Ergün ŞAKALAR

M.S. Thesis In Biology

July -2008

# A MOLECULAR GENETIC APPROACH TO THE QUANTITATIVE ANALYSIS OF PROCESSED MEAT BY REAL-TIME PCR TECNIQUES AND RAPID IDENTIFICATION OF THE SPECIES ORIGIN OF MEATS IN FOODSTUFF BY MULTIPLEX PCR

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Ergün ŞAKALAR

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**APPROVAL PAGE**

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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Head of Department

This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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July 2008

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## ABSTRACT

Recently the need of further information about the composition of food products, in particular of meat products, is increased, so identifying the species of origin of meat products and quantifying the amount of meat in mixed-processed products represent a substantial target for food inspection, underlined by the enforcement of community laws. Thus, specific, sensitive and easy analytical methods for the species detection and quantitation of food are necessary in order to verify the compatible with labelling requirements. For this necessity in this study, DNA-based tecniques, CM-PCR(conventional multiplex PCR, QRT-PCR(quantitative real-time PCR), MRT-PCR(multiplex real-time PCR) were developed and optimised for the analysis of origin and quantification of meats in complex food matrix. And also these tecniques were used to evaluate the ratios and presence of fraudulently added meat.

CM-PCR assay was applied to precessed and raw meats for the identification of the most used species in foodstuffs such as ruminant, poultry, fish and pork meterials. Specific-species primers designed in different regons of mitocondrial DNA were used after alignment of the available sequences in the GenBank database. The primers generated specific fragment of 183, 224, 290 and 374 bp length for poultry, fish, pork, and ruminant, respectively. The optimised CM-PCR assay was applied to 93 commercial meat products and it showed the presence of poultry meat in %50 of the analyzed products contain raw or processed red meat, evidenced the presence of animals species not indicated on the label. And also, overall multiplex results showed that 25 (35.1%) among 71 tested samples gave unexpected results (Table 12) not indicated in their labels. Clearly, we couldn't decide whether they are contamination or intentional admixture at the moment of the manufacturing of these products. Because of this drawback we developed QRT-PCR assay for poultry and ruminant using SYBR Green based-detection system.

The minimum effective quantification levels of QRT-PCR were 0,00006 ng/µl, 0.000076 ng/µl 0,000045 ng/µland 0,000045 ng/µl for ruminant , poultry ,fish and pork respectively. Also, processed 9 meat tested in commercially purchesed 100 meat products using this assay. The ruminant meat proportions were predicted after comparing with the standart dilution series. The results for proportion of ruminant showed significantly ruminant meat defectived and there was DNA of poultry especially instead of mammals DNA in the mixed-processed meats as the main component. Because, the amount of poultry meat was more than the ratios given on it's label in processed-mixed meat products on the contrary ruminant meat proportion was less than.

Also MRT-PCR was developed here was done to improve an assay that can combine the two advantages of real-time PCR and multiplex PCR together for animal gene detection and identification more quickly. The objective of this part of study was to design a rapid, specific and accurate MRT-PCR assay by using SYBR Green fluorescence dye cheaper than duble labeled probes to detect a group of mixed meat simultaneously. Our results indicate that our multiplex real-time PCR assay can be used to more quickly identify DNAs isolated from complex foods. We should tell that our both QRT-PCR and MRT-PCR assays applied on the DNAs of the complex meat matrix using SYBR Green florescence was first one in the field of molecular food analysis.

**Keywords;** PCR, Quantitative real-time PCR, Multiplex PCR, quantitative analysis of meat, identification of origin of meats, SYBR Green

# GERÇEK ZAMANLI PZR TEKNİKLERİ İLE İŞLENMİŞ ETLERİN KANTİTATİF ANALİZLERİNE MOLEKÜLER GENETİK YAKLAŞIM VE ÇOKLU PZR İLE GIDA MADDELERİNDEKİ TÜR ORİJİNLERİNİN HIZLI BELİRLENMESİ

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## ÖZ

Son zamanlarda gıda ürünlerinin özellikle de et ürünlerinin kompozisyonuyla ilgili çok daha ötede bilgi ihtiyacının olması karışık-işlenmiş gıdaların içinde bulunan etlerin tür orijinlerinin belirlenmesi ve miktarlarının ölçülmesi, toplum yasalarının uygunluğuyla altı çizilen gıda denetlemeleri için önemli bir hedefi temsil etmektedir. Bu yüzden gıdanın etiketleme gereksinimiyle uyumluluğunu doğrulamak, türünü tanımlamak ve miktarını ölçmek için sipesifik, hassas ve kolay analitik metotlara ihtiyaç vardır. Bu çalışmada bu gereklilikten dolayı compleks gıda matrikslerinde bulunan etlerin miktarı ve orijinlerinin analizleri için DNA temelli teknikler olan konvensiyonel çoklu zincir çoğaltma reaksiyonu (CM-PCR), kantitatif gerçek zamanlı zincir çoğaltma reaksiyonu (QRT-PCR) ve çoklu gerçek zamanlı zincir çoğaltma reaksiyonu (MRT-PCR), geliştirildi ve optimize edildi. Ve aynı zamanda kasıtlı olarak eklenen etlerin varlığını ve oranlarını elde etmek için bu teknikler kullanıldı.

CM-PCR tekniği gıda maddelerinde en çok kullanılan ruminant, kanatlı, balık ve domuz gibi türlerin identifikasyonu için çiğ ve işlenmiş etlere mitokondrial DNA'nın farklı bölgelerine dizaynedilmiş türe özgü primerler GenBank veri tabanlarında elde edilebilir sekansların sıraya konulmasından sonra kullanıldı. Bu primerler kanatlı için 183, balık için 224, domuz için 290 ve ruminant için 374 baz uzunluğunda spesifik frakmantlar üretti. Optimize edilen CM-PCR piyasadan satın alınan 93 et ürününe uygulandı ve analiz edilen çiğ ve işlenmiş kırmızı etlerin %50 sinde tavuk eti olduğu böylece içerisinde etiketinde işaret edilmeyen hayvan türlerinin varlığı kanıtlandı. Ve aynı zamanda bütün multiplex reaksiyonlaı test edilen 71 numunenin 25 (%35.1) inin beklenmeyen sonuçlar verdiğini gösterdi. Ancak bu varlığın üretim esnasındaki rasgele bir kontaminasyon mu yoksa kasten katılmış et mi olduğuna karar verilemedi. Bu dezavantajdan dolayı SYBR Green temelli bir algılama sistemi kullanılarak QRT-PCR geliştirildi.

QRT-PCR'ın minimum etkili kantitatif düzeyi ruminant için 0,00006 ng/µl,kanatlı için 0.000076 ng/µl, balık için 0,000045 ng/µl ve domuz için 0,000075 ng/µl idi. Bu teknik kullanılarak ticari olarak satın alınmış 93 et örneği içerisinde ki işlenmiş 9 et ürünü test edildi. Ruminant et oranları dilüsyon standart eğri serileriyle karşılaştırıldıktan sonra tahmin edildi. Ruminant et oranları için sonuçlar karışık-şlenmiş etlerinin defolu olduğunu ve içerisinde, ruminant etlerinin yerine önemli ölçüde ana komponent olarak kanatlı etlerinin ikame edildiğini gösterdi. Çünkü test edilen karışık-işlenmiş et ürünlerininde ki kanatlı etlerinin oranları etiketlerinde verilen oranlarından daha fazlaydı bunun aksine ruminant etlerinin oranı ise daha azdı.

Burda aynı zaman da SYBR Green florasan boyası kullanarak hayvan genlerinin daha hızlı algılanması ve tanımlanması için CM-PCR ve QRT-PCR'ın avantajlarını kombine eden MRT-PCR geliştirildi. Çalışmanın bu kısmının amacı karışık etlerin bir gurubunu eşzamanlı olarak tespit etmek için çift etiketli problardan daha ucuz olan SYBR Green florasan boyası kullanarak hızlı, spesifik ve doğru MRT-PCR yi dizayn etmekti. MRT-PCR sonuçlarımız karışık gıda maddelerinden izole edilen 2 türe ait DNA'nın hızlı bir şekilde tespit edileceğini gösterdi. Şunuda söylemeliyiz ki; SYBR Green florasan boyası kullanarak karışık etlerden izole edilen DNA üzerine uyguladığımız hem QRT-PCR hemde MRT-PCR tekniklerimiz moleküler gıda analizi alanında tekti.

**Anahtar Kelimeler;** PZR, kantitatif gerçek zamanlı PZR, çoklu PZR, etlerin kantitatif analizi, etlerin orjinlerinin tayini, SYBR Green

**DEDICATIONS**

*Dedicated to my parents*

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### SYMBOL/ABBREVIATION

| | | |
|---|---|---|
| PCR | : | Polymerase Chain Reaction |
| S-PCR | : | Simplex PCR |
| CM-PCR | : | Conventional Multiplex PCR |
| QRT-PCR | : | Quantitative Real-time PCR |
| MRT-PCR | : | Multiplex Real-time PCR |
| MBM | : | Morphology-Based Method |
| DBM | : | DNA-Based Method |
| PBM | : | Protein-Based Method |
| QPT | : | Quantitative PCR Technique |
| SSCP | : | Single Strand Conformation Polymorphism |
| RFLP | : | Restriction Fragment Length Polymorphism |
| BSE | : | Bovine Spongiform Encephalopathy |
| CJD | : | Creutzfeldt–Jakob Disease |
| TSE | : | Transmissible Spongiform Encephalopathies |
| US | : | United State |
| EU | : | European Union |
| FDA | : | Food and Drug Administration |
| IEF | : | Isoelectric Focusing |
| ELISA | : | Enzyme-Linked Immunosorbent Assay |
| RAPD | : | random amplified polymorphic DNA |
| MGB | : | Minor Groove Binding |
| SDS-AGE | : | Sodium Dodecyl Sulfate Agarose Gel Electrophoresis |
| SDS-PAGE | : | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |

| | | |
|---|---|---|
| MtDNA | : | Mitochondrial DNA |
| Tm | : | Temperature |
| dNTP | : | Deoxynucleoside Triphosphates |
| CCD | : | Charge-Coupled Device |
| RT-PCR | : | Reverse Transcriptase PCR |
| Ct | : | Cycle Threshold |
| EtBr | : | Ethidium Bromide |
| TBE | : | Tris Boric EDTA |
| LED | : | Light Emitting Diode |
| RFU | : | Relative Fluorescence Units |
| RPM | : | Revolution Per Minute |
| RU | : | Ruminant |
| PO | : | Poultry |
| FS | : | Fish |
| PR | : | Pork |
| UK | : | Unknown |
| PM | : | Processed Meat (salami, sausages, ham, frankfurter, meat ball) |
| RMM | : | Raw mince meat; |
| RWM | : | Raw Meat; |
| RS | : | Reference Species; |
| RR | : | Raw Ruminant; |
| RF | : | Raw Fish; |
| RP | : | Raw poultry; |
| RU | : | Raw but species unknown; |
| MLK | : | Mix, labeled and concentration of species given; |
| MLU | : | Mix, labeled and concentration of species not given; |
| MU | : | Mixed but species unknown |

# CHAPTER 1

# INTRODUCTION

Meat and meat products include nutrients that human requires for growing up, physiological function, body and soul healthy. The consumption and production of food in convenient conditions is a vital phenomenon for human life. It is accepted by outstanding authorities in food sector that diet without meat and meat products is unhealthy nourishment for consumers.

There has been a very great in numbers growth in quality product consumption and a change position in this respect. Currently, consumers want to buy quality products which are good-labeled. However, false or accidental mislabeling still exits and may not be detected, resulting in poor-quality products. People with allergy against special meat and its products do not demand combined meat products having allergen materials owing to the fact that their health will be in danger [1].

The identity of the ingredients in processed or composite mixtures is not always readily apparent and verified that the components are authentic and from sources acceptable to the consumers may be required. In most countries, food manufacturers choose to use some products in stead of another one such as lard, as a substitute ingredient for oil, chicken meat as a substitute ingredient for red meat because they are cheaper and easily available. Biological complications and health risks may be associated with daily intake. Hence, it is an important task for food control laboratories to be able to carry out species differentiation of raw materials to be used for industrial food preparation and the detection of animal species in food products [2]

Legislative authority in Turkey reported that the companies must accurately label their processed or raw meat products regarding species content. The possible aims of this law are; (i) to prohibit the unfair competition between food companies due to the fraudulent substitution or adulteration, (ii) to reduce medical problems of consumers who have specific meat allergies and (iii) religious rules in who are Muslims and Jewish peoples are not allowed to consume pork meat and many Hindu do not consume beef.

There is a potential possibility that some malicious companies have not obeyed the rule above. That is why scientists have developed several quantitative and qualitative analytical methods to identify meat species used in food stuffs. The methods can be classified into three groups: morphology-based methods (MBM), protein-based methods (PBM) such as electrophoretical, chromatographic and immunological techniques and DNA-based methods (DBM). Although electrophoretical and chromatographic methods are very popular, they have many disadvantages such as difficult optimization procedures, time consuming, high cost and low specificity and sensitivity. On the other hand, different cells in an organism can have different protein profile and in addition, proteins start to denature and alter their epitopes, which are essential for immunological techniques after an animal's death, cooking and heavy processing.

On the other hand, DBMs are two major advantages over PBMs. DNA is more stable in extreme conditions and that is why samples heated to as high as 120oC can still be analyzed. Next, DBMs are very species-specific to discriminate between even much related species such as chicken and turkey.

Many DBMs such as hybridization, PCR-RFLP analysis, PCR assay, SSCP analysis are used for qualitative or semi-quantitative analysis of meat products. PCR, as one of the most popular detection techniques, is too specific that minute traces of material could produce a positive result. In food technology, there is always a possibility for products to be contaminated with a negligible amount of any contaminants. It results in a requirement to develop quantitative DBMs that exactly distinguish samples containing negligible level of contamination from ones that are deliberately mis-described or adulterated.

In contrast to conventional PCR, quantitative PCR techniques (QPT) such as non-real-time QPTs and real-time QPTs, are able to discriminate and measure even minute traces of different animal species in foodstuff. Non-real-time QPTs are often described as ‘semi-quantitative PCR techniques’. They have some limitations to be solved during the quantitative measurements of DNA samples. One limitation is that they analyze end-point amplification products which may cause inhibitory effects to the reaction. That is why it can be difficult to interpret their results.

On the other hand, real-time PCR allows users to real-time monitor the production of amplification products and quantify samples at an early stage. Because of the inhibitory effects of PCR products, the quantification at an early stage in PCR process is intrinsically more accurate than at the end point analysis naturally associated with agarose or polyacrylamide gel electrophoresis.

Real-time data collection is accomplished by fluorescent molecules providing a strong correlation between fluorescence intensity and PCR product concentration. The fluorescent based methods used in real time PCR can be classified in two categorizes: probe-based such as TaqMan and DNA intercalating dyes such as the SYBR Green. Although probe-based ones are more accurate and additionally sequence- specific, they are more expensive, time and labor-intensive and it is much more difficult to design and optimize them.

Alternatively, SYBR Green I, DNA binding dye, binds in the minor groove of double-stranded DNA in a sequence-independent way and its fluorescence increases over 100-fold. That is why it is used to detect PCR products during real-time PCR without the need for any probes. Optimization is easy because the protocols in use for classic PCR can be used with only slight modifications. Economically, it is almost seven folds cheaper than probe-based methods.

Fajardo et al. [3] optimized a real-time PCR technique with SYBR Green detection system for quantification of red, fallow and roe deer in meat mixtures. Sawyer also developed a similar method to quantify total meat content and beef content in mixed samples by using species specific and universal primers.

In this work, we focused on quantification and identification of mixed meat such as sausage, salami and raw meat by combination of multiplex and the real-time PCR techniques. We describe the development of a real-time PCR technique with SYBR Green detection system to quantify the percentage of DNA in processed meat samples. Another aim of the present study was to improve multiplex real-time (MRT-PCR) in order to analysis food products containing mixed-treated and raw meat by combination of multiplex PCR and real-time PCR for the rapid identification and quantification of ruminant, poultry, fish and pork materials.

## 1.2. REVIWE OF LITERATURE

### 1.2.1. Potential Problems of Meat Usage from the Aspect of Health, Economical and Religious

Bovine spongiform encephalopathy (BSE), commonly referred to as ‘‘mad cow disease,’’ has a human form termed vCJD that is a variant of Creutzfeldt–Jakob disease, a fatal neurodegenerative disease that has caused large in number deaths. [4]

CJD in humans, BSE in cattle and scrapie in sheep, are all transmissible spongiform encephalopathies (TSE). The BSE epidemic was first recognized in the England in 1986 and it was diagnosed in Ireland in 1989, with a total of 474 cases reported until the present time. The epidemic is thought to have occurred as a result of feeding scrapie infected substances to cattle. It has been reported that the incidence of scrapie cases in sheep with similar protein features to those of BSE are possibly more frequent than originally believed. In 1996, the identification of new (vCJD) lead to the proposal that this new disease was caused by the transmission of BSE to the human population.1988 saw the introduction of a ban in the EU and the US on the inclusion of ruminant derived protein in animal feed. [5, 6]

Incorrect labeling of animal foods represents not only a commercial fraud but also a potential health problem in the case of consumers who exhibit sensitivity to undeclared allergens [3]. It is now clear that beef allergy is not an infrequent

occurrence, with an incidence between 3.28% and 6.52% among children with atopic dermatitis, its incidence may be as much as 0.3% in the general population [7].

The risk associated with infectious transmissible BSE in humans has discouraged many individuals around the world from consuming beef. Hindu populations also choose not to eat beef, while Jewish and Muslim populations choose to avoid consumption of pork, even in minute quantities, due to their religious beliefs. Many consumers prefer to include more chicken in their diet instead of beef, sheep or pork. In addition to infectious disease and religious concerns, many individuals are altering their eating behavior to include more chicken simply to reduce dietary fat intake in accordance with health trends. Any conceivable ambiguity in the labeling practices of commercial suppliers or grocery stores is unacceptable to these populations. The need for sensitive detection and quantification of bovine, chicken, porcine, and species in food and mixed foods products is critical in response to this consumer demand [4].

Meat adulterations have been increased for last twenty years. Researches on identification of meat have shown that there is misuse in the meat industry worldwide. For example; in Australia it had been reported that sheep, horse and kangaroo meats had interspersed in the exported ruminant meats. In Florida it had been detected that 22.5% of processed meats, 15.9% of ground meats include the meat of different animal. Kang'ethe reported that beef products of two different enterprises include 78.6% and 6.3% of pig meat after his analyze. In Mexico, a research shows that horse meat is detected in 9 of 23 hamburgers and mixture of horse and pork meat in 5 of 17 Mexico sausages. In Turkey, in last years it were determined that smuggled buffalo meats were bought in domestic market illegally. According to İzmir Veterinary Presidency's analyses of in a dish made of minced meat (çiğ köfte) which must be prepared with cow meat other meats (pork, horse and donkey meat) were detected [8]. Cattle meat was detected in 410 samples, chicken meat and horse meat were detected in 85 (20.7 %) and 14 (4.3 %) samples, respectively. According to the statistics in ref 8, 67 of 410 (16.3 %) samples were not in line with their labels and 79 of 410 (19.2 %) samples were found incorrectly [9].

### 1.2.2. Legislative about Meat

In response to the BSE epidemic in Europe, the United States Food and Drug Administration (FDA) imposed strict guidelines in 1997, prohibiting the use of ruminant-derived protein in the manufacture of animal feed intended for cows or other ruminants. The need for sensitive detection of ruminant species remains causing disease in foodstuffs is a crucial issue [4].

The European Union has implemented a set of very strict procedures for the labeling of food. Throughout the whole legislative procedure, the EU ensures the European consumers' right to be fully informed. Thus analytical methods for the species detection of food are essential in order to verify compliance with labeling requirements [10].

In Turkey, according to 147$^{th}$ article of food law, the species' names of meats used to prepare the meat products have to be presented on the label of product. Moreover, selling the other species' meats with different labels to get more profit is held as imitation and prohibition according to the Foodstuff Laws [11].

### 1.2.3. Methods for Meat Origin Analysis

Methods to analysis meat products can be divided to three categories according to the type of the target they focused on.

- Organoleptic Methods
- Protein-based Methods
- DNA-based Methods

#### 1.2.3.1 Organoleptic Methods

In these methods meat products are examined by naked-eye or microscopy. The methods used for determining meat species in the mixture food have changed from organoleptic examinations based on sensory characteristics and anatomical differences, to histological differences of hair and physical properties of fat tissue [12].

Some of disadvantages of the methods by naked–eye (organoleptic), for example a microscopic method based on the analysis of animal bone fragments.This method has been recognized as the ‘official’ method in the European strategy against the BSE. However, the need to long time, specialized staff in order to apply this technique and only enables the detection of zoological classes (mammalian, avian and fish), while the species origin of bone fragments remains undetermined. This technique is limited because it has no widely area of application. Although DNA undergoes thermal denaturation like proteins, it has been observed that DNA can be still detected by short fragment amplification [13].

#### 1.2.3.2. Protein-Based Methods

Many analytical methods that rely on protein analysis have been developed for identification of species such as liquid chromatography [14], electrophoresis techniques [15] immunological methods such as the enzyme-linked immunosorbent assay (ELISA) test [16,17] Isoelectric focusing (IEF) [18], glycogen levels in the muscle tissue, electrophoretical profiles of sarcoplasmic proteins. These methods differ greatly in sensitivity and specifity. Also, some of the methods are labor demanding requiring expertise and sophisticated and necessary items. Another factor limiting the use of some advanced methods is processing factor. For example, identification of origin of meats by ELISA can fail due to denaturation of protein in high temperature. Hence, number of researchers has been emphasized that there is a need for simpler, accurate and rapid techniques in order to determine the species of meat in meat products, especially in cooked-meat products.

#### 1.2.3.3. DNA-Based Methods

PCR, a method for amplification of DNA in an artificial environment, has been successfully used for species identification of animals, plant and bacteria [12]. The dot-blot technique was the first genetic approach for determination of species identity [19, 20]. At present; however, polymerase chain reaction (PCR) is an important technique for species identification [21]. Some PCR approaches are RAPD-PCR (random amplified polymorphic DNA fingerprints) [22], DNA mitochondrial D-loop analysis and RFLP analysis of different PCR fragments [23, 24].

DNA hybridization methods are complicated and generally inadequate, but PCR easily amplies target regions of template DNA in a much shorter time and thus is suitable for meat identification [24]. All PCR techniques have both advantages and disadvantages according to application areas.

Matsunaga et al. [24] developed a simple method using multiplex PCR for simultaneous identification of six meats.

Polymerase Chain Reaction (PCR) has been applied for the detection of bovine tissue in animal feedstuffs [25, 26].

Lahiff et al. [27] developed a PCR to identify ovine, porcine and poultry DNA in feedstuffs.

Myers et al. [28] identified different species in feedstuffs by using universal primers coupled with restriction endonucleases.

More recently, Bottero et al. [29] developed a method which involved the ability of primers to amplify wider target sequences. This PCR based assay demonstrated to be highly sensitive and useful in routine feedstuff analysis for the detection of all vertebrates.

A.Di Pinto et al. [10] optimized a duplex PCR in order to identify pork meat in horse meat fresh sausages from Italian retail sources.

Dalmasso et al. [13] developed sensitive multiplex PCR. The detection limit was 0,004% for fish primer and 0,002% for ruminants, poultry and pork primers.

Marıa Lopez-Andreo et al. developed six TaqMan real-time polymerase chain reaction (PCR) systems using minor groove binding (MGB) probes for the detection quantitation of bovine, porcine, lamb, chicken, turkey, and ostrich DNA in complex samples [30].

Violeta Fajardo et al. [31] developed a rapid real-time polymerase chain reaction (PCR) technique using SYBR Green detection system for the quantification of red deer, fallow deer, and roe deer DNAs in meat mixtures.

When the conventional PCR techniques were compared with real-time PCR, quantitative real-time PCR approaches allow discrimination and measurement of even minute traces of different animal species in foods of complex composition. Specifically, real-time PCR refers to the process where the production of amplification products is directly monitored during each amplification cycle. The assay allows to quantification at an early stage in the PCR process which is essentially more accurate than at the end point analysis typically associated with gel agarose gel electrophoresis (SDS-AGE) or polyacrylamide gel electrophoresis (SDS-PAGE) [31].

The need to support food-labeling legislation has provided a motivation in the development of methods for the analysis of food ingredients. PCR has been utilized for animal species identification by several authors. The amplification potential of PCR means that the technique can be exceptionally sensitive giving scope for the analysis of samples which, because of their low levels of target DNA, could not be tested using other methods. This sensitivity is, however, potentially problematic since a low level of adventitious contamination is often permitted by food labeling legislation. A common argument against the use of PCR based techniques has been that they are too sensitive and that minute traces of material would produce a positive result. Therefore there is a requirement to improve quantitative PCR methods that are sufficiently accurate to distinguish samples containing adventitious levels of contamination from those that are deliberately mis-described or adulterated.

The use of real-time PCR also offers advantages by allowing measurement at an early stage in the PCR process which is inherently more accurate than the end point analysis typically associated with gel based measurement. To illustrate the principles of this approach, the development of a system for the quantitative determination of beef in mixed samples is described [32].

### 1.2.4. Mitochondrial DNA (MtDNA)

Mitochondria are small granular or filamentous intracellular organelles, which are referred to as ‘powerhouse of the cell’. The number of mitochondria in a cell

depends upon its metabolic activity. On average, around 800–1000 mitochondria have been found per cell in animals. Each mitochondrion contains two to six circular DNA molecules with a size of about 16,500 bp. Each DNA molecule contains 22 tRNAs, 2 rRNAs and 13 protein coding gene. (Figure 1) Cytochrome b. and rRNA genes are the commonly targeted mitochondrial (mt) gene for meat identification. The species differentiation of raw/ heat processed meats is investigated by use of sequence analysis of mitochondrial 12S rRNA gene [33].

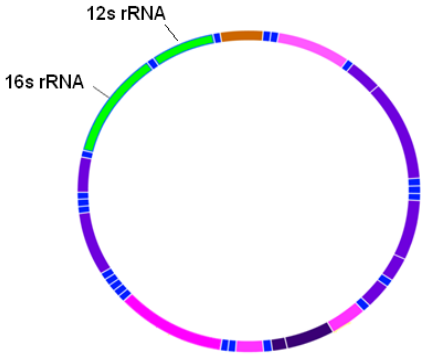



**Figure 1.** Mitochondrial DNA[1]

MtDNA is inherited from both parents and in which genes are rearranged in the process of recombination there is usually no change in mtDNA from parent to offspring. Although mtDNA also recombines, it does so with copies of itself within the same mitochondrion [34]. MtDNA is a powerful tool for tracking ancestry through females (matrilineage) and has been used in this role to track the ancestry of many species back hundreds of generations [35].

Segments of mitochondrial genome have proved to be useful for authentication of species origin of meat products. Mitochondrial genome has several advantages over nuclear genome for diagnostic studies of animals, because of a greater abundance in sample extracts and a higher copy number. In addition, mitochondrial genome is maternally inherited, and then sequence ambiguities from heterozygous genotypes are theoretically avoided [35].

[1]http://images.google.com/images

### 1.2.5. Primer

In recent years, methods for species detection based on amplification of target DNA regions through the use of species-specific primers and universal primers have been applied. The definition of mono-locus-specific primers is quite reliable. The results from the use of universal primers can be affected by the existing intraspecific polymorphisms and, normally, the identification of the amplicons requires the application of additional and more complex analytical techniques, such as sequencing, hybridization and single-strand conformational polymorphism determination (SSCP-PCR) [36].

Jason Sawyer et al. [32] improved a real-time PCR for quantitative meat species testing with the combines the use of real-time PCR with species-specific and universal primers to measure individual species content and total meat content respectively.

## 1.3. POLIMERASE CHAIN REACTION (PCR)

Polymerase Chain Reaction (PCR) [37, 38], allowed the production of large quantities of a specific DNA from a complex DNA template in a simple enzymatic reaction. PCR is a recently developed procedure for the *in vitro* amplification of DNA. PCR has transformed the way that almost all studies requiring the manipulation of DNA fragments may be performed as a results of its simplicity and usefulness [39]. In the 1980s, Kary Mullis and a team of researchers [40] at Cetus Corporation understood a way to start and stop a polymerase's action at specific points along a single strand of DNA. This DNA amplification procedure was based on an in vitro rather than an in vivo method [37, 38, 39]. Cell-free DNA amplification by PCR was able to simplify many of the standard procedures for cloning, analyzing, and modifying nucleic acids [41]. Previous techniques for isolating a specific piece of DNA relied on gene cloning slow procedure and a tedious PCR. When other scientists eventually succeeded in making the polymerase chain reaction perform as desired in a reliable fashion, they had an immensely powerful technique for providing essentially unlimited

quantities of the precise genetic *material* [39]. The first report related to PCR was in1985, more than 5000 scientific papers were published by 1992 [41].

### 1.3.1. General Principles of the PCR

PCR amplification mechanism shows not only its simplicity but also its practical function. (Figure 2). Oligonucleotide primers are first designed to be complementary to the ends of the sequence to be amplified, and then mixed in molar excess with the DNA template and deoxyribonucleotides in an appropriate buffer. Following heating to denature the original strands and cooling to promote primer annealing, the oligonucleotides each bind to a different strand of the target fragment. The primers are positioned so that when each is extended by the action of a DNA polymerase, the newly synthesized strands will overlap the binding site of the opposite oligonucleotide.

As the process of denaturation, annealing, and polymerase extension is continued the primers repeatedly bind to both the original DNA template and complementary sites in the newly synthesized strands and are extended to produce new copies of DNA. The end result is an exponential increase in the total number of DNA fragments that include the sequences between the PCR primers, which are finally represented at a theoretical abundance of 2n, where n is the number of cycles [41, 39, 42].

### 1.3.2. PCR Steps

#### 1.3.2.1. Initialization Step

This step consists of heating the reaction to a temperature of 94-96°C (or 98°C if extremely thermostable polymerases are used), which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR [39].

### 1.3.2.2. Denaturation Step

This step is the first regular cycling event and consists of heating the reaction to 94-98°C for 20-30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

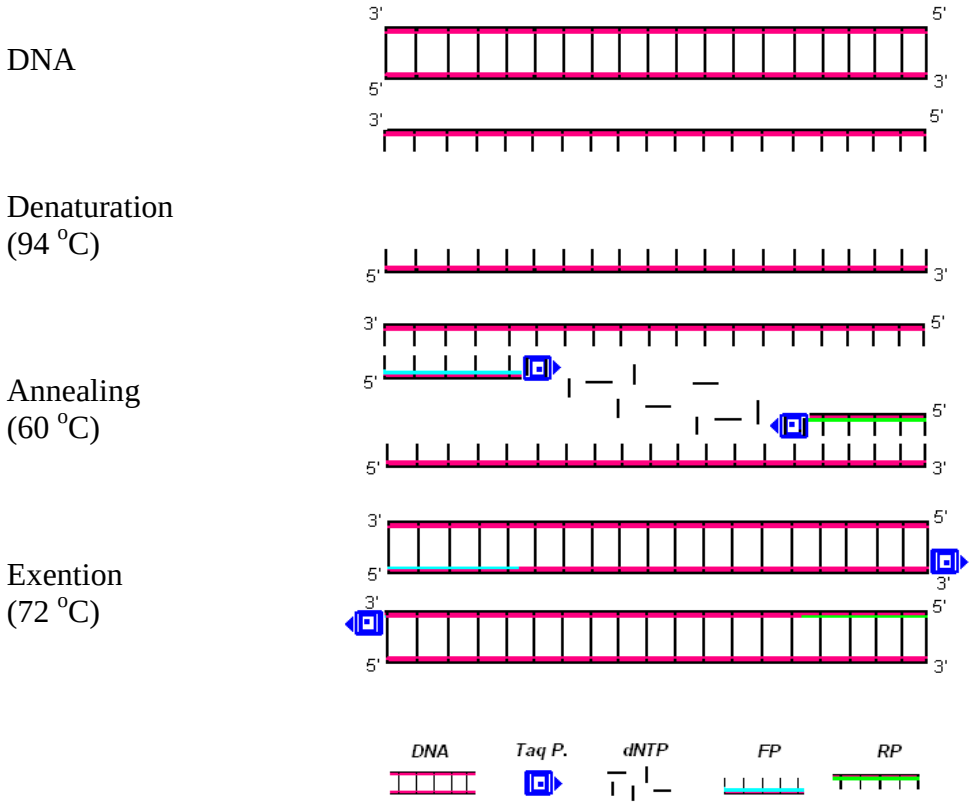



**Figure 2.** PCR amplification mechanisms.

#### 1.3.2.2.1 Denaturing Temperature and Time

The specific complementary association due to hydrogen bonding of single-stranded nucleic acids is referred to as "annealing": two complementary sequences will form hydrogen bonds between their complementary bases (G to C, and A to T or U) and form a stable double-stranded, anti-parallel "hybrid" molecule. One may make nucleic acid (NA) single-stranded for the purpose of annealing - if it is not single-stranded already, like most RNA viruses - by heating it to a point above the "melting

temperature" of the double- or partially-double-stranded form, and then flash-cooling it: this ensures the "denatured" or separated strands do not re-anneal. Additionally, if the NA (nucleic acide) is heated in buffers of ionic strength lower than 150mM NaCl, the melting temperature is generally less than 100$^{o}$C - which is why PCR works with denaturing temperatures of 91-97$^{o}$C.

Taq polymerase is given as having a half-life of 30 min at 95$^{o}$C, which is partly why one should not do more than about 30 amplification cycles: however, it is possible to reduce the denaturation temperature after about 10 rounds of amplification, as the mean length of target DNA is decreased: for templates of 300bp or less, denaturation temperature may be reduced to as low as 88$^{o}$C for 50% (G+C) templates [43] which means one may do as many as 40 cycles without much decrease in enzyme efficiency.

"Time at temperature" is the main reason for denaturation / loss of activity of Taq thus, if one reduces this, one will increase the number of cycles that are possible, whether the temperature is reduced or not. Normally the denaturation time is 1 min at 94$^{o}$C: it is possible, for short template sequences, to reduce this to 30 sec or less. Increase in denaturation temperature and decrease in time may also work: Innis and Gelfand [44] recommend 96$^{o}$C for 15 sec.

#### 1.3.2.3. Annealing Step

The reaction temperature is lowered to 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

##### 1.3.2.3.1. Annealing Temperature and Primer

Designing of primer length and sequence is of critical importance in designing the parameters of a successful amplification: the melting temperature of a DNA duplex

increases both with its length, and with increasing (G+C) content: a simple formula for calculation of the Tm is. Tm = 4(G + C) + 2(A + T) $^{o}$C.

Thus, the annealing temperature chosen for a PCR depends directly on length and composition of the primer(s). One should aim at using an annealing temperature (Tm) about 5$^{o}$C below the lowest Tm of their pair of primers to be used [44]. A more rigorous treatment of Ta is given by Rychlik *et al*. [45]: they maintain that if the Tm is increased by 1$^{o}$C every other cycle, specificity of amplification and yield of products <1kb in length is both increased. One consequence of having too low a Tm is that one or both primers will anneal to sequences other than the true target, as internal single-base mismatches or partial annealing may be tolerated: this is fine if one wishes to amplify similar or related targets; however, it can lead to "non-specific" amplification and consequent reduction in yield of the desired product, if the 3'-most base is paired with a target.

A consequence of too high a Ta is that too little product will be made, as the likelihood of primer annealing is reduced; another and important consideration is that a pair of primers with very different Ta may never give appreciable yields of a unique product, and may also result in inadvertent "asymmetric" or single-strand amplification of the most efficiently primed product strand. Annealing does not take long: most primers will anneal efficiently in 30 sec or less unless the Ta is too close to the Tm, or unless they are unusually long.

#### 1.3.2.4. Extension/Elongation Step

The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75-80°C [45, 46] and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTP's that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases in one minute.

#### 1.3.2.4.1. Elongation Temperature and Time

This is normally 70 - 72$^{o}$C, for 0.5 - 3 min. Taq actually has a specific activity at 37$^{o}$C which is very close to that of the Klenow fragment of *E coli* DNA polymerase I, which accounts for the apparent paradox which results when one tries to understand how primers which anneal at an optimum temperature can then be elongated at a considerably higher temperature - the answer is that elongation occurs from the moment of annealing, even if this is transient, which results in considerably greater stability. At around 70$^{o}$C the activity is optimal, and primer extension occurs at up to100 bases/sec. About 1 min is sufficient for reliable amplification of 2kb sequences [44]. Longer products require longer times: 3 min is a good bet for 3kb and longer products. Longer times may also be helpful in later cycles when product concentration exceeds enzyme concentration (>1nM), and when dNTP and / or primer depletion may become limiting.

#### 1.3.2.5. Final Elongation

This single step is occasionally performed at a temperature of 70-74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

#### 1.3.2.6. Final Hold

This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

(a) 1.3.3. Cycle Number

The number of amplification cycles necessary to produce a band visible on a gel depends largely on the starting concentration of the target DNA: Innis and Gelfand [44] recommend from 40 - 45 cycles to amplify 50 target molecules, and 25 - 30 to amplify 3x105 molecules to the same concentration. This non-proportionality is due to a so-called *plateau effect*, which is the attenuation in the exponential rate of product accumulation in late stages of a PCR, when product reaches 0.3 - 1.0 nM. This may be caused by degradation of reactants (dNTPs, enzyme); reactant depletion (primers, dNTPs - former a problem with short products, latter for long products); end-product

inhibition (pyrophosphate formation); competition for reactants by non-specific products; competition for primer binding by re-annealing of concentrated (10nM) product [44].

### 1.3.4. Taq Polymerase

Taq polymerase is a thermostable DNA polymerase named after the thermophilic bacterium *Thermus aquaticus* from which it was originally isolated [46]. It is normally abbreviated to "*Taq* Pol," or simply "*Taq*", and frequently used in PCR.

*T. aquaticus* is a bacterium that lives in hot springs and hydrothermal vents, and Taq was identified as an enzyme able to withstand the protein-denaturing conditions, namely, high temperature, required during PCR. [46] Therefore it replaced E.coli DNA polymerase in PCR. Taq's temperature optimum for activity is 75-80 °C with a halflife of 9 min at 97.5 °C [49].

One of Taq's drawbacks is its low replication fidelity since it lacks a 3' to 5' exonuclease proofreading activity; thus it has an error rate of about one in 9,000 nucleotides [50]. It can amplify a 1-kb strand of DNA in roughly 30-60 seconds at 72 °C. Some thermostable DNA polymerases, such as Pfu DNA polymerase that have been isolated from other thermophilic bacteria possess 3'-5' exonuclease proofreading activity.

### 1.3.5. Deoxynucleoside Triphosphates (dNTP)

A nucleotide is a chemical compound that consists of 3 portions: a nitrogenous base, a sugar, and one or more phosphate groups. In the most common nucleotides the base is a derivative of purine or pyrimidine, and the sugar is the pentose deoxyribose or ribose. Nucleotides are the monomers of nucleic acids, with three or more bonding together in order to form a nucleic acid. *Deoxynucleoside triphosphates* (dNTPs; also very commonly and erroneously are called deoxynucleotide triphosphates), the building blocks from which the DNA polymerases synthesizes a new DNA strand.

### 1.3.6. Magnesium Chloride Concentrations

Varying the $MgCl_2$ concentration is thought to affect primer annealing and template denaturation, as well as enzyme activity and fidelity. Note that the presence of EDTA or other chelators will lower the effective concentrations of $MgCl_2$. *Taq* DNA polymerase and dNTPs also bind free $Mg_2^{+}$ Generally, excess $Mg^{2+}$ will result in the accumulation of nonspecific amplification products, although insufficient $Mg^{2+}$ will reduce the yield [44].

### 1.3.7. Reaction Buffer

Buffer solution was provided for suitable chemical environment and optimum activity and stability of the DNA polymerase. Recommended buffers generally contain:

- 10-50mM Tris-HCl pH 8.3,
- up to 50mM KCl, 1.5mM or higher MgCl2,
- primers 0.2 - 1uM each primer,
- 50 - 200uM each dNTP,
- gelatin or BSA to 100ug/ml,
- and/or non-ionic detergents such as Tween-20 or Nonidet P-40 or Triton X-100 (0.05 - 0.10% v/v)

Modern formulations may differ considerably, however - they are also generally proprietary [44, 45, 51].

### 1.3.8. Instruments for PCR

Thermocyclers which automatically regulate temperatures for PCR cycling were introduced in 1986. In addition to the advances in PCR reagents, new instruments for automated thermal cycling and for analyzing PCR products have been developed. New thermal cyclers have increased rates of heating, cooling, and heat transfer to modified reaction vessels. The reaction vessels accommodated by the first generation thermal cyclers (or even water baths and heating blocks) were standard plastic microfuge tubes. PCR amplification in thin capillary tubes allowed rapid thermal cycling, and DNA synthesis to 20s. The speed of the temperature changes achieved in these systems has allowed the precise definition of temperature optima for each individual

step in the PCR cycle. The new generation thermal cyclers also accommodate more samples, have more precise thermal profiles, and are programmable [42].

### 1.3.9. PCR Today

PCRs can now be performed enabling the amplification of DNA fragments up to several kilobases in length by more than one million times their initial abundance. The procedure is highly automatable and requires just a few hours from beginning the thermocyling to product analysis. This was not the case previously, and the practical requirements for performing a PCR have been greatly simplified since the first manuscripts of the method [42]. Today, most of the initial hitches or inefficiencies of the PCR have been worked out. Furthermore, PCR has expanded to include more than 313619 articles [52].

## 1.4. CONVENTIONAL MULTIPLEX POLYMERASE CHAIN REACTION (CM-PCR)

Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. Since its first description in 1988 this method has been successfully applied in many areas of DNA testing, including analyses of deletion mutations and polymorphisms or quantitative assays and reverse transcription PCR [53]. The use of multiple, unique primer sets within a single PCR mixture to produce amplicons of varying sizes specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes, i.e., their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis.

### 1.4.1. Advantages of CM-PCR

#### 1.4.1.1. Internal Controls

Potential problems in PCR include false negatives due to reaction failure or false positives due to contamination. False negatives are often revealed in multiplex amplification because each amplicon provides an internal control for the other amplified fragments. For example, multiple exons may be amplified in assays that survey for gene deletion. Unless the entire region scanned by the multiplex PCR is deleted, amplification of some fragment(s) indicates that the reaction has not failed (Fig. 1A). Furthermore, because major deletions are usually contiguous, results that suggest noncontiguous deletions based on the absence of bands usually reflect artifactual failure of some fragments to amplify. Complete PCR failure can be distinguished from an informative no-amplification result by adding a control amplicon external to the target sequence to the reaction. In addition to monitoring PCR failure and artifacts, internal control amplicons can be designed to verify the presence of target template. In multiplex assays where closely related templates such as pathogen strains are distinguished by amplifying differing sequence, primers for a sequence common to all templates provide a positive control for amplification [54, 55].

#### 1.4.1.2. Indication of Template Quality

The quality of the template may be determined more effectively in multiplex than in single locus PCR. Degraded templates give weaker signals for long bands than for short. A loss in amplification efficiency due to PCR inhibitors in the template samples can be indicated by reduced amplification of an abundant control sequence in addition to the amplification of rarer target sequences in an otherwise standardized reaction [56].

#### 1.4.1.3. Indication of Template Quantity

The exponential amplification and internal standards of multiplex PCR can be used to assess the amount of a particular template in a sample. To quantity templates accurately by multiplex PCR, the amount of reference template, the number of reaction cycles, and the minimum inhibition of the theoretical doubling of product for each cycle

must be accounted. In the simplest method of quantification, the gene multiplexes for major deletions detect carriers or duplications in propounds when the band intensity of abnormal amplicons is compared with that of normal, homozygous fragments in the multiplex. Cycling conditions for carrier testing must be determined carefully, because the variation in amplicon intensities will be masked if the reaction is allowed to cycle until the signal is saturated. The diagnosis is most accurate when at least two other fragments are used in the comparison r and the analysis is performed by densitometry, fluorescent scanning on an automated DNA sequencer, or by analysis of charge coupled device camera images. Preliminary studies suggest that signal intensities of fluorescent multiplex PCR products may reflect relative amounts of mixed, disproportionate DNAs in forensic samples. The majority of multiplex quantification assays compare the signal intensity of a reference sequence to the signal from another sequence in the same reaction, either directly or by extrapolating the result to standard curves. There are numerous coamplification assays based on this principle in the literature of competitive PCR with RNA or DNA standards [57].

#### 1.4.1.4. Efficiency

The expense of reagents and preparation time is less in multiplex PCR than in systems where several tubes of uniplex PCRs are used. A multiplex reaction is ideal for conserving costly polymerase and templates in short supply. For maximum efficiency of preparation time, the reactions can be prepared in bulk, randomly tested for quality, and stored frozen without enzyme or template until use [58].

## 1.5. REAL-TIME PCR

### 1.5.1. History of Real-Time PCR

History of Real-Time PCR Techniques Higuchi *et al.* [59, 60] pioneered the analysis of PCR kinetics by constructing a system that detects PCR products as they gather. This “real-time” system includes the intercalating ethidium bromide in each amplification reaction, an adapted thermal cycler to irradiate the samples with ultraviolet light, and detection of the resulting fluorescence with a computer-controlled cooled CCD camera. Amplification produces increasing amounts of double-stranded

DNA, which binds ethidium bromide, resulting in an increase in fluorescence. By plotting the increase in fluorescence versus cycle number, amplification plots that provide a more complete picture of the PCR process than assaying product accumulation after a fixed number of cycles are produced by system.

### 1.5.2. Introduction to Real Time PCR

As the name suggests, real time PCR is a technique used to monitor the progress of a PCR reaction in real time with the combination of software and thermal cycler. At the same time, a relatively small amount of PCR product (DNA, cDNA or RNA) can be quantified. Real Time PCR is based on the detection of the fluorescent produced by reporter molecules which are like florescence dye, dual labeled probs which their light increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green) or sequence specific probes (i.e. Molecular Beacons, TaqMan® Probes or Scorpion Probes). Real time PCR facilitates the monitoring of the reaction as it progresses. One can start with minimal amounts of nucleic acid and quantify the end product accurately. Moreover, there is no need for the post PCR processing such as electrophoresis, gel preparing or monitoring under the  UV light because the resources are saved the and the time.

Real time PCR assays are now easy to perform, have high sensitivity, more specificity, and provide scope for automation. Real time PCR is also referred to as real time RT-PCR which has the additional cycle of reverse transcription that leads to formation of a DNA molecule from a RNA molecule. This is done because RNA is less stable as compared to DNA

Real-time PCR is the technique of collecting datas throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity [60]. Reactions are characterized by the point in time (or PCR cycle) where the target amplification is first detected. This value is usually referred to as cycle threshold ($C_t$), the time at which fluorescence intensity is greater than back ground fluorescence. Consequently, the greater the quantity of target DNA in

the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower $C_t$.

Real-time PCR assays are 10,000- to 100,000-fold more sensitive than RNase protection assays, 1000-fold more sensitive than dot blot hybridization. [61] The major disadvantage to real-time PCR is that it requires expensive equipment and reagents. In addition, due to its extremely high sensitivity, sound experimental design and an in-depth understanding of normalization techniques are imperative for accurate conclusions.

### 1.5.3. Fluorescent Reporter Molecules of Real-time PCR

In a real time PCR procedure, a fluorescent reporter molecule is used to monitor the PCR as it progresses. The fluorescence emitted by the reporter molecule manifolds as the PCR product accumulates with each cycle of amplification. Based on the molecule used for the detection, the real time PCR techniques can be categorically placed under two heads:

#### 1.5.3.1. Specific Probes

Specific detection of real time PCR is based on some oligonucleoside probes labeled with both a reporter fluorescent dye and a quencher dye. Probes are done with different chemistries are available for real time detection, these include: Molecular Beacons, TaqMan® Probes, FRET Hybridization Probes, Scorpion® Primers et cetera.

#### 1.5.3.2. Double-Stranded DNA Binding Dyes

Small molecules that bind to double-stranded DNA can be divided into two classes: Intercalators and minor groove binders [62]. Higuchi *et al.* [60] used the intercalator ethidium bromide for their real-time detection of PCR. Regardless of binding mechanism, there are two requirements for a DNA binding dye for real-time detection of PCR: a) increased fluorescence when bound to double-stranded DNA; b) no inhibition of PCR.

### 1.5.4. SYBR® Green

The mechanism of SYBR® Green I dye's interaction with DNA is not known. In real time PCR, DNA binding dyes are used as fluorescent reporters to monitor the real time PCR.The fluorescence of the reporter dye increases as the product accumulates with each successive cycle of amplification. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR during exponential phase.

In the solution, the unbound dye exhibits very little fluorescence. This fluorescence is substantially enhanced when the dye is bound to double strand DNA. SYBR® Green remains stable under PCR conditions and the optical filter of the thermocycler can be affixed to harmonize the excitation and emission wavelengths. (Figure 3)

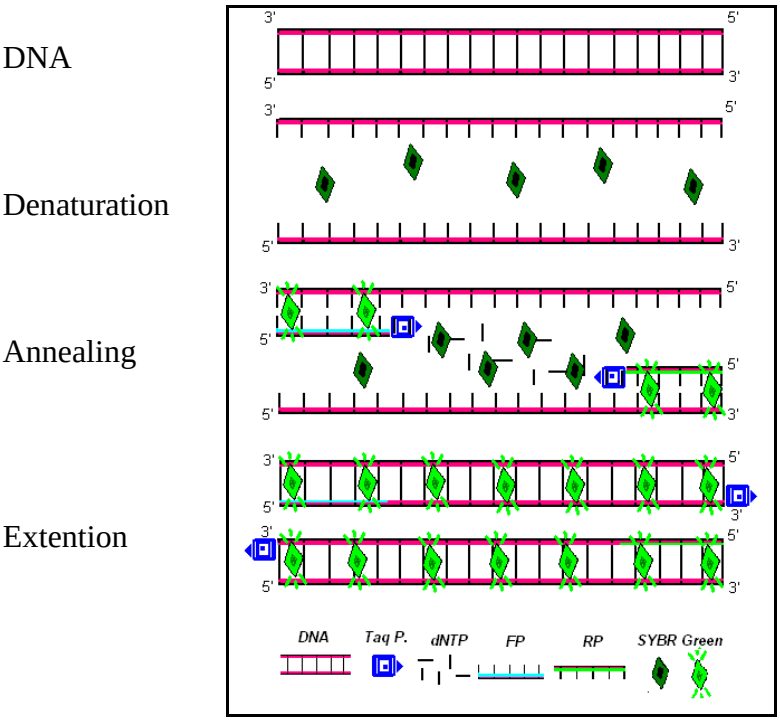



**Figure 3.** The binding of SYBR Green fluorescence dye between bases

While probe-based chemistries provide additional sequence-specificity to that given by the PCR primers, they are generally more difficult to design and optimize and add significantly to the overall costs of the analysis. In contrast, a DNA binding dye like SYBR green, which adheres to the minor groove of the double stranded DNA in a sequence-independent way, provides a flexible method without the need for individual probe design and optimization steps [30].

SYBR Safe is a variant of SYBR Green that has been shown to have low enough levels of mutagenicity and toxicity to be deemed nonhazardous waste under U.S. Federal regulations. It has similar sensitivity levels to EtBr. [63]. The advantages of SYBR Green are that it is inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer dimers and other nonspecific reaction products, which results in an overestimation of the target concentration. For single PCR product reactions with well-designed primers, SYBR Green can work extremely well, with spurious nonspecific back ground showing up only in very late cycles [63].

### 1.5.5. Optical System of Real-time PCR

Figure 4 shows how sample tubes are illuminated and signals detected from within the reaction chamber. All tubes pass the detector every revolution (150 milliseconds), enabling high-speed data capture. Up to six separate LED light sources can be used in combination with six different detection filters [65]. Figure 6 shows that results of the amplification can observed at the real-time.

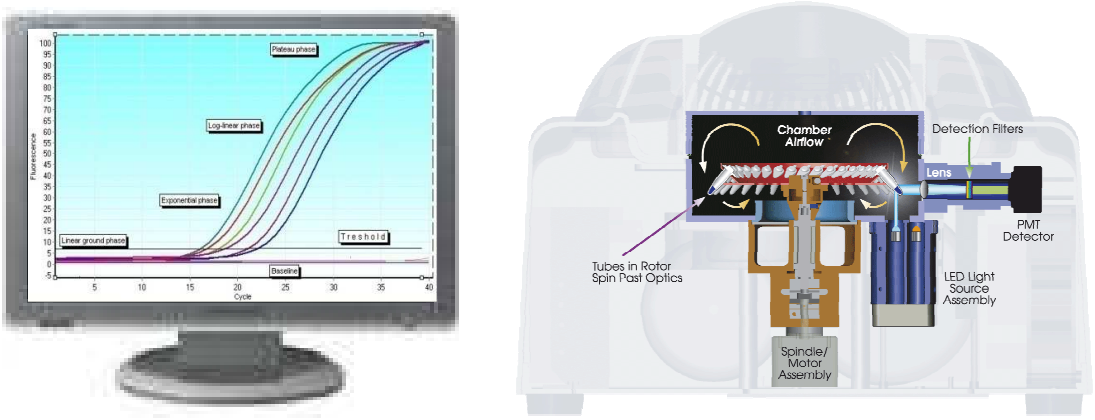



**Figure 4.** Cross-section of reaction chamber and appearance of curves of application.

### 1.5.6. Phases of Real-time PCR

PCR can be formed into four major phases (Figure 5): the linear ground phase, early exponential phase, log-linear phase, and plateau phase [66]. During the linear ground phase (usually the first 10–15 cycles), PCR is just beginning, and fluorescence emission at each cycle has not yet risen above background. Baseline fluorescence is calculated at this time. At the early exponential phase, the amount of fluorescence has reached a threshold where it is significantly higher than background levels. This value is representative of the starting copy number in the original template and is used to calculate experimental results [67]. During the log-linear phase, PCR reaches its optimal amplification period with the PCR product doubling after every cycle in ideal reaction conditions. Finally, the plateau stage is reached when reaction components become limited and the fluorescence intensity is no longer useful for data calculation [68].

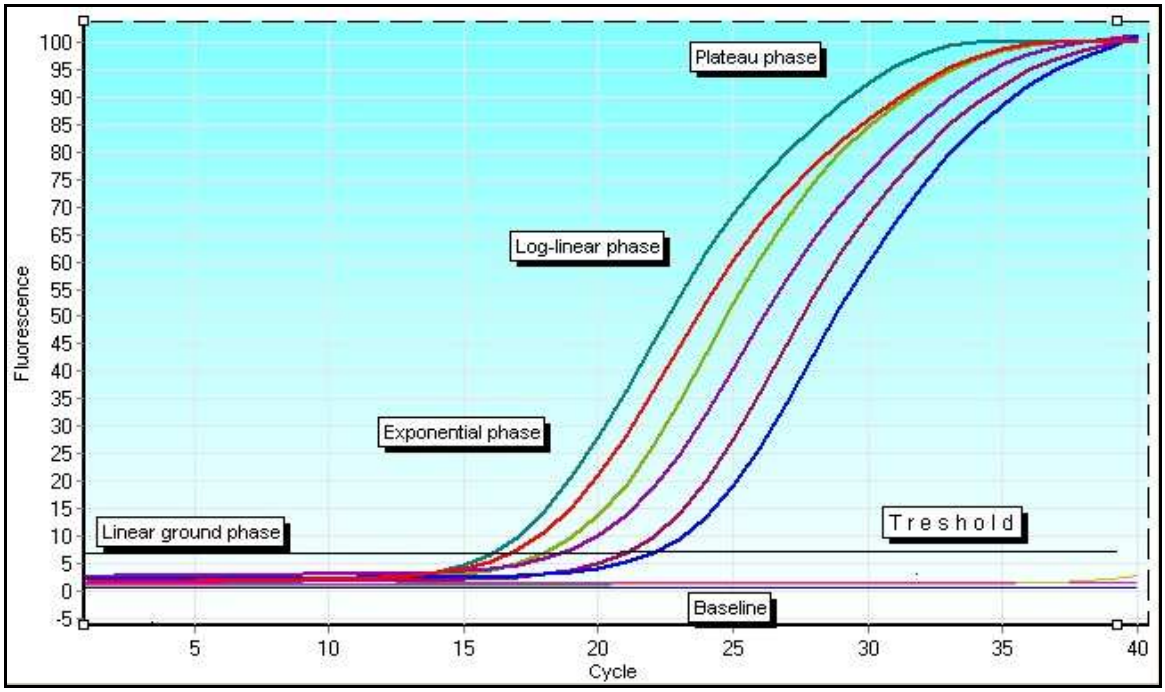



**Figure 5.** Phases of the PCR amplification curve.

The PCR amplification curve makes a graph the accumulation of fluorescent emission at each reaction cycle. The curves can occur into four different phases: the linear ground, early exponential, log-linear, and plateau phases. Data gathered from these phases are important for calculating background signal, cycle threshold (Ct), and

amplification efficiency. This graph was generated with Rotor-Gene 6000 series software, version 1.7.

## 1.6. QUANTITATIVE REAL-TIME PCR (QRT-PCR)

The ability to monitor the real-time progress of the PCR completely revolutionizes the way one approaches PCR-based quantification of DNA and RNA. Reactions are characterized by the point in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

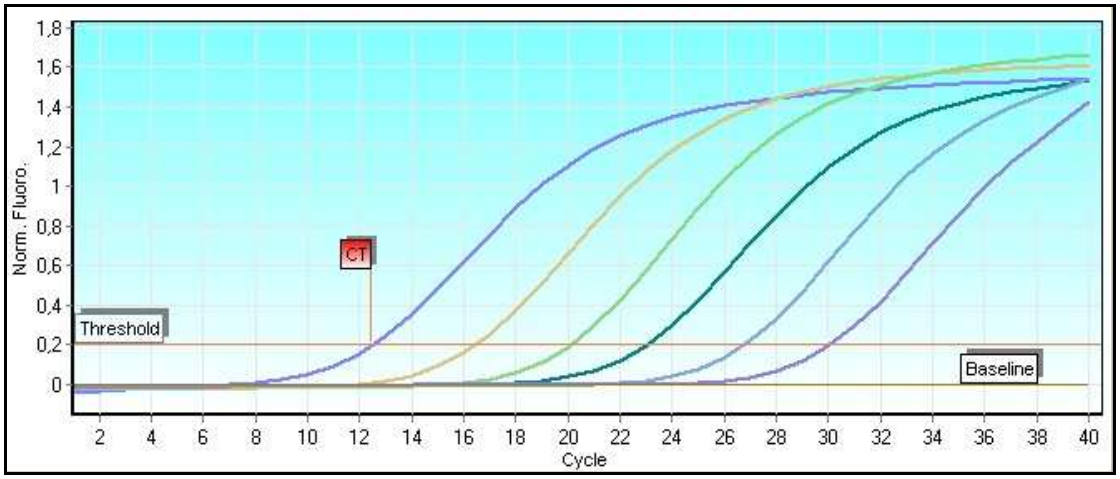



**Figure 6.** Amplification curves of quantification analysis

Figure 6 shows a representative amplification plot and defines the quantification analysis. An amplification plot is the plot of fluorescence signal versus cycle number. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter CT (cycle threshold) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. As shown by Higuchi et al. [60], a plot of the log of initial target copy number for a set of standards versus CT is a straight line. Quantification of the amount of target in unknown

samples is accomplished by measuring CT and using the standard curve to determine starting copy number.

### 1.6.1. Standard Curve

A standard curve is a research tool of quantification, a method of plotting assay data that is used to determine the concentration of a substance, particularly proteins and DNA. It can be used in many biological experiments. The assay is first performed with various known concentrations of a substance similar to that being measured. For example a standard curve for protein concentration is often created using known concentrations of bovine serum albumin. The assay procedure may measure absorbance, optical density, luminescence, fluorescence, radioactivity, et cetera.

Greater absorbance is for the higher the concentration. This data is used to make the standard curve, plotting concentration on the X-axis, and assay measurement on the Y axis. The same assay is then performed with samples of unknown concentration. To analyze the data, one locates the measurement on the Y-axis that corresponds to the assay measurement of the unknown component and follows a line to intersect the standard curve. The corresponding value on the X-axis is the concentration of substance in the unknown sample [69].

The standard curve method simplifies calculations and avoids practical and theoretical problems currently associated with PCR efficiency assessment. This technique is used in many laboratories because it is simple and reliable. Moreover, at the price of a standard curve on each PCR plate it also provides the routine validation for methodology.

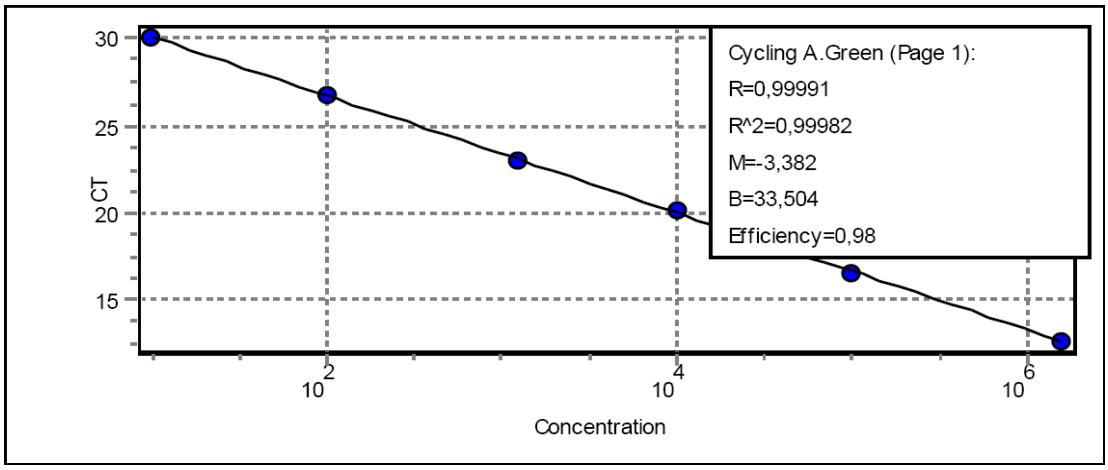



**Figure 7.** Standard curve of DNA amplifications.

The CT values are plotted versus the log of the initial amount of genomic DNA to give the standard curve shown in Figure 6.

### 1.6.2. Standard curve calculation

A standard curve is derived from the serial dilutions by a customary way. Relative concentrations are expressed in arbitrary units. Logarithms (base 10) of concentrations are plotted against crossing points.

### 1.6.3. R-value (square root of correlation coefficient)

The R-value of the calculation is the square root of the R^2 value. Unless you have a specific statistical application, the $R^2$ value is more useful in determining correlation [65].

### 1.6.4. R^2-value (correlation coefficient)

The R^2 value, or $R^2$ value (as displayed with the superscript), is the percentage of the data which is consistent with the statistical hypothesis. In the quantitation context, this is the percentage of datas which matches the hypothesis that the given standards form a standard curve. If the $R^2$ value is low, then the given standards cannot be easily fit onto a line of best fit. This means that the results obtained (ie. the calculated concentrations) may not be reliable. A good $R^2$-value is around 0.99 [70].

### 1.6.5. Slope, Amplification, Reaction Efficiency and B value

**Slope**: The slope (M) of a reaction (shown in the standard curve window), can be used to determine the exponential amplification and efficiency of a reaction.

The slope is calculated of being the change in $C_T$ divided by the change in log input (for example copy number). A 100% efficient amplification means a doubling of amplification product in each cycle resulting in an M value of -3.322, an amplification factor of 2 and a reaction efficiency of 1.

Given an M value of –3.322, the calculations are as follows:

**Amplification value**: $10^{(-1/-3.322)} = 2$

**Reaction efficiency**: $[10^{(-1/-3.322)}] - 1 = 1$

Here are two examples for two different slope values.

An M value 3.8 means that the reaction has an amplification value of ~1.83 and a reaction efficiency of 0.83 (or 83%).

There could be several reasons for this value. If the value needs to be improved, optimization steps like primer or probe concentrations, $MgCl_2$- or SYBR-Green I concentrations could be improved, or cycle times increased

An M value 3 means that the reaction has more than 100% efficient. A reason for this could be a disproportionate digestion of probe compared to the amplicon produced. In addition, if the R-value is low, then statistical error can cause unexpected reaction efficiency.

**B-value:** In a formula describing the relation between two variables, the intercept is expressed with the letter "B" (Y = MX + B). The intercept is also sometimes referred to as the Offset.

The B value represents the $C_T$ for a given concentration of 1 unit. By substituting 1 into the concentration formula as shown below:

$C_T = \log(1) * M + B$

$C_T = 0 * M + B$

We obtain $C_T = B$ as described above.

The intercept can change from run-to-run, and is less stable than the gradient. For this reason, most analysis techniques will analyze the gradient rather than the intercept [70].

### 1.6.6. Melting Curve Analysis

By using in real-time PCR, SYBR green binding to amplified DNA are simply measured the fluorescence increase as the dye binds to the increasing amount of DNA in the reaction tube. It is hoped that this increase in fluorescence is coming from the DNA. Is there any way to check that the correct fragments are amplified? One way to do some checking of the products is to do a melting curve. The fragments are controlled by melting curve analysis in order to understand if correct amplification or not.

The real-time machine not only monitors DNA synthesis during the PCR, it also determines the melting point of the product at the end of the amplification reactions. The melting temperature of a DNA double helix depends on its base composition (and its length if it is very short). All PCR products for a particular primer pair should have the same melting temperature - unless there is mispriming, primer-dimer, artifacts, contamination or some other problem. Since SYBR green does not distinguish between one DNA and another, an important means of quality control is to check that all samples have a similar melting temperature. After real time PCR amplification, the machine is programmed to do a melt curve, in which the temperature is raised by a fraction of a degree and the change in fluorescence is measured. At the melting point, the two strands of DNA will separate and the fluorescence rapidly decreases. The software plots the rate of change of the relative fluorescence units (RFU) with time (T) (-d (RFU)/dT) on the Y-axis versus the temperature on the X-axis, and this will be peak at the melting temperature (Tm). Figure-6 shows that are the melting curves for the sample; when long peaks represent long DNA fragments, short peaks show a primer-dimers artifact because they are such a short DNA and would give a peak with a lower melting temperature [71].

If the peaks are not similar, this might suggest contamination, mispriming, primer-dimer artifact etc. You need to be sure that the only thing you detect with SYBR green is the thing you want to detect; that is a specific DNA fragment corresponding to the size predicted from the position of the primers on the DNA. if you are looking at mRNA) or the genomic DNA, plasmid DNA, etc (according to what your target DNA is). You need to know DNA product size and Tm.

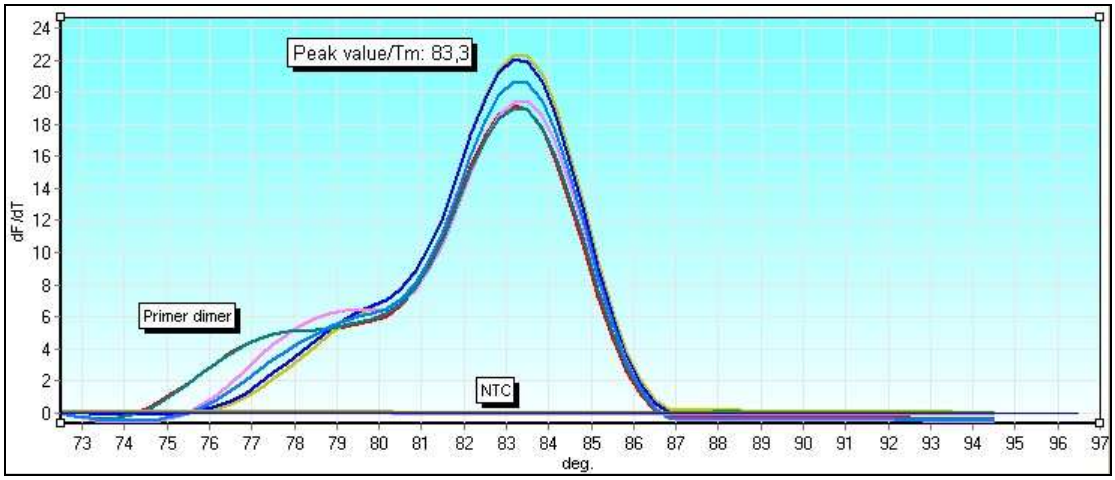



**Figure 8.** The graph of DNA melting curves.

In this melting curve graph, all samples are results of experiment made with the same primer pair.

## 1.7. MULTIPLEX REAL-TIME PCR (MRT-PCR)

Real-time PCR assay provided comparable sensitivity and superior reproducibility, precision and shorter performance time when compared to previous methods. This fluorescence-based real-time assay not only can quickly identify target genes independently but also allows for multiple PCR reactions in one tube by employing the unique melting curve analysis following DNA amplification [72].

Multiplex PCR allows amplification several target genes simultenously within a single reaction tube using several primers [MRT-PCR]. The templates are amplified in the PCR followed by melting curve analyisis: In figure 26 (a,b,c), the chart represents

the change in fluorescence as a function of time (dF/dT) versus the temperature of the reaction products.

Since SYBR Green fluorochrome effectively is bound all amplicons without establishing a direct differentiation between ruminant and poultry specific products, multiplex MRT-PCR fragments are detected by melting curve analysis. Hence, ruminant and poultry amplicons can be easily distinguished by specific Tm values due to the different length and compositions of two amplicons.

Some scientists shared the same opinion with SYBR Green fluorescence dye can not do multiplexing for example, Although multiplexing reactions cannot be performed with SYBR Green, specificity can be achieved by careful primer design and reaction optimization, which can be confirmed from dissociation (or melt) curve analysis [3, 73, 74].

Normally SYBR green is used in simplex reactions, however when coupled with melting curve analysis, it can be used for multiplex reactions [75].

# CHAPTER 2

# EXPERIMENTAL PART

## 2.1 MATERIALS

### 2.1.1 Meat Samples

During the research, 93 meat samples categorized were collected (Figure 9, Table 1).

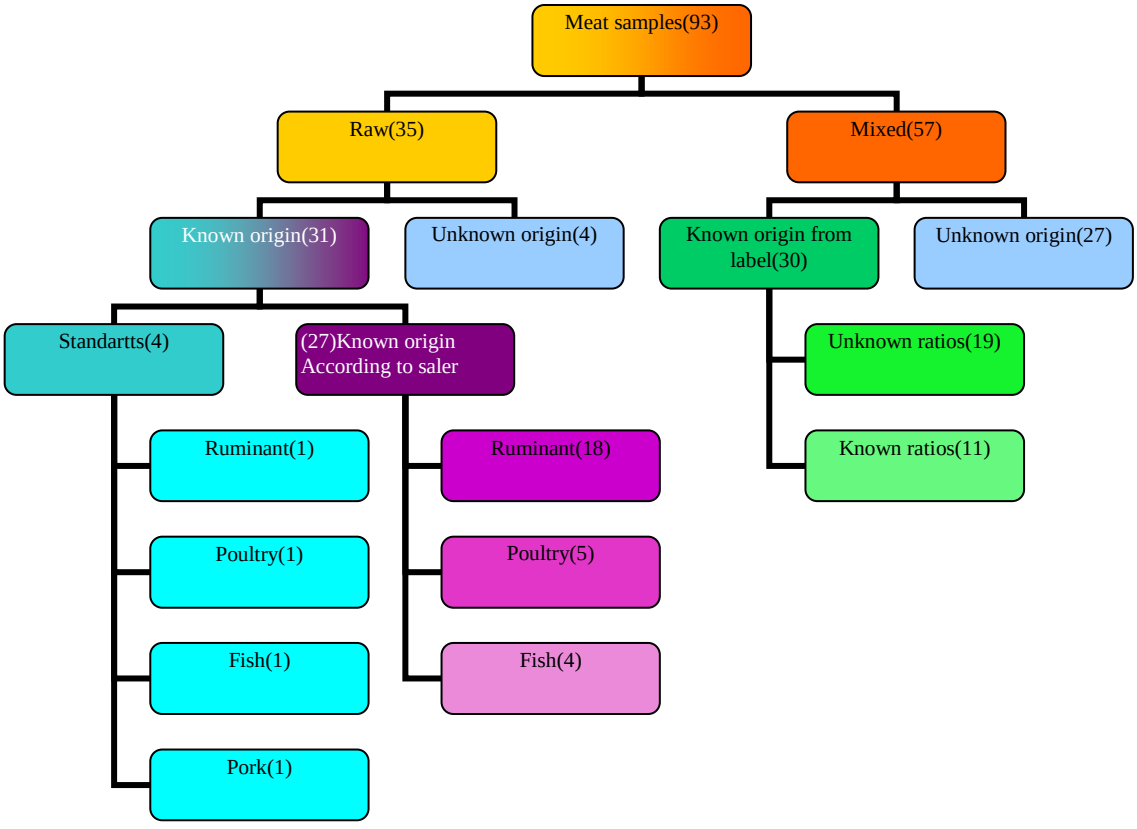


**Figure 9.** Meat samples collected from shops located in the different points of Istanbul

57 of 93 were processed and mixed meat products bought from supermarkets or ordinary local shops. 30 and 31 among them were labeled and unlabelled samples respectively. There was no any information about the ratio of the meat species used in the processed-mixed meat in the labels of 19 samples.

There were 35 raw samples (mince etc.) included in the research. 4 of 34 were standards which were cut directly from the body of the animals (ruminant, poultry, fish, and pork). Although the origin of one of 34 samples was unknown, those of the rest were known and they were obtained from ordinary local shops and supermarkets.

**Table 1.** Grouping of Samples according to the criteria given below

| Criteria | Groups | # | (%) |
|---|---|---|---|
| Reference species | RU | 1 | (1.1) |
| | PO | 1 | (1.1) |
| | FS | 1 | (1.1) |
| | PR | 1 | (1.1) |
| Species given on label | RU | 42 | (45.2) |
| | RU+PO | 13 | (14.0) |
| | PO | 10 | (10.8) |
| | FS | 5 | (5.4) |
| | PR | 1 | (1.1) |
| | UK | 22 | (23.7) |
| Processing Type | PM | 57 | (61.3) |
| | RMM | 21 | (22.6) |
| | RWM | 15 | (16.1) |
| Categorized according to the processing and origin | RS | 4 | (4.3) |
| | RR | 18 | (19.4) |
| | RF | 4 | (4.3) |
| | RP | 5 | (5.4) |
| | RU | 5 | (5.4) |
| | MLK | 11 | (11.8) |
| | MLU | 19 | (20.4) |
| | MU | 27 | (29.0) |

RU: Ruminant; PO: Poultry; FS: Fish; PR: Pork; UK: Unknown; PM: Processed Meat (salami, sausages, ham, frankfurter, meat ball); RMM: Raw mince meat; RWM: Raw Meat; RS: Reference species; RR: Raw Ruminant; RF: Raw Fish; RP: Raw poultry; RU: Raw but species unknown; MLK: Mix, labeled and concentration of species given; MLU: Mix, labeled and concentration of species not given; MU: Mixed but species unknown.

### 2.1.2 Equipments

The list of the machines and equipments used in the study was shown below.

| | |
|---|---|
| Thermocyclers | : TECHNE TC-512(UK), Corbet Rotor-Gene 6000 Rotary Analyzer (AUSTRALIA) |
| Software | : Rotor-Gene Software |
| Autoclave | : CERTO CLAW A-4050 Traun, Austria |
| Camera | : Sony Cybershot DSC T520 |
| Centrifuges | : Hettich, Mikro 22 |
| Deep freezers | : BEKO, Turkey |
| Electrophoretic Equipment | : Bio-Rad Sub Cell, GT |
| Magnetic Stirrers | : Chiltern Hotplate Magnetic Stirrer |
| Power supplies | : Bio-Rad Power PAC-300 |
| Refrigerator | : Philips, +4$^{o}$C, -20$^{o}$C |
| Transilluminator | : Bio-Rad GelDoc 2000 |
| Vortex | : IKA LABORTECHNIK |
| Water Purification System | : Millipore, Water Purification System, Ultra Pure Water |
| Balance | : Sartorius, Wender Landstrasse 94-108 D-37075 Goettingen, Germany |
| Pipets | : Nichipet EX |
| Tips | : Neptune BT brand barrier tips |
| Spectrophotometer | : UNICAM UV-VISIBLE, VISION SOFTWARE V3.41 |

### 2.1.3 Chemicals

Many of the chemicals were supplied from either MERCK [Germany] or SIGMA [USA]. Alcohols were purchased from RIEDEL DE-HAEN [Germany].

### 2.1.4 Buffers and Solutions

During DNA isolation and visualization of the PCR products, different buffers were used.

- **DNA Isolation:** Solutions and buffers for DNA isolation were obtained by the commercial kit called Qiagen DNeasy® Blood & Tissue Kit (QIAGEN, Germany)
- **PCR:** One buffer (Taq polymerase buffer (Takara,Biogen): 10x Taq Buffer + [NH4]2SO4 – MgCl2 (Fermentas, Germany) for standart PCR and real-time PCR and one master mix solution (SYBR® Green PCR Master Mix (Takara, Germany) just only for real-time PCR were used during the study.
- **Gel Electrophoresis:** Agarose gel electrophoresis was performed for visualization of PCR products. The buffers and solutions for the method were given in the list below.
    - 10 x TBE buffer: 54 g Trisbase, 27.50 g Boric acid, 4.650 g NaEDTA dissolved in 500ml distilled water.
    - 10 x loading buffer : 2.5mg/ml bromophenol blue, 1% SDS in 2 ml of glycerol
    - Ethedidum Bromide : 10 mg/ml (Merck, Germany)
    - 3% agarose: 3 gram agarose (Merck, Germany) was dissolved in 100 ml boiling 0.5xTEB buffer.

### 2.1.5 Oligonucleoside Primers

Oligonucleotide primers were used to amplify specific gene loci, which are called 16S rRNA, 12S rRNA-tRNA Val and 12S rRNA. The detailed information about primers' design was given in the section called 2.2.2. DNA Extraction and Primer Design.

**Table 2.** Design of oligonucleotides of the different animal species

| Primers | Species | Genes | Positions | Oligonucleotides primers | Amplicons (bp) |
|---|---|---|---|---|---|
| Ruminant | *Bos taurus* | 16S rRNA-tRNA | *Bos taurus* EU177870 | 5' GAA AGG ACA AGA GAA ATA AGG 3'<br>5' TAG CGG GTC GTA GTG GTT CT 3' | 374 |
| Pork | *Sus scrofa* | 12S rRNA-tRNA Val | *Sus scrofa* bNC 000845 | 5' CTA CAT AAG AAT ATC CAC CAC A 3'<br>5' ACA TTG TGG GAT CTT CTA GGT 3' | 290 |
| Fish | *Sardinops melanostictus* | 12S rRNA | *Sardinops mel.* bNC 002616 | 5' TAA GAG GGC CGG TAA AAC TC 3'<br>5' GTG GGG TAT CTA ATC CCA G 3' | 224 |
| Poultry | *Meleagris meleagridis* | 12S rRNA | *Gallus gallus* bNC 001323 | 5' GGG CTA TTG AGC TCA CTG TT 3'<br>5' TGA GAA CTA CGA GCA CAA AC 3' | 183 |

## 2.2 METHODS

### 2.2.1. Preparation and Collection of Meat Samples

Samples were obtained commercially from the different points of İstanbul. During the collection of meat samples, clean, non-used plastic boxes were used to prevent the cross-contamination. As soon as samples were gotten, they were directly transported to Molecular Biology and Genetic Research Laboratory of Fatih University at the Buyukcekmece Campus, Istanbul. Then, each one was weighed about 20 gr and homogenized by sterile the mixers in the falcon tubes manually. Exactly 25 mg of samples was placed in a 1.5 ml microcentrifuge tube and numbered E-1 to E-100. Finally, they were stored under -20$^{o}$C until the DNA isolation procedure.

On the other hand, information about the samples were loaded to an Excel File (Microsoft Excel, USA) and performed a raw table. The table contained the place where they were obtained, production date, expired date, their mark, origin and storage and packaging conditions, etc.

### 2.2.2 DNA Extraction, Quality and Quantity of DNA, Primer Design

#### i. DNA extraction:

DNA was extracted from 25 mg of meat samples using the DNeasy® Protocol provided with the DNeasy® Tissue Kit (Qiagen, Hilden, Germany).

- One hundred and eighty microlitres ATL buffer and 20 µl Proteinase K were added and vortexed.
- The mixture was incubated at 56 ºC in a water bath to disperse the sample until the tissue was completely lysed. The mixture was vortexed for 15 s.
- 200µl AL buffer was added to the sample, vortexed thoroughly.
- 200µl ethanol (96–100%) was added to mixture and vortexes to yield a homogenous solution.
- The homogenous solution was pipetted into the DNeasy® mini column in a 2 ml collection tube.

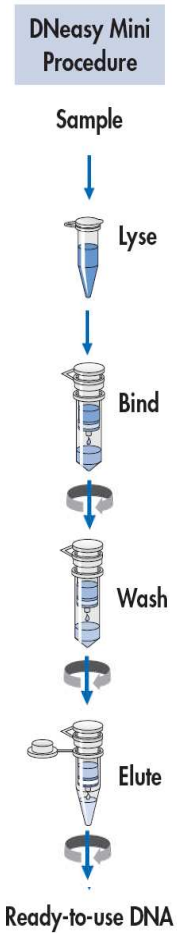



**Figure 10.** A simple domenstration of Dneasy DNA isolation procedure

- The homogenous solution was centrifuged at 8000 RPM for 1 min.
- The flow-through and collection tube was discarded and the DNeasy® mini column was put in a new 2 ml collection tube.
- 500 µl AW1 buffer was added and spun at 8000 RPM for 1 min.
- The flow-through and collection tube was discarded and the DNeasy® mini column was placed in another 2 ml collection tube.
- 500 µl AW2 buffer was added and centrifuged at 14,000 RPM for 3 min to dry the DNeasy membrane and then the flow-through and collection tube was removed.
- The DNeasy® mini column was placed in a clean 1.5 ml microcentrifuge tube. 200 µl AE buffer was pipetted directly onto the DNeasy® membrane and incubated at room temperature at 1 min.
- This was then spun at 8000 RPM for 1 min to elute. Elution was repeated to increase final DNA concentration. Last solution volume is 400 microliter in a microsentrifuge tube.
- After the amount of DNA was measured by spectrophotometer the DNA solution was stored at -20 ºC until used in processes of PCR.

### ii. Quantification of Nucleic Acids

DNA concentration was calculated by this formula: DNA concentration = OD260 X extinction coefficient (50µg/ml) X dilution factor.

A spectrophotometer device was used to determine the concentration of DNA in a solution. Samples were exposed to ultraviolet light at 260 nm and 280 nm. With the 260:280 ratio was determined qualification of nucleic acids.

### iii. Protein Contamination and the 260:280 ratio

The ratio of absorptions at 260nm vs 280nm is commonly used to assess the purity of DNA with respect to protein contamination, since tends to absorb at 280nm.(Table 2)

**Table 3.** Sensitivity for protein contamination in nucleic acids[3]

| % nucleic acid | % protein | 260:280 ratio |
|---|---|---|
| 100 | 0 | 2.00 |
| 95 | 5 | 1.99 |
| 90 | 10 | 1.98 |
| 70 | 30 | 1.94 |

The ratio of 260/280 ranged from 1.6 to 2.0, which was sufficiently pure for PCR reactions [76]. The method dates back to 1942, when Warburg and Christian showed that the ratio is a good indicator of nucleic acid contamination [77].

**Table 4.** The results of quantification and qualification for nucleic acids

| Name | | O.D.260 nm | O.D.280 nm | 260/280 | Concentration |
|---|---|---|---|---|---|
| Control | Ruminant | 1,200 | 0,589 | ± 2 | 60 ng/µl |
| | Pork | 1,514 | 0,714 | ± 2 | 75,7 ng/ µl |
| | Poultry | 1,520 | 0,728 | ± 2 | 76 ng/ µl |
| | Fish | 0,895 | 0,447 | ± 2 | 44,75 ng/ µl |
| Samples | E-42 | 1,400 | 0,677 | ± 2 | 70ng/ µl |
| | E-48 | 1,608 | 0,801 | ± 2 | 80 ng/ µl |
| | E-17 | 0,908 | 0,466 | ± 1,9 | 45,4 ng/ µl |
| | E-44 | 1,286 | 0,648 | ± 1,9 | 64,3 ng/ µl |
| | E-50 | 0,966 | 0,535 | ± 1,8 | 48,3 ng/ µl |
| | E-90 | 1,577 | 0,723 | ± 2 | 78,85 ng/ µl |
| | E-91 | 1,112 | 0,540 | ± 2 | 55,6 ng/ µl |
| | E-92 | 1,160 | 0,567 | ± 2 | 58 ng/ µl |
| | E-93 | 1,071 | 0,548 | ± 1,9 | 53,55 ng/ µl |

[3]http://en.wikipedia.org/wiki/ Quantification of nucleic acid

### iv. Primer Design and Production

We only designed ruminant primers (16S rRNA) using Primer3 (v. 0.4.0) primer design software which is free on internet and sensitivity and specificity of all primers (Table 1) were checked by using the BLAST of NCBI (National Center for Biotechnology Information). All the primers were synthesized by the company, Iontek, Istanbul, Turkey.

### 2.2.3. Polymerase Chain Reaction (PCR) Conditions

#### 2.2.3.1. Conventional Simplex PCR

PCR amplification was performed in a final volume of 25 µl containing 10x Taq Buffer + [NH4]2SO4, 1 unit of Platinum Taq DNA Polymerase, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 2 mM MgCl2, 0.1 mM of each primers and 60-80ng/µl of DNA template. Amplification was performed in a Thermocycler Techne with the following cycling conditions; after an initial heat denaturation step at 94 °C for 10 min, 35 cycles were programmed as follows: 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 5 min.

**Table 5.** Simplex PCR composition

| | **PCR compossition** | **Volume** | **Concantriation** |
|---|---|---|---|
| **Master Mix** | ddH2O | 15.875µl | _ |
| | Buffer | 2.5 µl | 1x |
| | dNTP | 2 µl | 0.2mM |
| | MgCl2 | 2 µl | 2mM |
| | Forward Primer | 0.5µl | 0.1mM |
| | Rreverse Primer | 0.5µl | 0.1mM |
| | Taq polymerase | 0.125µl | 1U/reaction |
| | Template DNA | 0.5µl | 60-80ng/µl |
| **Total** | | 25 µl | |

### 2.2.3.2. Conventional Multiplex PCR

For the simultaneous detection of each species, a one-step multiplex PCR was developed using each of the primer sets previously designed for the simplex PCR.

**Table 6.** Multiplex PCR composition

| | **PCR composition** | **Volume** | **Final Concant.** |
|---|---|---|---|
| **Master Mix** | ddH2O | 13.55µl | _ |
| | Buffer | 2.5 µl | 1x |
| | dNTP | 2 µl | 0,2mM |
| | MgCl2 | 2 µl | 2mM |
| | Forward Primer for Ruminant | 0.4µl | ±0.1mM |
| | Reverse Primer for Ruminant | 0.4µl | |
| | Forward Primer for Pork | 0.4µl | |
| | Reverse Primer for Pork | 0.4µl | |
| | Forward Primer for Poultry | 0.4µl | |
| | Reverse Primer for Poultry | 0.4µl | |
| | Forward Primer for Fish | 0.4µl | |
| | Reverse Primer for Fish | 0.4µl | |
| | Taq polymerase | 0.15µl | 1U/reaction |
| **Template Types** | Template DNA for Ruminant | 0.4µl | 60-80ng/µl |
| | Template DNA for Pork | 0.4µl | |
| | Template DNA for Poultry | 0.4µl | |
| | Template DNA for Fish | 0.4µl | |
| | **Total** | 25 µl | |

As for the simplex PCR, amplification was performed in a final volume of 25 µl containing 10x Taq Buffer + [NH4]2SO4, 1.5 unit of Platinum Taq DNA Polymerase (Iontek,Turkey), 0.2 mM each of dATP, dCTP, dGTP, dTTP (Iontek,Turkey), 2 mM $MgCl_2$, 20, 20, 12.5 and 10 pmol of ruminant, pork, fish and poultry primers, respectively, and 60-80ng/µl of DNA template. Thermal cycling was programmed following the same procedure used in simplex PCR.

Amplimers were resolved by electrophoresis on 3% agarose gel (MERCK) run in Tris Boric EDTA Buffer for 50 min at 110 V.

### 2.2.3.3 Agarose Gel Electrophoresis

A 3% agarose gel was performed for the detection of the PCR products.

Preparation of the gel:

1. 2.4 g of agarose (Sigma, St. Louis, USA) was added to a 80 ml of 0.5 M Tris-Borate EDTA (TBE) buffer.
2. The mixture was boiled.
3. The gel was cooled to 60 $^{o}$C and a 4 µl of Ethidium bromide was added. It was poured to its plate and then a comb was placed into the gel.

Loading the samples:

1. 2 µl loading dye (Fermentas, Germany) was mixed with PCR products as a tracking dye.
2. 10 µl of PCR products were put in each slot.
3. A 100 bp DNA Ladder (MBI Fermentas, Hanover, MD, USA) as a molecular marker was dropped into the side slot.
4. The gel was run at 110V in 0.5 M TBE buffer for 50 minutes.
5. The gel was illuminated by the transilluminator of the Gel Doc 2000 (Biorad, Milan, Italy).

0.5X TBE buffer was diluted from 10X TBE. To prepare 10X TBE buffer, 54 g Tris base, 27.50 g Boric acid, 4.650 g NaEDTA was dissolved in 500ml distilled water.

### 2.2.4. Real-time PCR

Real-time PCR amplification was performed in a final volume of 20 µl containing SYBR Green master premix (Takara, Japan), 0.2 Mm each of Datp, Dctp, Dgtp, Dttp (Takara, Japan) 0.1 Mm each of primers and 60-80ng/µl of DNA template (Table 6). Amplification was performed in a Corbet Rotor-Gene 6000 rotary analyzer (Corbett,

Australia) with the following cycling conditions; after an initial heat denaturation step at 94 °C for 10 min, 40 cycles were programmed as follows: 94 °C for 10 s, 60 °C for 20 s, 72 °C for 20 s and after that melting curve analysis was programmed its ramp was formed from 72 C° to 95 C° raising by 1 C° each step. Program waits for 90 s. of pre-melt conditioning on first step and for 5 s. for each step afterwards.

**Table 7.** Real-time PCR composition

| | **PCR composition** | **Volume** | **Concentration** |
|---|---|---|---|
| **Master Mix** | $ddH_2O$ | 7.2µl or 8.2µl | _ |
| | Forward Primer | 0.4µl | 0.1mM |
| | Reverse Primer | 0.4µl | 0.1mM |
| | SYBR premix ex tag | 10µl | 1x |
| | Template DNA | 2µl or 1µl | 60-80ng/µl |
| | **Total** | 25 µl | |

### 2.2.4.1. Identification of Species by Melting Curve Analysis

Melting Curve Analysis Tools of Rotor Gene Software Program (Corbett, Australia) at the end of each reaction was used to identify specific-species melting temperature (tm) value of the amplified region of the template DNA.

### 2.2.4.2. Preparation of Diluents of Standard DNA Samples

Diluents of four species (ruminant, pork, poultry and fish) standard samples were prepared according to the following ratio: 1, 1/10, 1/100, 1/1.000, 1/10.000, 1/100.000, 1/1000.000,1/10.000.000. For dilution, sterile water and DNA solution of four species was used as a solvent.

### 2.2.5. Quantitative Real-time PCR (QRT-PCR) Assay

After PCR, quantification of the DNA concentration in samples was performed by the Quantitative Analyzing Tools of Rotor Gene Software Program (Corbett, Australia) to quantify the ratio of meats of different species in processed meat. To do

that, the software itself determined cycle threshold (Ct) values of each DNA samples, compared them with the known concentration of diluents of standard DNA samples and made a concentration versus Ct values plot.

The PCR products were loaded to 3% agarose gel electrophoresis to check the size of PCR products.

#### 2.2.5.1. QRT-PCR's Sensitivity and Specificity

Progressive dilution of a mixed DNA template was diluted in DNA of other species respectively 1, 1/10, 1/100, 1/1000, 1/10.000, 1/100.000, 1/1.000.000,1/10.000.000. The results obtained from these dilutions at the end of PCR were shown both in raw data and melting curve analysis of software which is called Rotor-Gene. The detection limits were determined for ruminant, pork, poultry and fish. Real-time dilution products also were run on a 3% agarose gel, and stained with ethidium bromide. The results in agarose gel were compared with the result of QRT-PCR.

#### 2.2.5.2. Standard Curve

A lot of experiments were made to optimize standard curve data by using dilution values (from1 to 1/10.000.000) of DNA which are belong to ruminant, pork, poultry and fish in order to reach maximum reaction application efficiency and optimum $R^2$ value.

### 2.2.6. Multiplex Real-time PCR (MRT-PCR)

Multiplex real-time PCR was applied to identify ruminant, poultry, fish and pork materials in the same reaction. For this purpose different PCR conditions were used such as thermal gradients and amplification curves were compared with DNA bands in the results conventional multiplex PCR.

# CHAPTER 3

# RESULTS

## 3.1. Optimization of Simplex PCR

In a preliminary phase of the investigation, simplex PCRs were optimized and carried out on DNA samples extracted from raw meat to verify the sensitivity and specificity of the primers. The primers generated specific fragments of 374bp, 183bp, 224bp, 290bp for ruminants, poultry, fishes and pork respectively (Figure 11, 12). To detect possible cross-reactions, each set of primers was performed in simplex PCR with non-target species. In no case, a cross-reaction was observed.

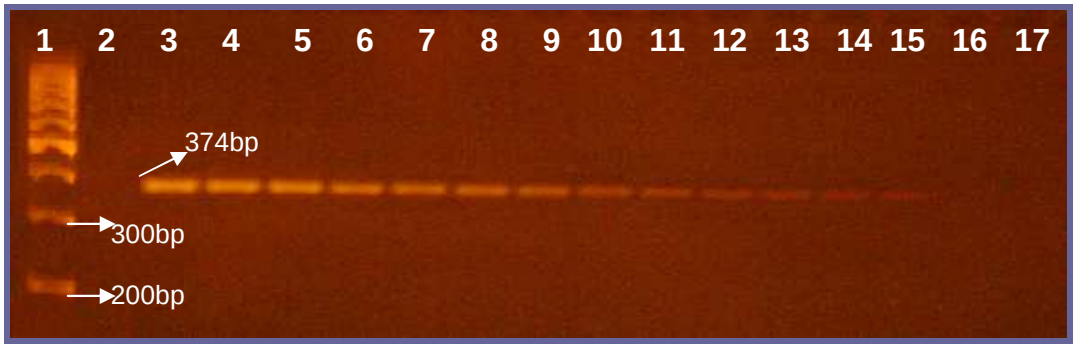



**Figure 11.** Evaluation of assay sensitivity progressive dilution of ruminant DNA template diluted in DNAs of pork, poultry and fish.

In figure 11; lane 1, M, 100-bp ladder; lane 2, control reagent; lane 3, 100%; lane 4, 100%; lane 5, 100%; lane 6, 20%; lane 7, 10%; lane 8, 2%; lane 9, 1%; lane 10, 0.2%; lane 11, 0.1%; lane 12, 0.02%; lane 13, 0.01%; lane 14, 0.002%; lane 15, 0.001%; lane 16, 0.0002%; lane 17, 0.0001%.

### 3.2. Conventional Multiplex PCR Specificity and Optimization

When multiplex PCR was carried out on analogous samples, the set of primers retained the same specificity (Figure 11). The electrophoretic pattern clearly shows the absence of cross-reaction. In fact, only the species specific band is evident.

We optimized multiplex PCR at the same conditions of simplex PCR in order to check mixed meat products and showed the applicability of multiplex PCR (Fig. 3) on the commercial meat.

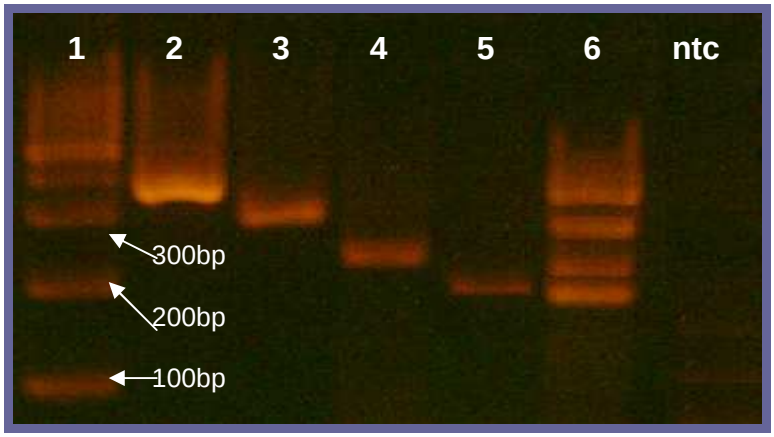



**Figure 12.** Specificity of multiplex PCR of DNA from raw meat

In figure 12, it is represented lane 1; M, 100-bp ladder. Lane 2, ruminant (374bp); lane 3, pork (290bp); lane 4, poultry (183); lane 5, fish (224bp); lane 6, mixture of all animal (ruminant, pork, poultry, and fish) DNA; lane ntc, no template control reagent.

The size of PCR products was as expected with no additional fragment from a target species. This result showed that the species-specific primers amplified only one size fragment from a target species. Primer specifity to the other species was examined by multiplex PCR using the same primer mixture in the method. Figure 11 showed the result of an optimized multiplex PCR which resulted in a single band of target size from one meat species and no fragment produced by non-specific amplification

### 3.2.1. The results of Raw and Mixed Meat Samples by CM-PCR

The applicability of the assays to commercial products, raw meat and mixed meat has been demonstrated. The optimized multiplex PCR method was performed at once to all samples collected from the different points of Istanbul. The some of the actual experimental results were shown in four different figures (Figures 13, 14, 15, and 16) and four different tables (Tables 8, 9, 10 and 11) that compared the true species composition of the samples and the results. With regard to commercial meat products, the species claimed in label have been researched by the modern DNA analysis methods.

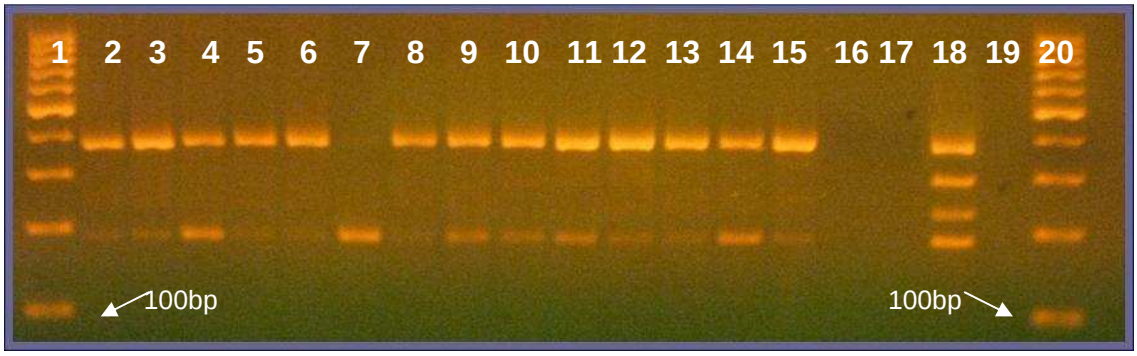



**Figure 13.** Conventional multiplex PCR assay 1 for controlling of samples

**Table 8.** Conventional multiplex PCR assay 1 results

| Num.&Name | | Given Composition | Reveal Composition | Num.&Name | | Given Composition | Reveal Composition |
|---|---|---|---|---|---|---|---|
| 1 | M | 100-bp Ladder | | 11 | RU3 | Ruminant | Rum./Poultry |
| 2 | RR2 | Ruminant | Rum./Poultry | 12 | RU4 | Ruminant | Rum./Poultry |
| 3 | RR4 | Ruminant | Rum./Poultry | 13 | RU5 | Ruminant | Rum./Poultry |
| 4 | E-17 | Rum./Poultry | Rum./Poultry | 14 | ML5 | Rum./Poult. | Rum./Poultry |
| 5 | ML4 | Ruminant | Rum./Poultry | 15 | E-42 | Ruminant | Rum./Poultry |
| 6 | RR5 | Ruminant | Rum./Poultry | 16 | NTC | Negative Template Control | |
| 7 | RP1 | Poultry | Poultry | 17 | NTC | Negative Template Control | |
| 8 | RR8 | Ruminant | Rum./Poultry | 18 | PCT | Positive Control Template | |
| 9 | RR10 | Ruminant | Rum./Poultry | 19 | NTC | Negative Template Control | |
| 10 | RR10 | Ruminant | Rum./Poultry | 20 | M | 100-bp Ladder | |

In table 8, multiplex PCR assay 1 results show that many ruminant origin products were unintentionally contaminated or intentionally mixed by poultry meat (lane 2, 3, 5, 6, 8, 9, 10, 11, 12, 13, and 15).

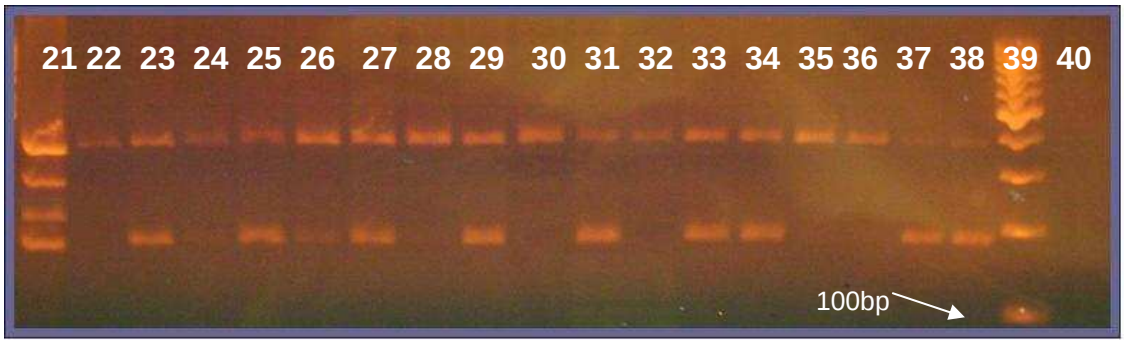



**Figure 14.** Conventional multiplex PCR assay 2 for controlling of samples

**Table 9.** Conventional multiplex PCR assay 2 results

| Name | | Given Composition | Reveal Composition | Name | | Given Composition | Reveal Composition |
|---|---|---|---|---|---|---|---|
| 21 | PCT | | | 31 | ML8 | Rum./Poultry | Rum./Poultry |
| 22 | ML4 | Ruminant | Rum./Poultry | 32 | RR18 | Ruminant | Ruminant |
| 23 | ML1 | Rum./Poultr | Rum./Poultry | 33 | E-44 | Rum./Poultry | Rum./Poultry |
| 24 | MU2 | Ruminant | Rum./Poultry | 34 | E-44 | Rum./Poultry | Rum./Poultry |
| 25 | MU3 | Unknown | Rum./Poultry | 35 | RR13 | Ruminant | Ruminant |
| 26 | MU4 | Ruminant | Rum./Poultry | 36 | ML10 | Ruminant | Ruminant |
| 27 | MU5 | Ruminant | Rum./Poultry | 37 | E-91 | Rum./Poultry | Rum./Poultry |
| 28 | RR11 | Ruminant | Ruminant | 38 | E-92 | Rum./Poultry | Rum./Poultry |
| 29 | MU6 | Unknown | Rum./Poultry | 39 | M | 100-bp DNA Ladder | |
| 30 | ML6 | Ruminant | Ruminant | 40 | NTC | No Template Control | |

In Figure 14, multiplex PCR assay 2 results shows that many ruminant origin products were unintentionally contaminated or intentionally mixed by poultry meat (lane 22, 24, 26 and 27). Some of the samples were dually tested such as the one, E-44 lane which lane 33 and 34 belong to.

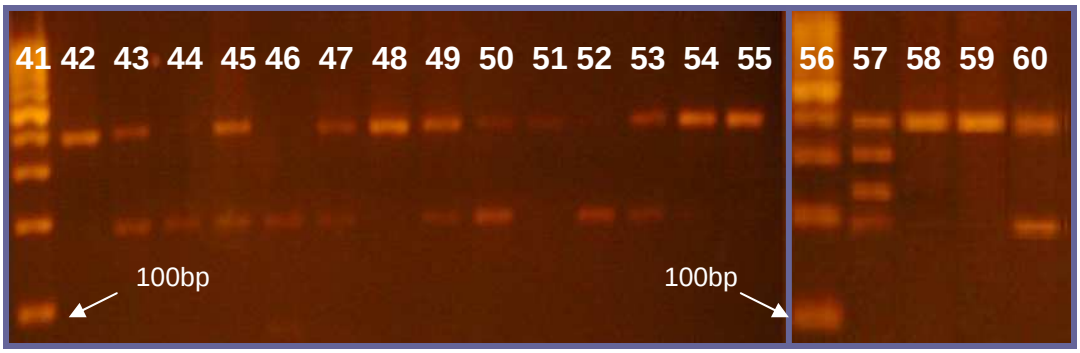



**Figure 15.** Conventional multiplex PCR assay 3

**Table 10.** Conventional multiplex PCR assay 3 results

| Name | | Given Composition | Reveal Composition | Name | | Given Composition | Reveal Composition |
|---|---|---|---|---|---|---|---|
| 41 | M | | | 51 | E-50 | Rum./Poultry | Ruminant |
| 42 | ML7 | Ruminant | Ruminant | 52 | E-52 | Rum./Poultry | Rum./Poultry |
| 43 | ML9 | Ruminant | Rum./Poultry | 53 | MU9 | Rum./Poultry | Rum./Poultry |
| 44 | ML12 | Poultry | Rum./Poultry | 54 | MU10 | Unknown | Rum./Poultry |
| 45 | ML13 | Ruminant | Rum./Poultry | 55 | MU10 | Unknown | Rum./Poultry |
| 46 | ML18 | Ruminant | Poultry | 56 | M | | |
| 47 | ML19 | Ruminant | Poultry | 57 | PCT | | |
| 48 | MU1 | Unknown | Rum./Poultry | 58 | E-59 | Ruminant | Ruminant |
| 49 | MU8 | Unknown | Rum./Poultry | 59 | MU12 | Unknown | Ruminant |
| 50 | E-48 | Rum./Poultry | Rum./Poultry | 60 | MU13 | Ruminant | Rum./Poultry |

In figure 15, multiplex PCR assay 3 results shows that many ruminant origin products were unintentionally contaminated or intentionally mixed by poultry meat (lane 43, 44, 45, 46, 47, 51 and 58).

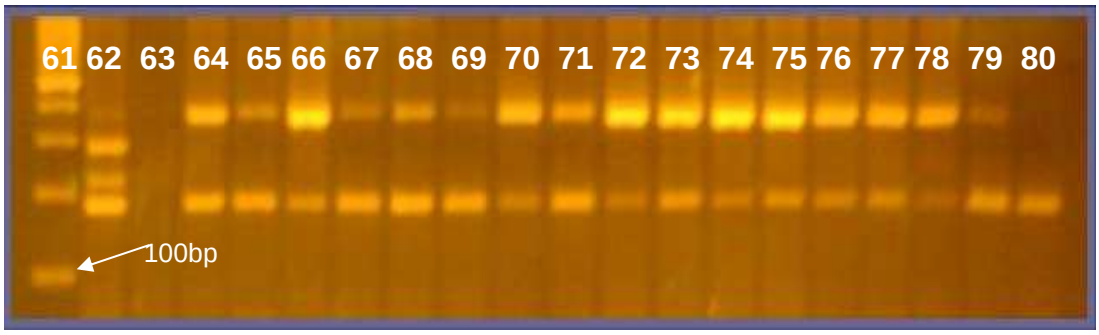



**Figure 16.** Conventional multiplex PCR assay 4

**Table 11.** Conventional multiplex PCR assay 4 results

| Name | | Given Composition | Reveal Composition | Name | | Given Composition | Reveal Composition |
|---|---|---|---|---|---|---|---|
| 61 | M | | | 71 | MU19 | Unknown* | Rum./Poultry |
| 62 | PCT | Positive Contr ol Template | | 72 | MU21 | Unknown* | Rum./Poultry |
| 63 | NTC | Negative Te | | 73 | MU22 | Unknown* | Rum./Poultry |
| 64 | ML14 | Ruminant | Rum./Poultry | 74 | MU23 | Unknown* | Rum./Poultry |
| 65 | ML16 | Poultry | Rum./Poultry | 75 | MU24 | Unknown* | Rum./Poultry |
| 66 | MU15 | Unknown* | Rum./Poultry | 76 | MU25 | Unknown* | Rum./Poultry |
| 67 | MU16 | Unknown* | Rum./Poultry | 77 | MU26 | Unknown* | Rum./Poultry |
| 68 | MU17 | Unknown* | Rum./Poultry | 78 | MU27 | Unknown* | Rum./Poultry |
| 69 | ML17 | Poultry | Rum./Poultry | 79 | E-90 | Rum./Poultry | Rum./Poultry |
| 70 | MU18 | Unknown* | Rum./Poultry | 80 | E-42 | Rum./Poultry | Poultry |

*mixed meat

In table 11, multiplex PCR results shows that many ruminant origin products were unintentionally contaminated or intentionally mixed by poultry meat (lane 64, 65, 69 and 80).

**Table.12** Overall multiplex results

| Given Species | (b) # | *(%)* | Reveal Results | # | *(%)* |
|---|---|---|---|---|---|
| RU | 42 | *(45.2)* | RU | 21 | *(50)* |
| | | | RU+PO | 19 | *(47.5)* |
| | | | PO | 2 | *(2.5)* |
| RU+PO | 13 | *(14.0)* | RU+PO | 12 | *(92.3)* |
| | | | PO | 1 | *(7.7)* |
| PO | 10 | *(10.8)* | PO | 7 | *(70.0)* |
| | | | RU+PO | 3 | *(30.0)* |
| FS | 5 | *(5.4)* | FS | 5 | *(100.0)* |
| PR | 1 | *(1.1)* | PR | 1 | *(100.0)* |
| UK | 22 | *(23.7)* | PO | 1 | *(4.5)* |
| | | | RU | 1 | *(4.5)* |
| | | | RU+PO | 20 | *(91.0)* |

Overall multiplex results showed that 25 (35.1%) among 71 (RU+ (RU+PO) +PO+FS+PR) samples gave unexpected results (Table 12) not indicated in their labels. It was seen that 19 of 42 RU samples were contaminated with PO whereas 3 of 10 PO were contaminated with RU. On the other hand, 2 of 42 RU were found as only PO though it was stated as only RU on their labels.

We had 30 commercial labeled meat mixture products (salami, frankfurter, sausages, etc) manufactured by high quality outstanding companies. It was revealed that one company had used only poultry meat whereas they stated they used mixture meat of poultry and ruminant. Overall, it was observed that only 18 of 30 products obeyed their labels. That is, 40% of commercially labeled products were carrying different meat species not indicated in their labels (table 8)

**Table 13.** Results of commercial labeled mixed meat products

| Given Species | (c) # | *(%)* | Reveal Results | # | *(%)* |
|---|---|---|---|---|---|
| RU | 14 | *(46.6)* | RU | 6 | *(42.9)* |
| | | | RU+PO | 6 | *(42.9)* |
| | | | PO | 2 | *(14.3)* |
| RU+PO | 12 | *(40.0)* | RU+PO | 11 | *(91.7)* |
| | | | PO | 1 | *(8.3)* |
| PO | 4 | *(13.3)* | PO | 1 | *(25.0)* |
| | | | RU+PO | 3 | *(75.0)* |

In addition to ruminant meat, the presence of poultry has been evidenced in many meat products although no poultry meat usage has been given in their official labels. In our experiment, no fish and pork meat were detected.

### 3.3. Identification of Species by Real-time PCR Melting Curve Analysis

Real time PCR technique was optimized. All of the reference strains (ruminant, pork, fish, and poultry) were amplified. Real-time results of PCR amplification products were shown using Rotor-Gene 6000 system employing SYBR Green I. (Fig.1)

All of the reference strains were amplified several times and melting curve chart belonging to ruminant, pork, fish and poultry (Figure 2 a) was obtained by using real-time PCR melting curve analysis program in the Rotor-Gene Software. Temperature values belonging to four species were identified according to melting curve peaks. It was seen that each species has a different characteristic Tm value. Test results were considered positive when their melting Tm was within the average Tm ± 0.3 SD for each class of species. By looking at the characteristic Tm value which is specific to the species, it can be easily identified that the sign as a result on the machine is a false positive primer-dimer or a true positive PCR product. Alternatively, one can easily say what the origin of the amplicon belongs to.

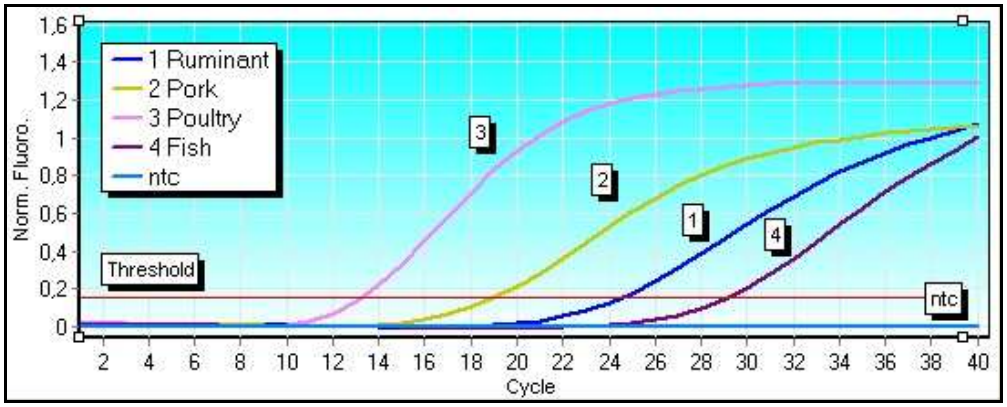
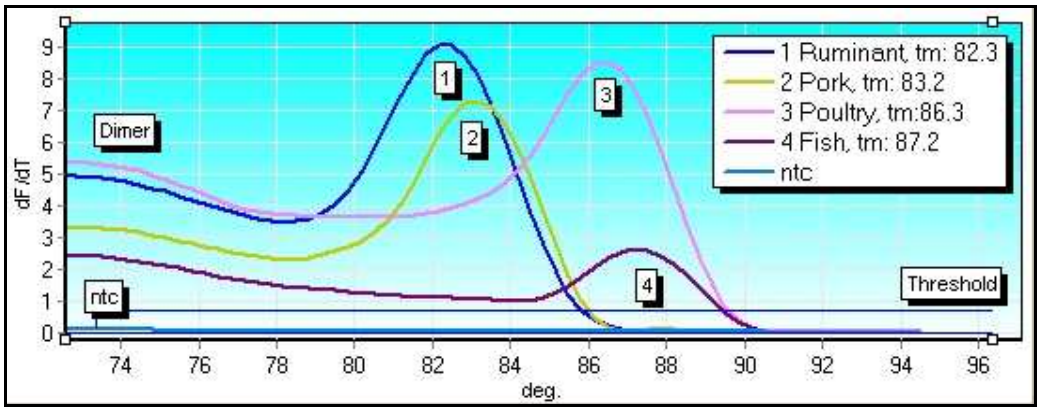



**Figure 17**. Quantification raw data chart of species

The figure 16 shows raw data analysis of four different species. Curve 1, ruminant; curve 2, pork; curve 3, poultry; curve 4, fish; ntc, no template control.

a




b

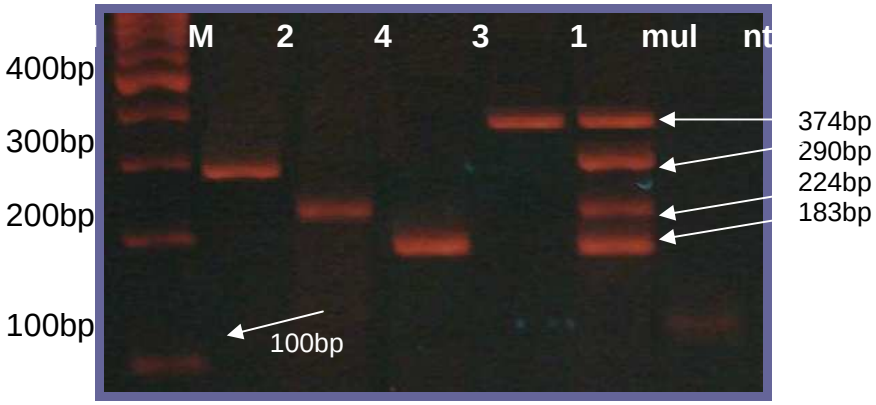



**Figure 18**. Melting curves of species (a) and gel analysis (b)

Figure 18 is for gene classes of curve 1, ruminant; curve 2 pork; curve 3, poultry; curve 4, fish in SYBR Green I real-time PCR with Rotor-Gene 6000. The templates were amplified in the PCR followed by melting curve analysis: (a) the chart represents the change in fluorescence as a function of time (dF/dT) versus the temperature of the reaction products. (b) PCR products from control strains were run on a 3% agarose gel, and stained with ethidium bromide. Lane M, 100bp DNA ladder; lane 1, ruminant (374bp, tm: 86.3); lane 2, pork (290bp, tm: 82.3); lane 3, poultry (183bp, tm: 86.3); lane 4, fish (224bp, tm: 87.2); lane mul, multiplex control, it was produced by conventional multiplex PCR; lane ntc, negative template control.

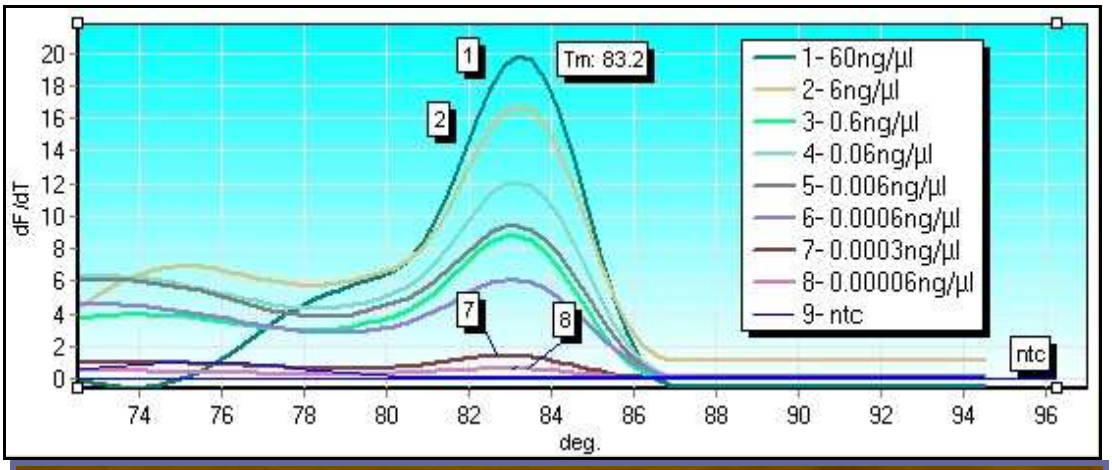
### 3.4. Identification of Real-time PCR Assay Sensitivity

Serial diluted reference DNA samples were amplified and their melting curves were analyzed to find out the least detectable diluents. It showed that almost 1 million fold diluted samples even could be detected by the method. By the way, SYBR green Real-time PCR could detect the amplicon in pork's 2 million times diluted tube. The amplicon then were separated by electrophoresis and visualized (Figure 19 b). It was revealed that SYBR-green Real-time PCR system is at least twice more sensitive than conventional visualization system, gel electrophoresis.

#### 3.4.1. Ruminant PCR Assay Sensitivity

The templates were amplified in the PCR followed by melting curve analyses: (a) the chart represents the change in fluorescence as a function of time (dF/dT) versus the temperature of the reaction products. The detection limit was 0.0006% for ruminant which has tm: 83.2

a




b

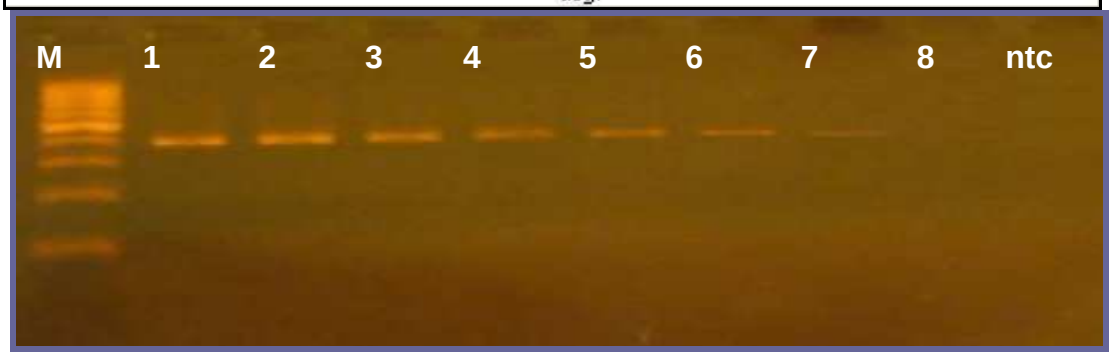


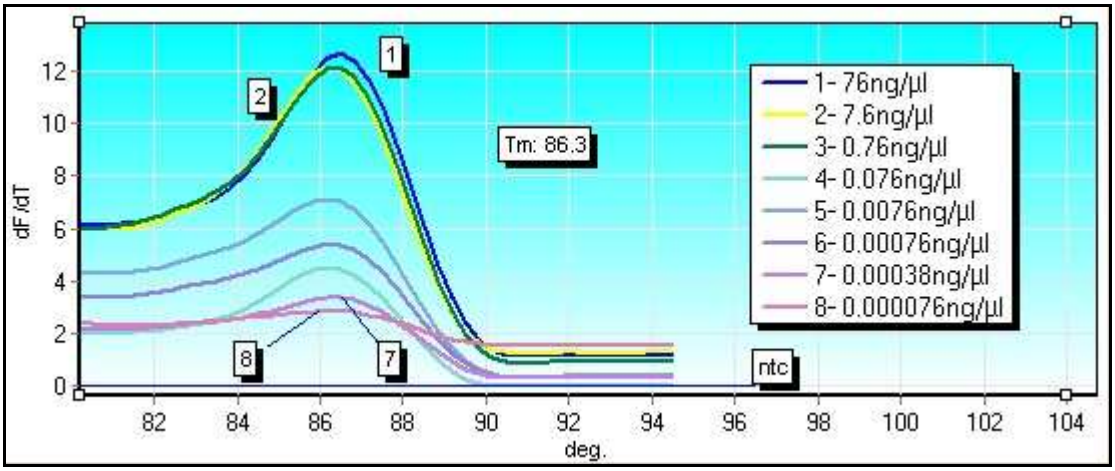

**Figure 19**. Melting curves of ruminant DNA diluted (a) and gel analysis (b)

Figure 19 is for ruminant sensitivity in SYBR Green I real-time PCR with Rotor-Gene 6000. (b) PCR products from control ruminant were run on a 3% agarose gel, and stained with ethidium bromide. Lane M, 100bp DNA ladder; lane 1, 100%; lane 2, 10%; lane 3, 1%; lane 4, 0.1%; lane 5, 0.01%; lane 6, 0.001%; lane 7, 0.0001%; lane 0.00001%;lane ntc, negative template control.

### 3.4.2. Poultry PCR Assay Sensitivity

The templates were amplified in the PCR followed by melting curve analyisis: (a) the chart represents the change in fluorescence as a function of time (dF/dT) versus the temperature of the reaction products. The detection limit was 0.00076% for poultry which has tm: 86.5

a




b

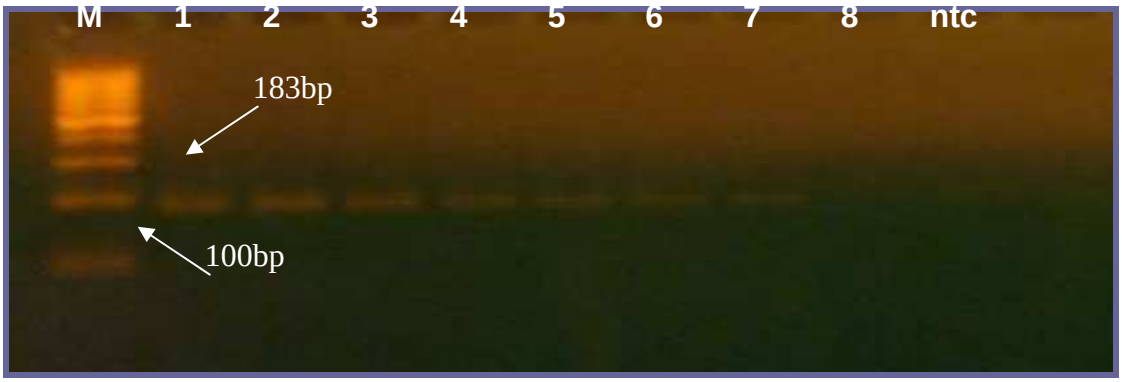



**Figure 20**. Melting curves of poultry DNA diluted (a) and gel analysis (b)

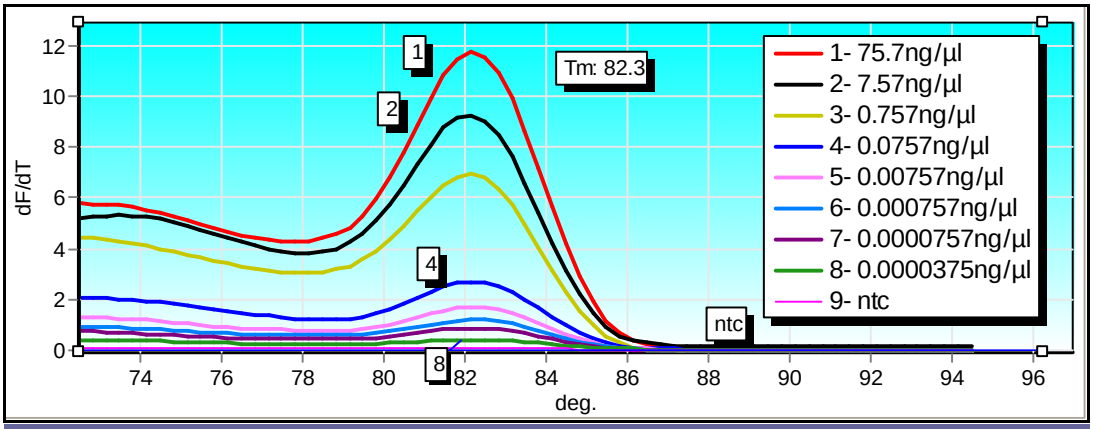
Figure 20 is for poultry sensitivity in SYBR Green I real-time PCR with Rotor-Gene 6000. PCR products from control poultry were run on a 3% agarose gel, and

stained with ethidium bromide. Lane M, 100bp DNA ladder; lane 1, 100%; lane 2, 10%; lane 3, 1%; lane 4, 0.1%; lane 5, 0.01%; lane 6, 0.001%; lane 7, 0.0001%; lane 0.00001%;lane ntc, negative template control.

### 3.4.3. Pork PCR Assay Sensitivity

The templates were amplified in the PCR followed by melting curve analysis :( a) the chart represents the change in fluorescence as a function of time (dF/dT) versus the temperature of the reaction products. The detection limit was 0.0000375% for pork which has tm: 83.2

a




b

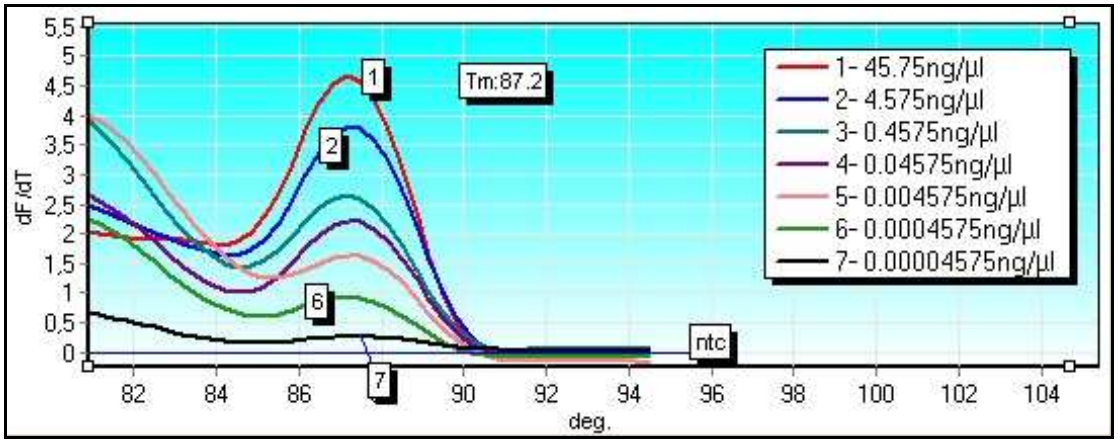

**Figure 21**. Melting curves of pork DNA diluted (a) and gel analysis (b)

Figure 21 is for sensitivity in SYBR Green I real-time PCR with Rotor-Gene 6000). PCR products from control pork were run on a 3% agarose gel, and stained with ethidium bromide. Lane M, 100 bp DNA ladder; lane 1, 100%; lane 2, 10%; lane 3, 1%; lane 4, 0.1%; lane 5, 0.01%; lane 6, 0.001%; lane 7, 0.0001%; lane 8, 0.00005%;lane ntc, negative template control.

### 3.4.4. Fish PCR Assay Sensitivity

The templates wer amplified in the PCR followed by melting curve analysis: (a) the chart represents the change in fluorescence as a function of time (dF/dT) versus the temperature of the reaction products The detection limit was 0.0001% for fish which has tm: 86.5

a




b


**Figure 22.** Melting curves of fish DNA diluted (a) and gel analysis (b)

Figure 22 is for fish sensitivity in SYBR Green I real-time PCR with Rotor-Gene 6000. PCR products from control fish were run on a 3% agarose gel, and stained with ethidium bromide. Lane M, 100 bp DNA ladder; lane 1, 100%; lane 2, 10%; lane 3, 1%; lane 4, 0.1%; lane 5, 0.01%; lane 6, 0.001%; lane 7, 0.0001%; lane 8, 0.00001%;lane ntc, negative template control.

### 3.5. Applicability of QRT-PCR on the Mixed Meat Commercially

QRT-PCR was optimized and applied on processed/treated meat samples. During optimization, we made Ct values of serial diluted reference samples versus

concentration plot. Ct values were determined automatically by the device's software by using a threshold line drawn on the exponential phase of DNA curves as seen in figure Figure 23 a&b. The plot showed a linear decrease. Linear-regression analyzing unit in the package of the software developed a formula such as Y=AX + B. The linear regression analyzing software calculated that R and $R^2$ values are more than 1.9 which shows significance of the formula. By using the formula it produced, Corbett Rotor-gene software estimated the concentration of unknown samples with a negligible experimental error. Then, we did quantify all the samples and calculated their ratio in the mixture meat products even contamination.

After we apply QRT-PCR, with the primer sets of only poultry and ruminant, on the commercial good quality and high prices labeled mixed meat nine products whose experimental numbers are E-17, E-42, E-44, E-48, E-50, E-90, E91, E-962, E-93 it was seen that most of the products contained excess amount of poultry meat regarding to their labels.

### 3.51. Ruminant Meat Quantification in Mixed Meats

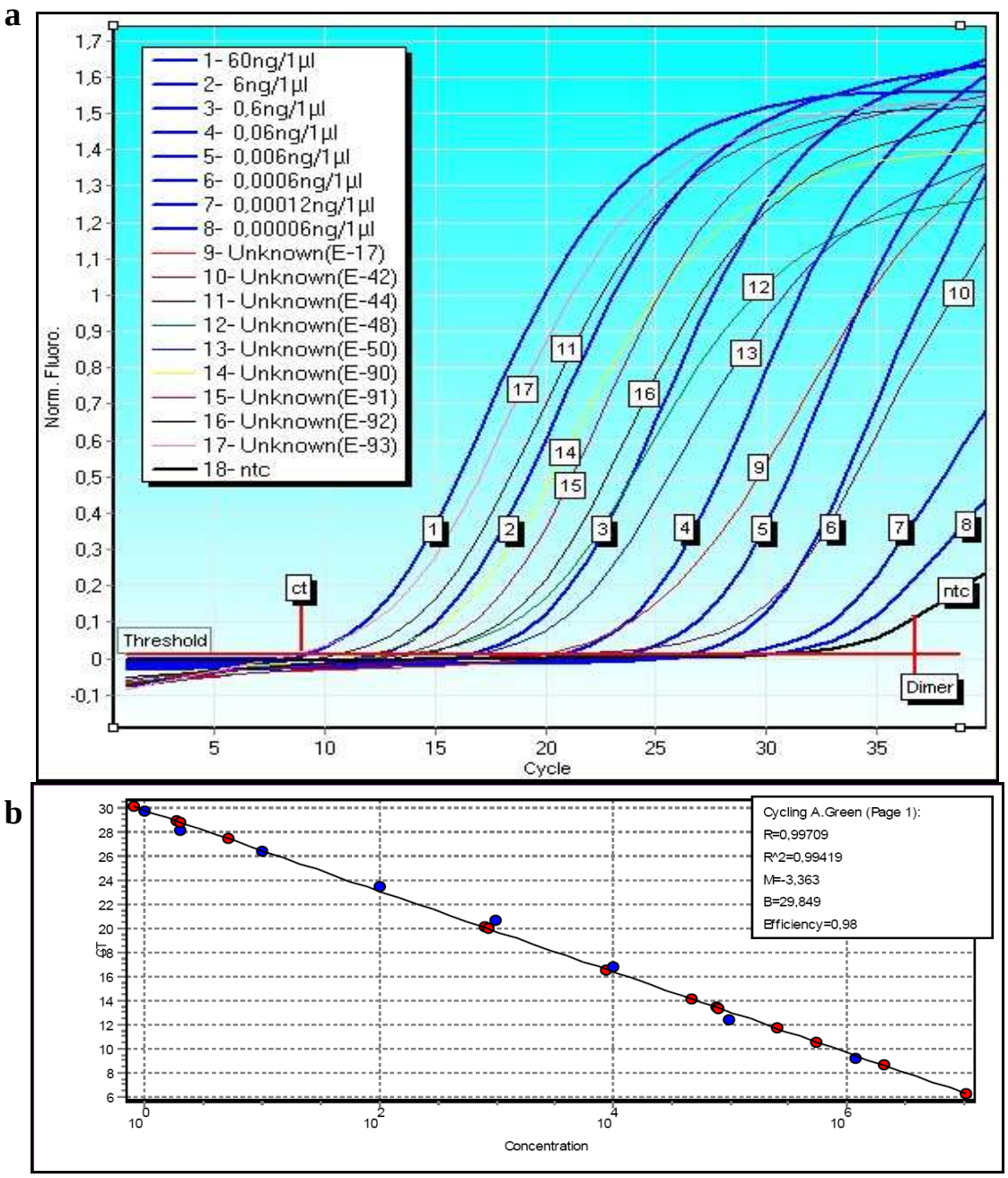



**Figure 23**. Ruminant quantification analysis in the processed meats (a) and calibration ruminant standard curve (b) for quantification in SYBR Green I real-time PCR with Rotor-Gene 6000.

In figure 23, the templates were amplified in the PCR followed by quantification analysis: (a) The chart represents the change in normal fluorescence as a function of time (dF/dT) versus the cycle of the reaction products. Curve 1,2,3,4,5,6,7,8, ruminant

standard amplification; curve 9,10,11,12,13,14,15,16,17, unknown amplification; curve 18, ntc, negative template control (b) The chart represents standard cure for ruminant standard amplification.

**Table.14** Results of ruminant quantification assay

| No. | Name | Type | Ct | Calc Conc (ng/ul) | Expexted (ng/ul) | | Spectro Conc (ng/ul) | |
|---|---|---|---|---|---|---|---|---|
| *1* | *Ruminant* | *Standart* | *9,16* | *78* | According to 78ng/ul | According to spectro | *60* | Given Concentration |
| *2* | *Ruminant* | *Standart* | *12,33* | *9* | | | *6* | |
| *3* | *Ruminant* | *Standart* | *16,81* | *0,43* | | | *0,6* | |
| *4* | *Ruminant* | *Standart* | *20,73* | *0,3* | | | *0,06* | |
| *5* | *Ruminant* | *Standart* | *23,52* | *0,004* | | | *0,006* | |
| *6* | *Ruminant* | *Standart* | *26,43* | *0,0006* | | | *0,0006* | |
| *7* | *Ruminant* | *Standart* | *28,14* | *0,00019* | | | *0,00012* | |
| *8* | *Ruminant* | *Standart* | *29,8* | *0,00006* | | | *0,00006* | |
| 9 | E-17(20%) | Sausage | 19,97 | 0,025 | 16 | 10 | 45,4 | Samples |
| 10 | E-42(50%) | Salami | 20,09 | 0,023 | 39 | 35 | 70 | |
| 11 | E-44(70%) | Sausage | 10,55 | 15,2 | 55 | 45 | 64,3 | |
| 12 | E-48(60%) | Salami | 13,37 | 2,3 | 47 | 48 | 80 | |
| 13 | E-50(70%) | Sausage | 16,6 | 0,25 | 55 | 34 | 48,3 | |
| 14 | E-90(60%) | Unknown | 11,67 | 7,1 | 47 | 42 | 78,8 | |
| 15 | E-91(60%) | Sausage | 13,42 | 2,15 | 47 | 33 | 55,6 | |
| 16 | E-92(80%) | Frankfurter | 14,15 | 1,3 | 62 | 46 | 58 | |
| 17 | E-93(100%) | Sausage | 9,46 | 56,5 | 78 | 54 | 54 | |
| *18* | NTC | | *30,17* | *0,00002* | | | | |

In Table 11, the column entitled ‘‘*Name*’’ indicates the standard origin of ruminant and ratio of ruminant meat in the mixed meat. The column entitled ‘‘*Type*’’ indicates standard samples and control samples. ‘‘Ct’’ column gives cycle threshold values and shows that there are correlation between ct values and amount of target DNA quantification. For example high Ct value means low concentration of target DNA in the reaction. The column declared as ``*Calculation concentration''* shows values of DNA concentration calculated by Corbett Rotor-Gene analyzer automatically itself. These values are original datum of PCR machine and we need to compare the amount of control samples with calculation concentration values. In the ‘‘*Expected* ’’column datum was calculated as follows: Because calculated concentration of template DNA of the standard sample-1 was 78ng/ul, the calculation concentrations of control samples (sausage, salami…) were estimated in accords with their given ratios. For instance,

expected value of E-17 whose label declares that the percentage of ruminant meat used in the product is 20%, is 78X20/100=16ng/ul. As for expected concentration according to spectrophotometric measurement, spectrophotometeric concentration of control samples were examined in order to find similarities and differences in accord with their given ratios For example, expected value of E-17 45.4X20/100= ±10ng/ul. ‘‘*Given concentration*’’ datum in the ’’*spectro concentration*’’ column were used for both standard (positive control) and PCR assay sensitivity.

As a result, ratios of ruminant samples given on labels were not fitted to the actual results (too far from the fact). For instance, the amount of ruminant meat in the samples, E-17, E-42 and E-50, was negligibly small that we propose ruminant meat were actually not added to the products. May be, this was a contamination in processed-mixed meat products. On the contrary, only value of E-93 in calculation concentration column in table 11 was near to ratio given on its label. We also propose that samples, E-17, E-42, E-50, having lower ruminant meat than 1%, might be just contaminated by ruminant meat.

### 3.5.2. Poultry Meat Quantification in Mixed Meats

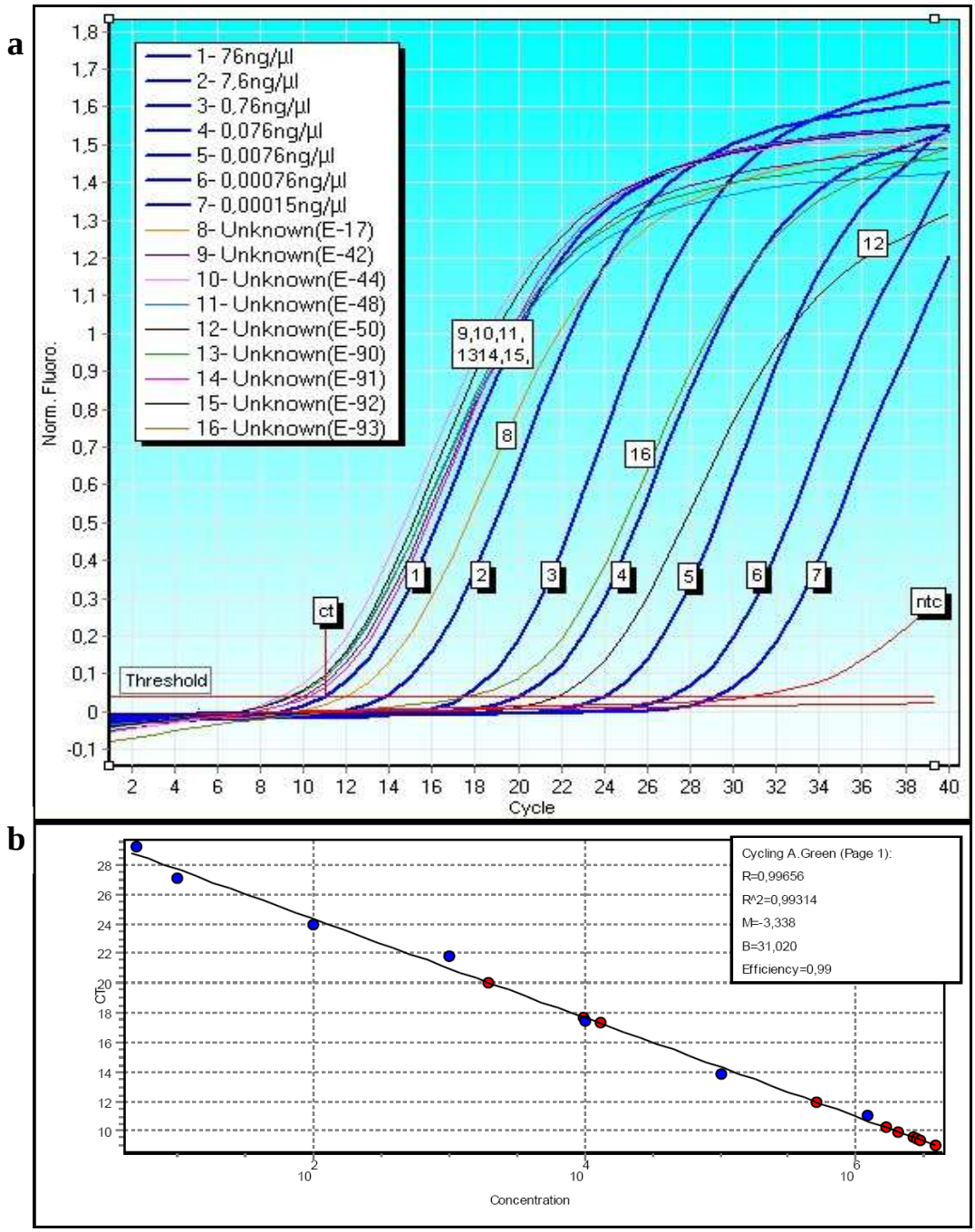



**Figure 24.** Poultry quantification analysis in the processed meats (a) and calibration poultry standard curve (b) for quantification in SYBR Green I real-time PCR with Rotor-Gene 6000.

The templates were amplified in the PCR followed by quantification analysis: (a) The chart represents the change in normal fluorescence as a function of time (dF/dT)

versus the cycle of the reaction products. Curve 1,2,3,4,5,6,7,8 poultry standard amplifications; curve 9,10,11,12,13,14,15,16,17 unknown amplifications; curve 18 ntc, negative template control (b) The chart represents standard cure for poultry standard amplification.

**Table15.** Results of poultry quantification assay

| No. | Name | Type | Ct | Calc Conc. ((ng/ul)) | Expexted (ng/ul) | | Spectro Conc. (ng/ul) | |
|---|---|---|---|---|---|---|---|---|
| 1 | Poultry | Raw-Standard | 11,01 | 78 | According to 78ng/ul | According to spectro. | 76 | Given Concentr. |
| 2 | Poultry | Raw-Standard | 13,82 | 10 | | | 7,6 | |
| 3 | Poultry | Raw-Standard | 17,5 | 0,78 | | | 0,76 | |
| 4 | Poultry | Raw-Standard | 21,86 | 0,03 | | | 0,08 | |
| 5 | Poultry | Raw-Standard | 23,95 | 0,0079 | | | 0,0076 | |
| 6 | Poultry | Raw-Standard | 27,08 | 0,0009 | | | 0,00076 | |
| 7 | Poultry | Raw-Standard | 29,23 | 0,0002 | | | 0,00015 | |
| 8 | E-17(80%) | Sausage | 11,96 | 20 | 62 | 36 | 45,4 | Samples |
| 9 | E-42(50%) | Salami | 9,97 | 85 | 39 | 35 | 70 | |
| 10 | E-44(30%) | Sausage | 9,05 | 159 | 23 | 19 | 64,3 | |
| 11 | E-48(40%) | Salami | 9,43 | 120 | 31 | 32 | 80 | |
| 12 | E-50(30%) | Sausage | 20,06 | 0,6 | 23 | 15 | 48,3 | |
| 13 | E-90(40%) | Unknown | 9,6 | 107,5 | 31 | 31 | 78,8 | |
| 14 | E-91(40%) | Sausage | 10,24 | 67,5 | 31 | 22 | 55,6 | |
| 15 | E-92(20%) | Frankfurter | 9,49 | 116,5 | 15 | 12 | 58 | |
| 16 | E-93(0%) | Sausage | 17,71 | 33,5 | 0 | 0 | 53,5 | |
| 17 | NTC | | 31,75 | 0,00003 | | | | |

In Table 12, the column entitled ‘‘*Name*’’ indicates the standard origin of poultry and ratio of poultry meat in the mixed meat. The column entitled ‘‘*Type*’’ indicates that standard samples are raw and control samples which are type. ‘‘*Ct*’’ column gives cycle threshold values and shows that there are correlations between ct values and amount of target DNA quantification. For example high ct value means low concentration of target DNA. The column labeled ``*Calculation concentration*'' shows values of DNA concentration calculated as follows: Because calculated concentration of template DNA of the standard sample-1 was 78ng/ul, the calculation concentrations of control samples (sausage, salami…) were estimated in accords with their given ratios. For instance, expected value of E-17 whose label declares that the percentage of ruminant meat used in the product is 80%, is 78X80/100=62ng/ul. As for expected concentration according to spectrophotometric measurement, spectrophotometric concentration of control samples were examined in order to find similarities and differences in accord with their

given ratios For example, expected value of E-17 45.4X80/100= ±36ng/ul.45. ‘‘*Given concentration*’’ datum in the ’’*spectro concentration*’’ column were used for both standard (positive control) and PCR assay sensitivity.

As a result, ratios of poultry given on label were not suitable even went too far from fact. For instance, the amount of poultry meat was more than the ratios given on it’s label in the samples, E-42, E-44, E-48, E-90, E-91, E-92. In spite of this, value of E-17 and E-50 in calculation concentration column in table 12 was less than ratio given on its label. Nevertheless different of other control samples contain E-42, E-44, E-48, E-90, E-91, E-92 and E-93 from positive sample (78ng/ul) were more two folds than ratios on the label. This result showed that poultry meat could be substituted ruminant meat if we took account of calculation concentration for ruminant values in table 11.

We used dilution series as a contamination marker and compared with concentration of control samples and we saw that samples lower than 1% contain E-50 might be contamination.

### 3.6. Optimization of Multiplex Real-time PCR (MRT-PCR) by SYBR Green

The reaction condition including reagents for MRT-PCR assay were optimized for making small modifications, SYBR Green I added, to the protocol of CM-PCR. The primers for poultry and ruminant were combined for the multiplex reaction. Using SYBR Green I and the Corbett Rotor Gene Analyzer system, the accumulation of amplicons in the reaction was monitored over time. The templates were amplified in the Real-time PCR followed by melting curve analysis.

Figure 24 a, b and c is the results of different experiments. They represent the changes in fluorescence as a function of time (dF/dT) versus the temperature of the reaction products.

Since SYBR Green florescence effectively was bound all amplicons without establishing a direct differentiation between ruminant and poultry specific products, multiplex MRT-PCR fragments were detected by melting curve analysis. Hence, ruminant and poultry amplicons were easily distinguished by specific Tm values due to the different length and compositions of two amplicons.

In each of charts on figure 24a, b and c, melting curves of positive templates can be clearly seen. Chart c had especially the melting curves of both poultry and ruminant positive controls separately.

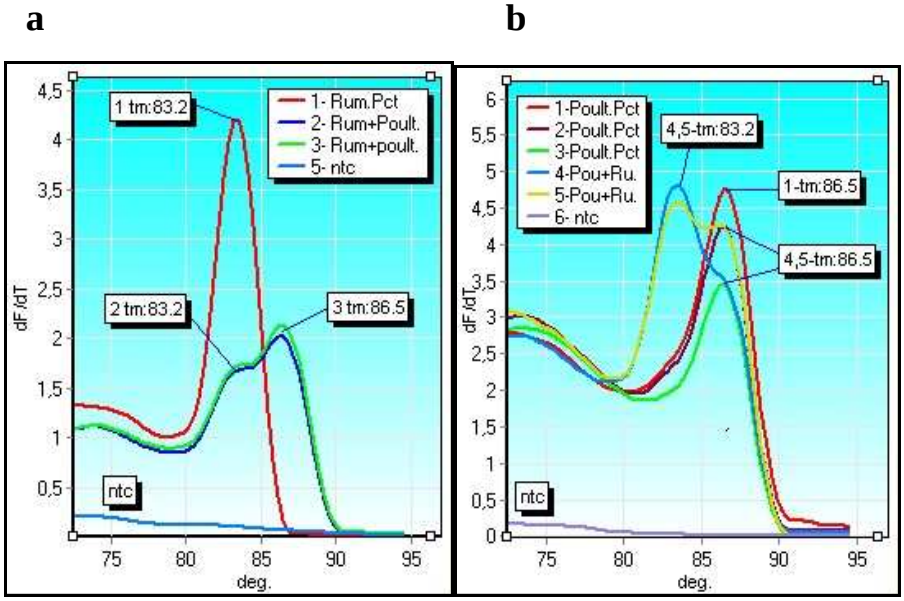
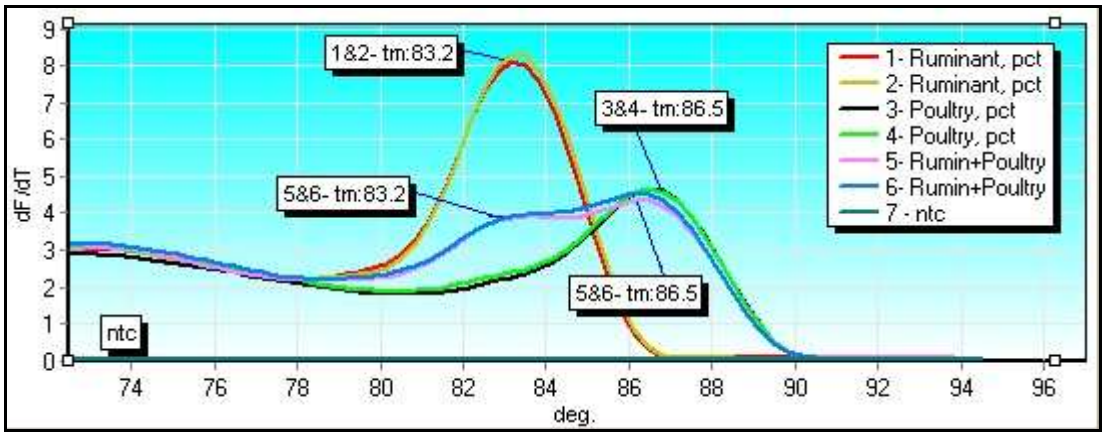



**Figure 25.** Specifity of multiplex real-time PCR assay, fluorescence melting curve for ruminant and poultry in SYBR green I multiplex real-time PCR with Corbett Rotor Gene.

In figure 25, (a) Melting temperature profiles of ruminant and poultry as follows: 1: Ruminant, pct(positive template control), tm: 83.2; 2,3: Ruminant+Poultry (multiplex amlication), tm: 83.2, tm: 86.5; ntc (negative template control).(b) 1, 2, 3: Poultry, pct; 4, 5: Poultru+Ruminant (multiplex amplication), tm:86.5, tm: 83.2, ntc

c




d

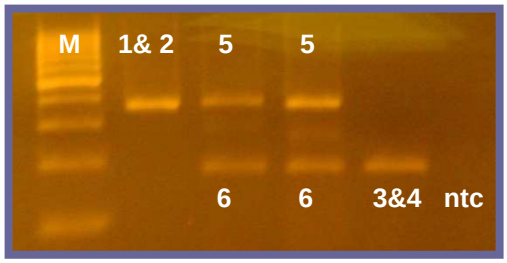



**Figure 26.** Specifity of multiplex real-time PCR assay, fluorescence melting curve for ruminant and poultry in SYBR green I multiplex real-time PCR with Corbett Rotor Gene and gel image of products 2.

In figure 26, (c) Melting temperature profiles of ruminant and poultry as follows: 1&2: Ruminant, pct, tm: 83.2; 3&4: Poultry, pct, tm: 86.5; 5&6:Ruminant+Poultry(multiplex amlication); ntc. (d)MRT-PCR products were run on a %3 agarose gel, and stained with ethidium bromide. Lane M, 100bp DNA ladder; lane 1&2: Ruminant, pct, 374bp; 5 and 6: Ruminant+Poultry (multiplex amplication); 2&3: Poultry, pct, 183bp, positive template control; ntc, negative template control.

The MRT-PCR resulted in a two peaks in a single curve shown in the figure 24. These peaks were mounted from their specific location on temperature axis 83.2 for ruminant and 86.5 for poultry.

SYBR Green I obviates the need to examine PCR products on time-consuming agarose gels. In spite of this, MRT-PCR products were run on the agarose gel and we saw that the sizes of the amplicons were as expected: 183bp and 374 bp for poultry and ruminant respectively. (Figure 25, d)

# CHAPTER 4

# DISCUSSION

Accurately labeling for food products is a must in many companies. In food industry, meat and its products are one of the essential ingredients in foodstuffs. The possible aims of the law above are; (i) to prohibit the unfair competition between food companies due to the fraudulent substitution or adulteration , (ii) to reduce medical problems of consumers who have specific meat allergies and (iii) religious rules in whose Muslims and Jewish peoples are not allowed to consume pork meat and many Hindu do not consume beef.

Some of low qualified companies can not obey the rule above and use different types of species in different quantity in meat products although they do not decelerate it in their labels. There are not so much low priced and qualified analytical techniques and it encourages the companies to select illegal ways to produce food. That is why scientists have developed several quantitative and qualitative analytical methods to identify meat species used in food stuffs.

The methods can be classified into three groups: morphology-based methods (MBM), protein-based methods (PBM) such as electrophoretic, chromatographic and immunological techniques and DNA-based methods (DBM).

DBMs are two major advantages over others. DNA is more stable in extreme conditions and that is why samples heated to as high as 120$^{o}$C can still be analyzed. Next, DBMs are very species-specific to discriminate between even much related species such as chicken and turkey.

Polymerase chain reaction-restriction length polymorphism (PCR-RFLP), as one of DBMs, of the molecular methods applied in the past for meat species identification commonly. However, applicability of this method in mixed-adulterated meats was found not to be satisfactory. Results of these techniques were not representative of the actual amount of the different components present in the mixture. This could be due to the disparity in the quantity of PCR product amplified and the amounts of target DNA present [33].

In this thesis, we optimized conventional simplex and multiplex PCR and developed a novel QRT-PCR method and MRT-PCR using SYBR Green dye.

One of DBMs is conventional simplex PCR technique. Conventional PCR has been applied for the detection of origin of different species by a lot of scientists [10, 13, 24, 25, 27, 28, 29]. Jerilyn A. Walker et al, also used it to make a quantitative analysis according to the gel electrophoresis results of amplicons. They found out that bovine DNA was detected at 0.005 %( 0.5 pg), porcine DNA was detected at 0.0005% (0.05 pg), and chicken DNA was detected at 0.05% (5 pg) in a 10-ng mixture of bovine, porcine, and chicken DNA templates by this method. We also optimized a similar method and could detect 0,001% of amplicon by ruminant (Figure 11), pork, poultry and fish and. We also scanned all samples with fish and pork primers and saw that none of the samples except fish or pork samples declared on the labels was contaminated by pork and fish meats.

The CM-PCR in present thesis depicts the development and application of a multiplex PCR to detect ruminant, poultry, fish and pork materials in foodstuffs in a single reaction step that highly decreases the cost of tests.

We used mitochondrial DNA for the detection and quantification of species in processed meat. Because of high copy number of small, circular mitochondrial DNA in cells, their chances of their survival under different processing conditions are higher, making it ideal for processed meat species identification [78].

The conventional multiplex PCR described in this thesis proved to be very useful when DNA mixtures were tested. The same assay, applied on commercial processed meat containing salami, sausage and frankfurter, showed its predictability was very good.

Many scientists used CM-PCR for the same purposes. Dalmasso et al. [12] applied CM-PCR on the 13 commercial labeled processed meals (pet food, baby food meat and blood meal). They found that 3 of 4 commercial meals were carrying meats belonging to the species not declared on their labels. The ratio in our study is 35.1% (25 over 71). From another point of view, 6 and 2 of 14 ruminant meat products manufactured by high quality companies were contaminated by poultry and carrying only poultry meat respectively ( Table 10, 11).

Briefly, our CM-PCR assay was applied to processed and raw meats for the identification of the most used species in foodstuffs such as ruminant, poultry, fish and pork materials. Specific-species primers designed in different regions of mitochondrial DNA were used after alignment of the available sequences in the GenBank database. The primers were generated specific fragment of 183, 224, 290 and 374 bp lengths for poultry, fish, pork, and ruminant, respectively. The optimized CM-PCR assay was applied to 93 commercial meat products and it showed the presence of poultry meat in 25/71 of the analyzed products contain raw or processed red meat, evidenced the presence of animals species not indicated on the label. In this study, as for samples shown in figure 12, 13, 14 and15 the results of conventional multiplex PCR performed on commercial meals in Istanbul, evidenced the presence of animals species not indicated on the label.

The results of conventional multiplex PCR assay on the commercial food have suggested an extension of the assay to other items from the retail trade, such as pet food, baby food etc. The test could be useful in the control of different products, such as baby food, to verify the origin of the raw materials, especially in products submitted to denaturing technologies, for which other methods cannot be applied [12] For instance, the Ouchterlony method cannot distinguish between closely-related species such as wild boar and pig, cattle and buffalo, sheep and goat. The effectiveness of ELISA and SDS-PAGE is hampered by the cumbersome process of isolating species-specific proteins. IEF presupposes that the protein composition of meat is similar within species, and has differences between, for instance, muscle proteins of sheep and goat. However, even the electrophoresis patterns of serum proteins and brain proteins could be different within the *same* species [76].

Disadvantageously, Clearly, CM-PCR could not detect whether they, unexpected results, are contamination or not at the moment of the manufacturing of these products. That is, it could be difficult to establish whether a fraud is presumable or an unintentional contamination occurred, in case of very small amounts of contaminating animal materials. Because of this drawback, we tried to develop a quantitative real-time PCR technique by SYBR Green to quantify the presence of animal material in foodstuff samples.

The developments and the availability of specific quantitative PCR-based methods for identification of small amounts of DNA are necessary as a support of an efficient surveillance system for species substitution lacking nowadays. The enforcements of legislation guidelines to guarantee public health associated to the improvements of detection methodologies appear to be necessary to differentiate between technically inevitable contamination or intentional admixture [18].

In spite of the extensive use of real-time PCR technology for gene expression analysis and identification of microorganisms [79, 80, 81, 82 ] there are relatively few published reports on its application for food species quantification such as that of beef, pork, lamb, chicken, and turkey [30, 32, 82, 3]. Only one of them is about real-time PCR detection and quantification of meat species by using SYBR Green fluorescence dye. The technique was optimized only to quantify dear meats in meat products [3].

In the study, quantification was performed to determine the proportions of species contained in the food sample (corbett rotor software system). That is because the main potential advantages of real-time PCR technology are the possibility of performing quantitative measurements. Nonetheless, other benefits are: (1) It categorizes DNA origin without the need for any additional time consuming and laborious steps such as sequencing, enzyme digestion, or conformational analysis; (2) Real-time PCR assays are fast, since multi-well plates (96 or more) can be assayed in a working day resulting in routine high-throughput screening of multiple samples; (3) data can be collected directly from a real-time PCR instrument or a fluorescence spectrophotometer, avoiding the need for electrophoresis; (4) Potential contamination of the PCR mixture with target DNA is greatly reduced because the reaction tubes remain closed throughout the assay; (5) Providing almost equivalent efficiency and specificity, SYBR Green assays have

almost 7 folds lower cost compared to fluorescent probe-based assays with Taqman probes or molecular beacons [3].

The drawbacks of most real-time PCR applications come from the fact that the accuracy of the method can be influenced by factors affecting to the DNA yield such as the extent of DNA degradation and, and the high cost derived of specific fluorescent probes [83]. To decrease this limitation, the use of species-specific primers and SYBR Green was introduced for the detection of PCR products without the need for probes linked to fluorescent molecules [83, 84].

During the optimization of protocols, the standard curve method was applied, as shown in Figs. 1 b and 2 b. The threshold cycle (CT) indicates the cycle number at which the fluorescence generated within a reaction crosses the threshold. The threshold should be put above any baseline activity and within the exponential increase phase. The CT value is related directly to the amount of PCR product and so related to the original amount of target DNA present in the reaction tube. (Figure 1 a and figure 2 a) A low CT value means high level, and a high CT value means low level of initial amount of the target one. A plot of the logarithm of the initial target copy number for a set of standards vs. CT is used for regression line. The quantification of the target amount in unknown samples is accomplished by measuring CT and using the standard curve to determine the starting copy number. [83] Both identification and quantification by real-time PCR assay can be completed in 1.5 h including 20 min for sample preparation, 70 min for PCR cycling.

Real-time PCR data obtained from different concentration of DNA are comparable only when the experimental efficiencies for the different reactions are similar and near to the value of 100%. [74] (Figure 22 b, 23 b). The efficiency (E) of a real-time PCR assay can easily be calculated from the Formula $E = [10^{(-1/slope)} - 1]$ X100 (Rotor-Gene 6000 operator manual). Our QRT-PCR experiments' efficiencies were 0.98 for ruminant, 0.99 for poultry and also R and $R^2$ values of experiments were 0.99709, 0.99419 for ruminant, 0.99656 and 0.9999314 for poultry respectively.

In the case of quantitative methods, the limit of quantification (LOQ) is the lowest level of analyte that can be reliably quantified, given a known concentration of target taxon genome. For this purpose, tenfold dilution series of DNA from ruminant, pork,

poultry and fish were prepared. Dilution series containing 60-0,00006ng of DNA for ruminant and 76-0,000076ng of DNA for poultry were tested in ten replicates, and compared with curve series of unknown DNA. The results of the quantification of the respective animal species including ruminant and poultry in the samples given in table 11, 12 showed that the sensitivity, the specificity and the reproducibility of the method was very high.

The minimum effective quantification levels of QRT-PCR were 0.00006 ng/µl, 0.000076 ng/µl 0.000045 ng/µl and 0.000045 ng/µl fơr ruminant, poultry, fish and pork respectively. This shows that this assay highly is sensitive, specific and ideal for he identification and quantification of ruminant, poultry, fish and pork DNA in mixed products. kötü Örnek ver. We saw that these results were almost overlapped by those of Jerilyn A. Walker [3]. In their study, they also found their minimum effective quantification level as Bovine DNA was detected at 0.005% (0.5 pg), porcine DNA was detected at 0.0005% (0.05 pg), and chicken DNA was detected at 0.05% (5 pg) in a 10-ng mixture of bovine, porcine and chicken DNA templates.

Nine commercial labeled meat products were tested by our QRT-PCR method and seen that none of the products carried meat ingredients as indicated in labels. The ruminant meat proportions were predicted after comparing with the standard dilution series. The results of ruminant products showed all ruminant meat products were defective and poultry meats had been added especially instead of ruminant meat which was the main component of the mixed-processed meats. On the other hand, in the amount of poultry meat was more than the ratios given on it’s label in processed-mixed meat products on the contrary ruminant meat proportion was less than. For example Jerilyn A. Walker [4] also tested six commercially purchased meat products using SYBR Green Real-time PCR assay. The most surprising finding of their meat analyses was that the chicken sausage they selected for testing contained significant amounts of both beef (0.06%) and pork (7,7%). However, the amount of pork in the sample, nearly 8%, would appear to be more than trace quantities. Since only a single sample of chicken sausage from a single grocery store appeared to be mislabeled these findings may not be indicative of a widespread problem with regard to ambiguous meat labeling practices. However, if an individual consumer had a strong objection to pork

consumption, or was allergic to pork, the consumption of this mislabeled meat product could potentially have devastating consequences.

By means of QRT-PCR even minute admixtures of different animal species can be identified in foodstuffs of complex composition and proportions of species can be determined by relating the concentration of detected species-specific sequences to the concentration of a suitable reference sequences. Statements about the absolute content of proportion of meat or plant are not possible yet. The amount of ingredients analyzed needs to be determined besides the other ingredient sugar, salt, oil and ice, which cannot be identified by DNA analytical methods, together with additives and flavoring agents [83].

The ruminant proportions were quantified after comparing with the standard dilution series. The results for proportion of ruminant showed significantly ruminant meat defective and there was DNA of poultry especially instead of mammals DNA in the mixed meat as the main component. The results obtained in this study from sausages, salami, frankfurter and minced meat samples highlighted that the substitution of meat species is rather frequent and probably due to both unavoidable contamination and intentional admixture for economic reasons.

Real-time PCR assay provided comparable sensitivity and superior reproducibility, precision and shorter performance time when compared to previous methods. This fluorescence-based real-time assay not only can quickly identify target genes independently but also allows for multiple PCR reactions in one tube by employing the unique melting curve analysis following DNA amplification [72].

The study of multiplex real-time PCR using SYBR Green dye we described here was done to develop an assay that can combine the two advantages of real-time PCR and multiplex PCR together for animal gene detection and identification. The objective of this study was to design a rapid, specific and accurate multiplex fluorogenic PCR assay to detect a group of mixed meat simultaneously. Our results indicate that our multiplex real-time PCR assay can be used to more quickly identify ruminant and poultry DNAs isolated from complex foods.

Using the intercalating fluorescence dye SYBR Green I and the Corbett Rotor Gene Analyzer system, the accumulation of amplicons in the reaction can be monitored over time. SYBR Green I obviates the need to examine PCR products on time-consuming agarose gels. After PCR amplification, the Corbett Rotor Gene Analyzer continuously monitors the decrease of fluorescence resulting from the release of SYBR Green I during DNA melting point analysis by slowly increasing the temperature. The Tm of specific amplicons and unique shape of the melting peak can be used to differentiate the target genes and identify them.

Some scientists proposed that MRT-PCR with SYBR Green fluorescence dye can not be possible. Although multiplexing reactions cannot be performed with SYBR Green, specificity can be achieved by careful primer design and reaction optimization, which can be confirmed from dissociation (or melt) curve analysis [3, 73, 74]. Taqman Real-time PCR was used by many scientiss in MRT-PCR [85]. The uniformity of each species-specific amplicon in conjunction with fluorophor-specific TaqMan probes would make these assays amenable to multicolor multiplex detection, whereas SYBR Green-based detection would not [73].

There are few reports describing the use of a multiplex flourogenic PCR however most of them are with bacteria [72]. There are no previous reports describing the use of a multiplex fluorogenic PCR assay using SYBR Green to identify meat genes, so it is not possible for direct comparison of our assay with others.

In briefly, we describe a multiplex SYBR Green I real-time PCR assay which appears to be a promising tool for rapid, sensitive, specific and accurate identification of ruminant and poultry gene in the processed-mixed meat products. The use of a simple and less expensive SYBR Green I format with Tm analysis of PCR products is easier and faster to perform compared with conventional PCR analytic approaches. This method readily distinguishes specific and nonspecific PCR products, even if they produce fragments of almost equal sizes in electrophoresis. Determination of melting points was very reproducible in our experiments.

The use of our assay for the identification of meat DNA within food products will provide additional molecular approach for outbreak investigation and surveillance.

Finally, this is a rapid and simple assay that could be used in molecular food analysis as well as for the surveillance.

# CHAPTER 5

# CONCLUSION

We developed QRT-PCR, MRT-PCR and optimized CM-PCR for quantitative and qualitative analysis of meat products. They are promising techniques for cheap, rapid, sensitive, specific and accurate identification of animal DNA in the processed-mixed meat products. These techniques are potentially reliable techniques for detection of illness, BSE, origin and amount of meat from other animals for labeling regulation and Halal authentication. In addition QRT-PCR is a method clarified the cause of a positive result whether it is due to adulteration of the product or inadequate handling during manufacture and useful tool in the food industry.

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