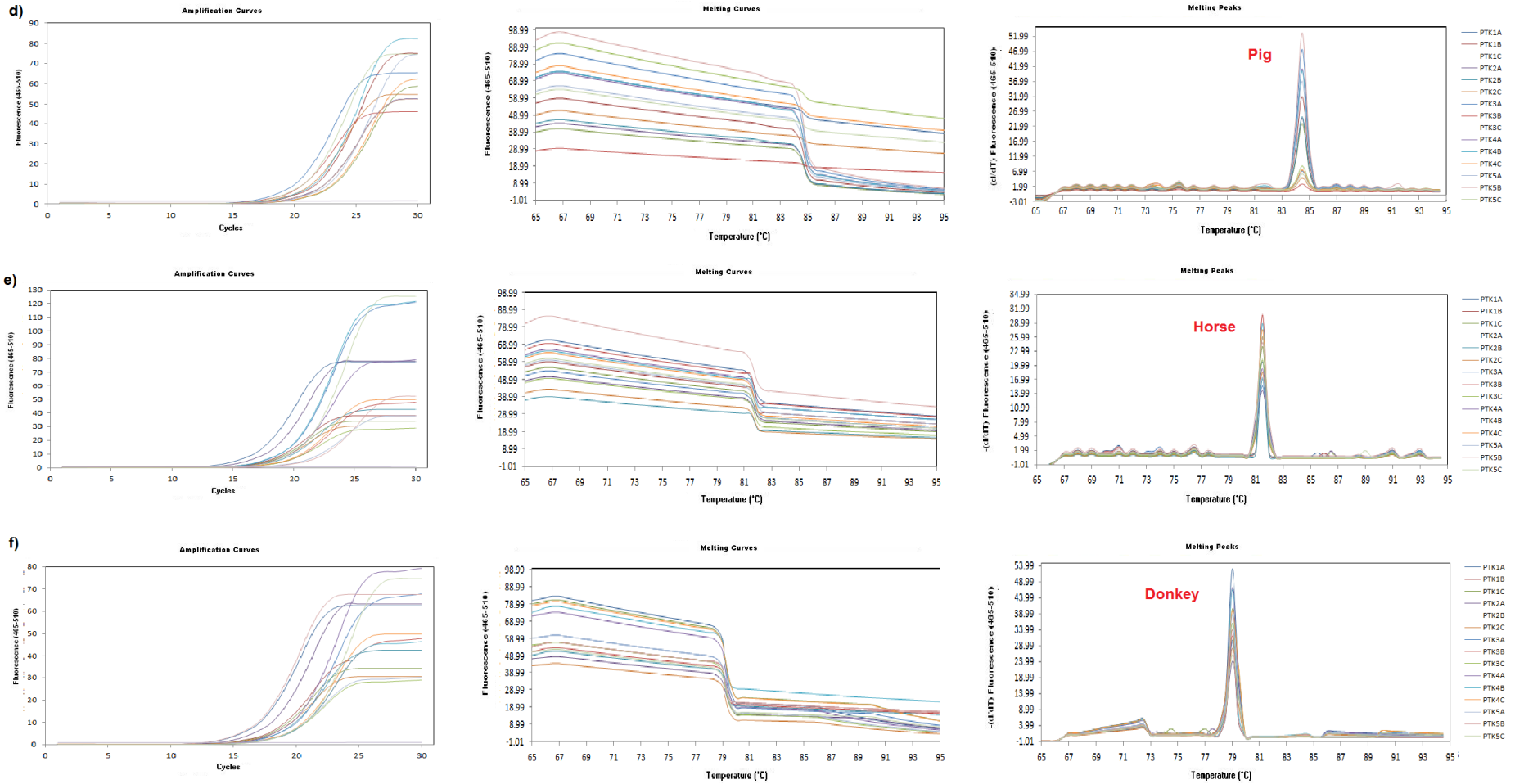


Figure A.1 (continued) : QPCR result obtained from the 5 different protocols of pig, horse and donkey sample. The color of the each data series was shown on the top right corner of the melting peak charts.

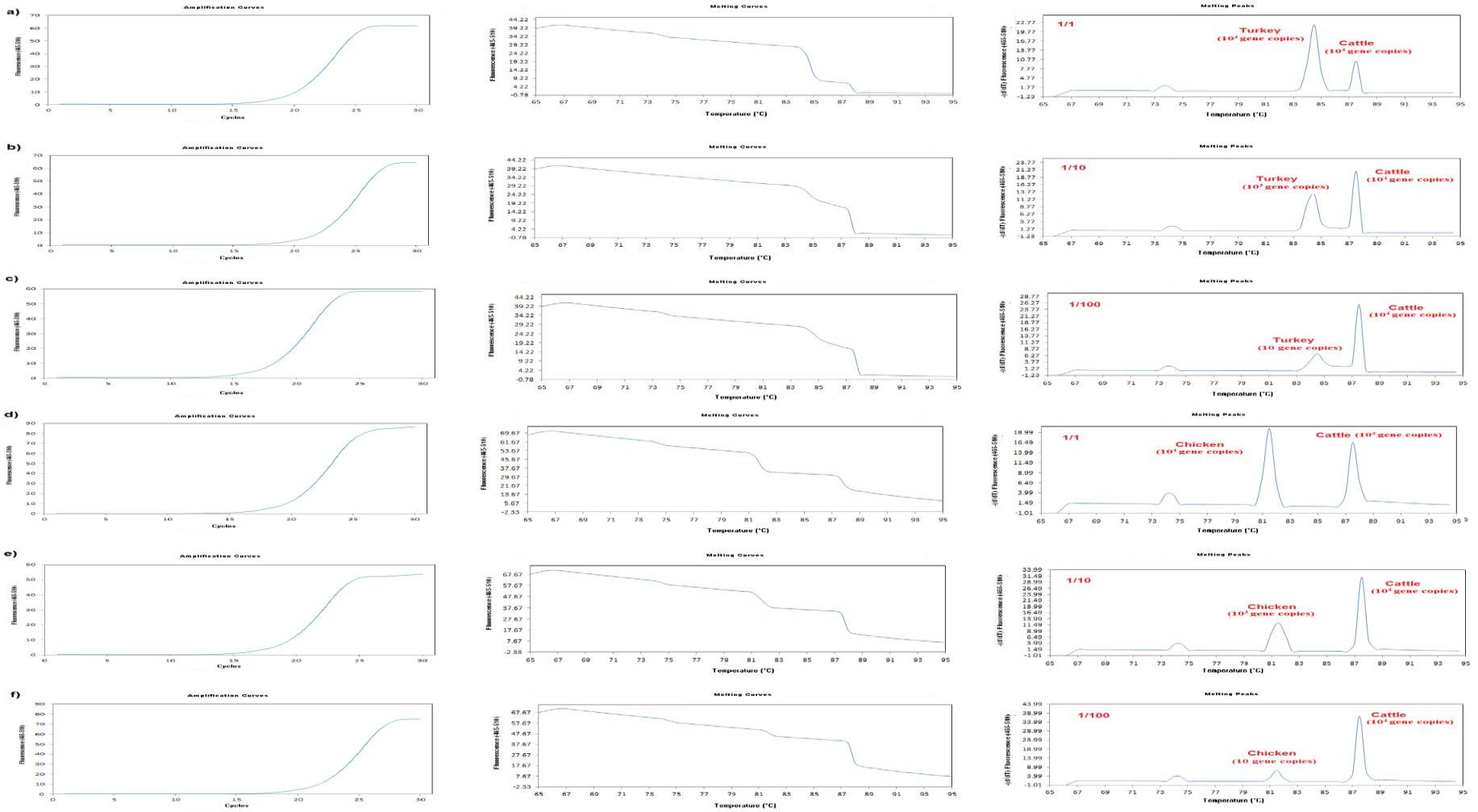


Figure A.2 : QPCR results of cattle, chicken, turkey when mixed at different ratios (1/1, 1/10, 1/100 and 1/1000).

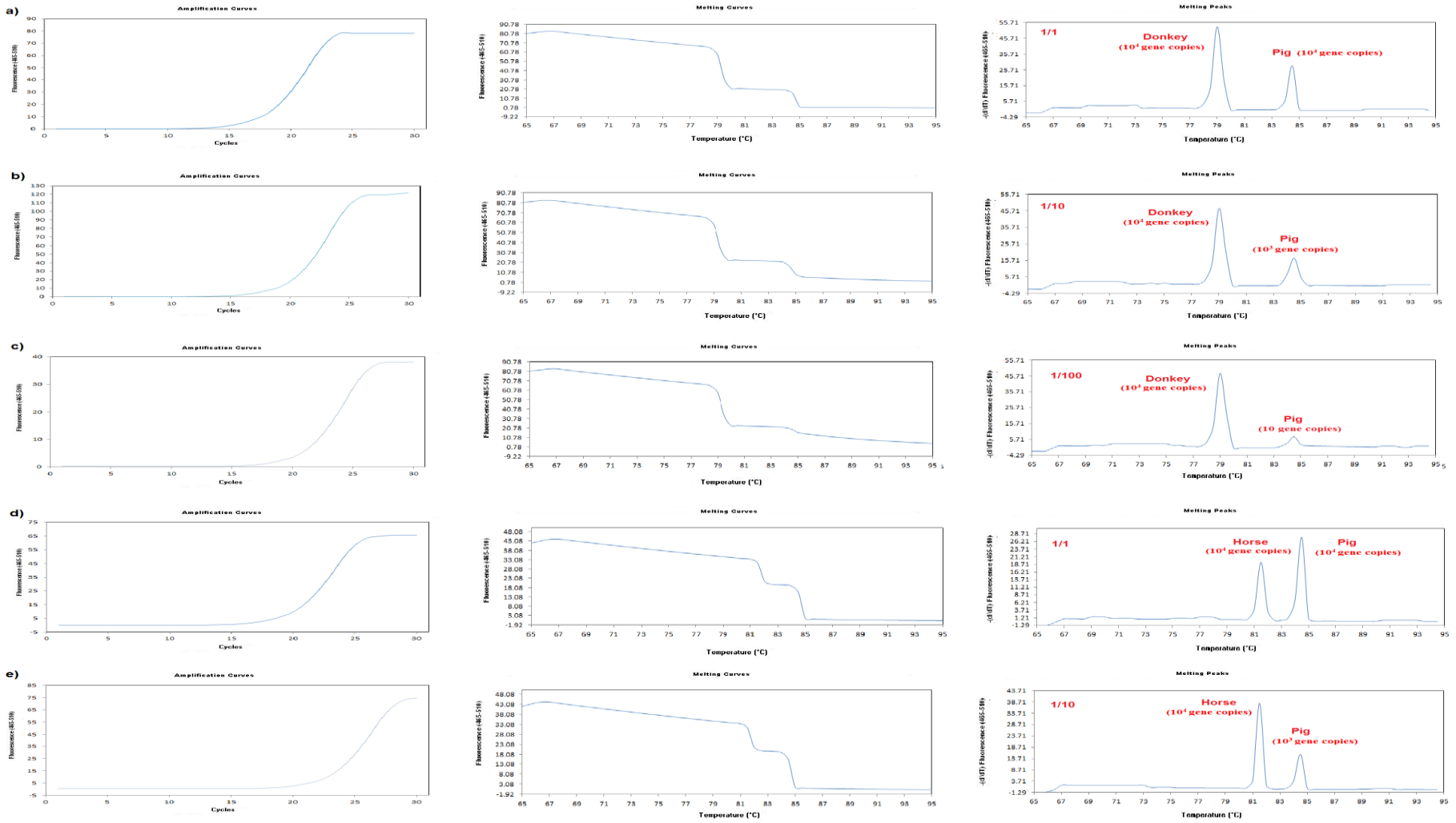


Figure A.3 : QPCR results of donkey, horse and pig when mixed at different ratios (1/1, 1/10, 1/100 and 1/1000).

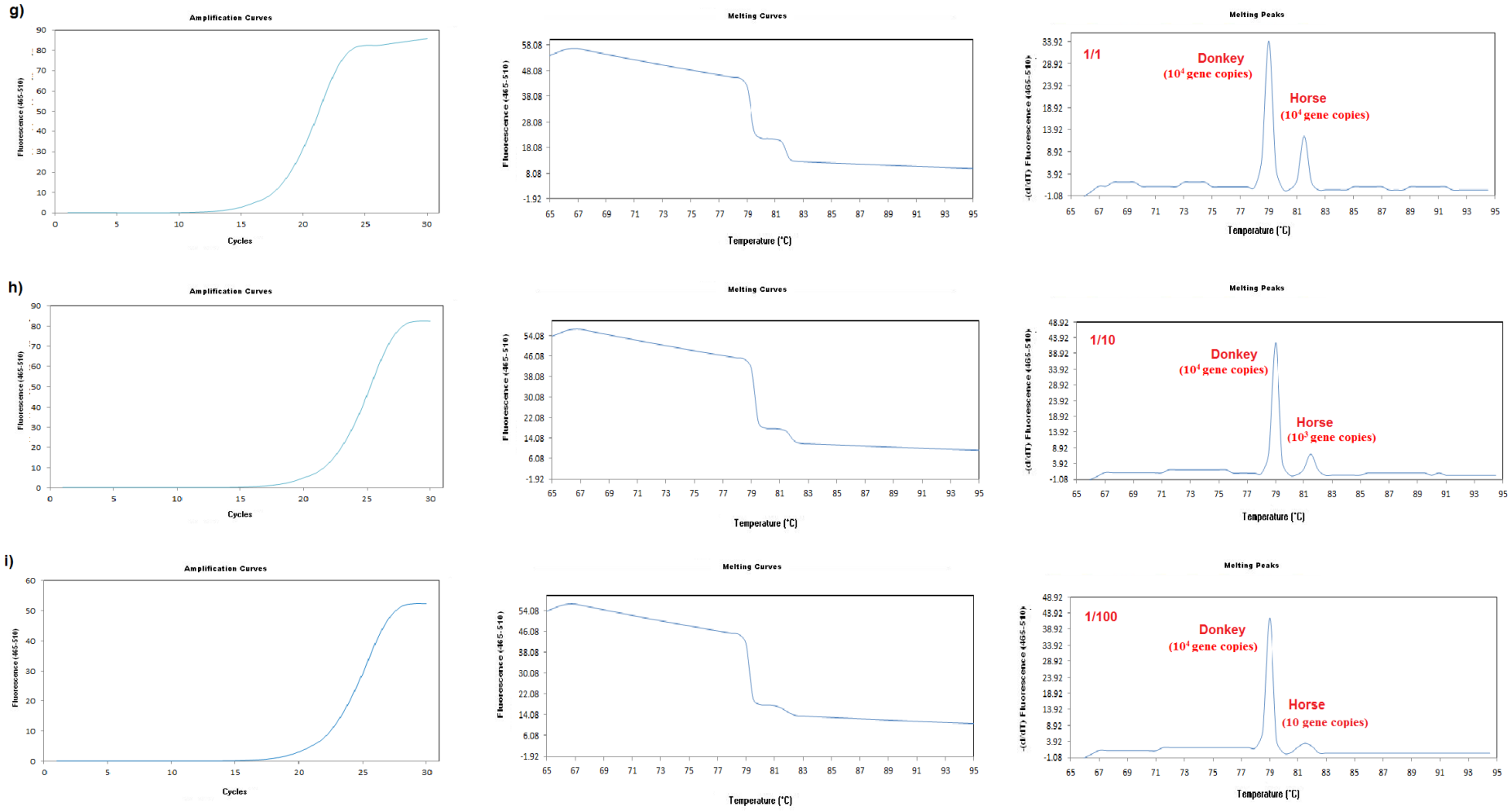
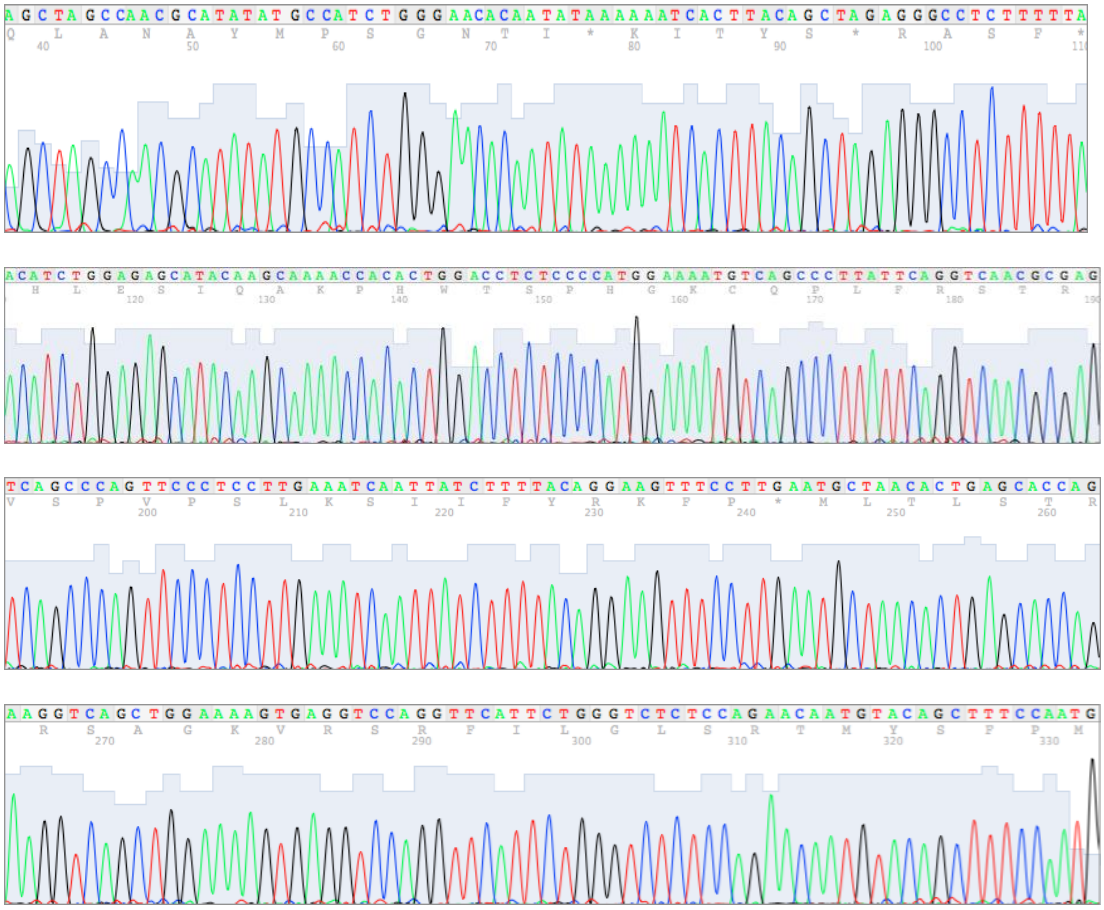
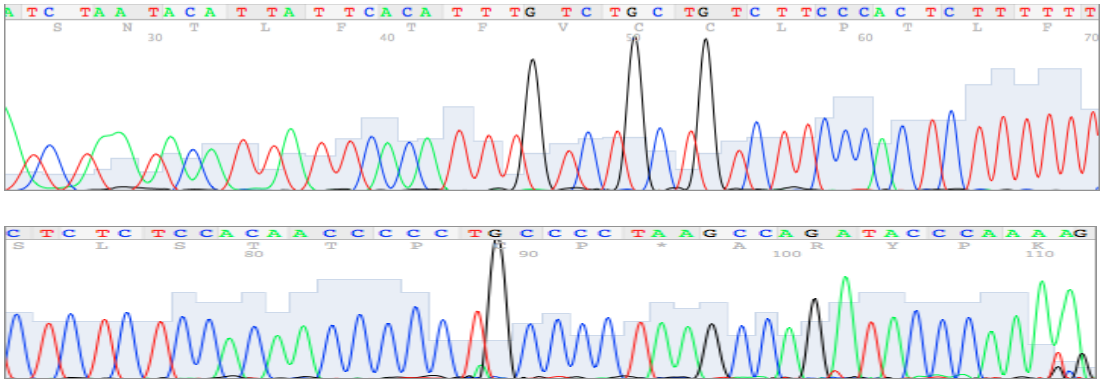


Figure A.3 (continued) : QPCR results of donkey, horse and pig when mixed at different ratios (1/1, 1/10, 1/100 and 1/1000).

a)



b)



c)

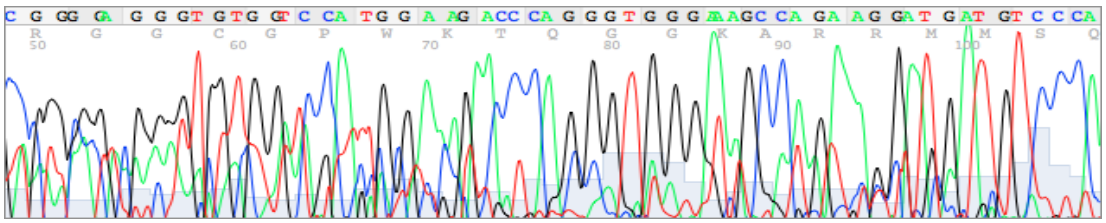
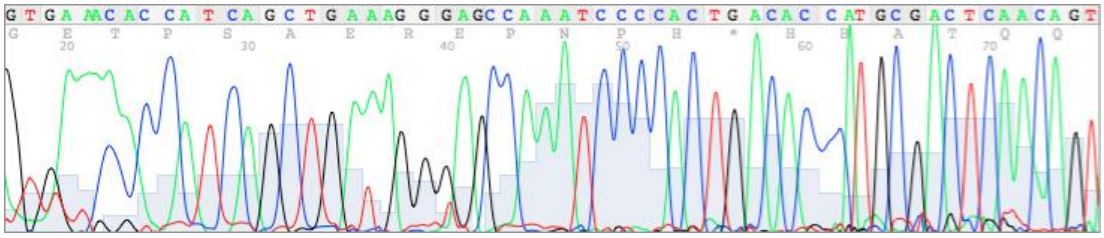
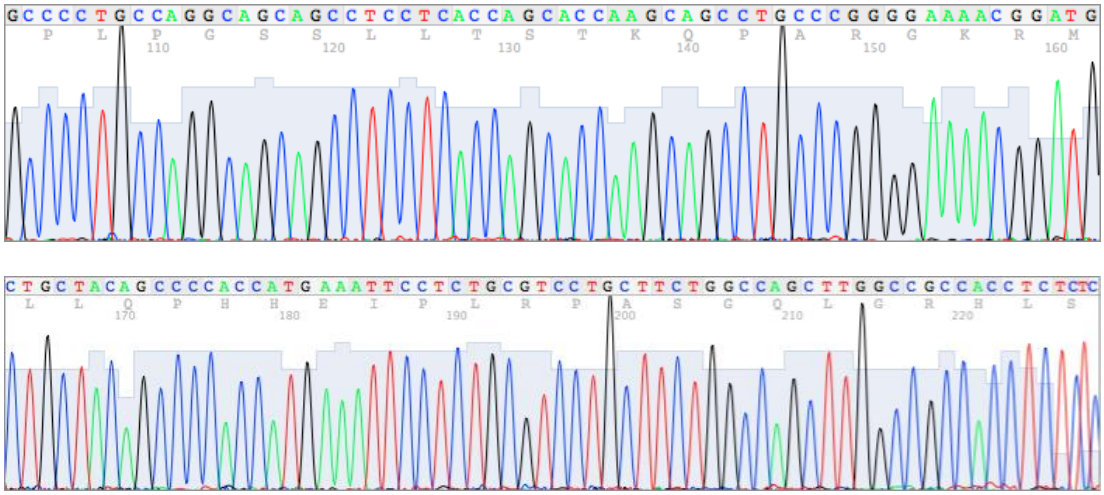


Figure A.4 : Sequence chromatograms: a(horse), b(donkey), c(pig), d(chicken), e(cattle), f(turkey).

d)



e)



f)

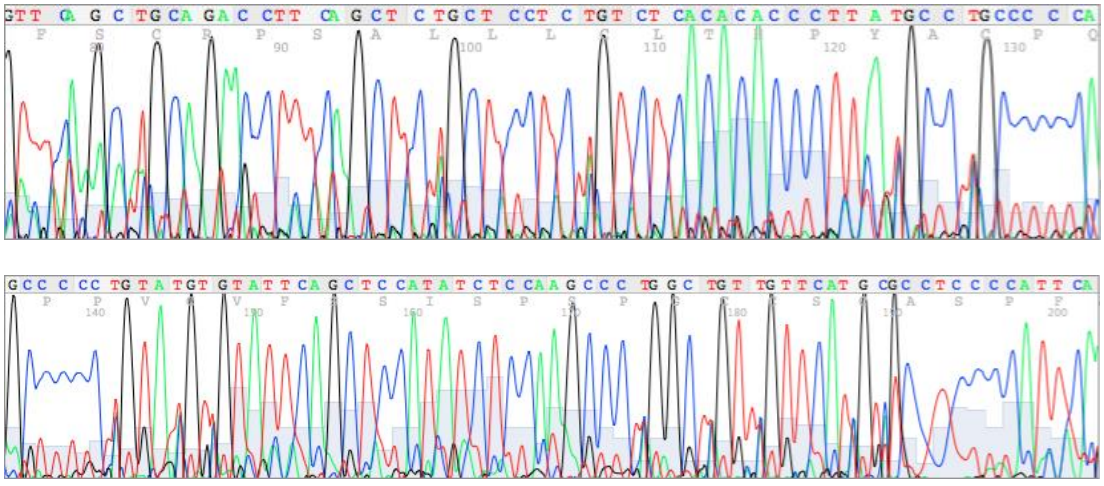


Figure A.4 (contiuned) : Sequence chromatograms: a(horse), b(donkey), c(pig), d(chicken), e(cattle), f(turkey).

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