Hatice Nur AYDIN

DEVELOPMENT OF A FAST SIMPLE PROFILING METHODFOR SAMPLE SCREENING USING HRM OF Y-STRs

SAMPLE SPINE

M.S.
2015

FATİH UNIVERSITY

The Graduate School of Sciences and Engineering

Master of Science in
Genetics and Bioengineering

DEVELOPMENT OF A FAST SIMPLE PROFILING METHOD FOR SAMPLE SCREENING USING HRM OF Y-STRs

by

Hatice Nur AYDIN

January 2015

DEVELOPMENT OF A FAST SIMPLE PROFILING METHOD FOR SAMPLE SCREENING USING HRM OF Y-STRs

by

Hatice Nur AYDIN

A thesis submitted to

the Graduate School of Sciences and Engineering

of

Fatih University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Genetics and Bioengineering

January 2015
Istanbul, Turkey

APPROVAL PAGE

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

_____
Assoc. Prof. Mustafa Fatih ABASIYANIK
Thesis Supervisor

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

_____
Assoc. Prof. Mustafa Fatih ABASIYANIK
Head of Department

Examining Committee Members

Assoc. Prof. Mustafa Fatih ABASIYANIK _____

Asst. Prof. Gönül SCHARA _____

Asst. Prof. Bakytzhan BAKHAUTDIN _____

It is approved that this thesis has been written in compliance with the formatting rules laid down by the Graduate School of Sciences and Engineering.

_____
Prof. Nurullah ARSLAN
Director

January 2015

DEVELOPMENT OF A FAST SIMPLE PROFILING METHOD FOR SAMPLE SCREENING USING HRM OF Y-STRs

Hatice Nur AYDIN

M.S. Thesis – Genetics and Bioengineering
January 2015

Thesis Supervisor: Assoc. Prof. Mustafa Fatih ABASIYANIK

ABSTRACT

Human genome varies between individuals at a rate of approximately 0.03% and this small difference makes people unique individuals. These variable markers are being located and characterized at the specific sites in the genome and used for human identification. The polymorphisms are classified in two groups; sequence and length polymorphisms. STR markers we use in this thesis are length polymorphisms which vary based on the number of nucleotide repetitions within a particular locus. STRs have been frequently used in forensics, population genetics, and genetic genealogy. STR haplotyping is mainly done by gel based and capillary electrophoresis methods which are labor intensive and time consuming.

In this study we developed high resolution melting (HRM) method as a screening tool to discriminate Y-STR male haplotypes. Twelve unrelated male individuals were screened for variation at nine commonly used Y-chromosome specific STR loci (DYS19, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, and DYS448). The nine Y-STR loci were amplified with their specific primers in the presence of EVA Green dye, and then the HRM analysis was performed.

Different haplotypes of the four loci DYS393, DYS390, DYS439, and DYS 448 were distinguished by HRM analysis. These loci can be added to the list of STRs that had been studied for HRM screening as sex determining loci.

Keywords: Polymorphism, haplotype, HRM, screening, STR, Y-STR.

Y-STR'LERİN YÜKSEK ÇÖZÜNÜRLÜK ERİME (HRM) TEKNİĞİ KULLANILARAK ÖRNEK TARAMA İÇİN HIZLI VE BASİT PROFİLLEME YÖNTEMİ GELİŞTİRİLMESİ

Hatice Nur AYDIN

Yüksek Lisans Tezi –Genetik ve Biyomühendislik
Ocak 2015

Tez Danışmanı: Doç. Dr. Mustafa Fatih ABASIYANIK

ÖZ

İnsan genomu bireyler arasında yaklaşık %0.03 oranında farklılık gösterir ve bu küçük fark insanları eşsiz bireyler yapar. Bu değişken kısımların genom içindeki spesifik alanlardaki konumları ve özellikleri belirlenebilir ve bu belirteçler insan identifikasyonu için kullanılabilirler. Bu polymorfizmler iki grupta sınıflandırılırlar; sekans ve uzunluk polymorphizmleri. Bu tezde STR belirteçleri uzunluğu belirli bir lokustaki nukleotid tekrarlarının sayısına göre değişen uzunluk polymorfizmleridir. STRlar adli tıp, popülasyon genetiği ve genetik soy bilim çalışmalarında sıklıkla kullanılmaktadır. STR haplotipleme ağırlıklı olarak yoğun iş gücü gerektiren ve zaman alıcı olan jel bazlı kapiler elektroforez yöntemleri ile gerçekleştirilir.

Bu çalışmada Y-STR erkek haplotiplerini ayırt etmek için bir tarama aracı olarak, Yüksek Çözünürlüklü Eritme (HRM) yöntemi geliştirdi. On iki akraba olmayan erkek birey, yaygın biçimde kullanılan Y-kromozomuna özgü dokuz STR lokusunun (DYS19, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, and DYS448) varyasyonu açısından taranmıştır. Bu dokuz Y-STR lokusu EVA yeşil boya varlığında spesifik primerler ile amplifiye edilmiş ve daha sonra HRM analizi gerçekleştirilmiştir.

Dört lokusun: DYS393, DYS390, DYS439 ve DYS448, farklı haplotipleri HRM analizi ile ayırt edilmiştir. Bu lokusların cinsiyet tayin edici lokuslar olarak HRM taraması için önceden çalışılmış olan STRlar listesine eklenebileceği gösterilmiştir

Anahtar Kelimeler: Polymorfizm, haplotip, HRM, tarama, STR, Y-STR.

To my family

ACKNOWLEDGEMENT

I express sincere appreciation to Assoc. Prof. Mustafa Fatih ABASIYANIK for his guidance and insight throughout the research.

The technical assistance especially of Derya Sultan KARABULUT, Muammer DERVİSEVİC, Zeynep Aydın SİNİRLİOĞLU, Elif GÜNAL and Gülhan YAŞAR are gratefully acknowledged.

I would also thank to the Scientific Research Fund of Fatih University for supporting my thesis financially under the project number P500051401_B.

I express my thanks and appreciation to my family and my friend Zehra EKİNCİ for their understanding, motivation and patience. Lastly, but in no sense the least, I am thankful to all colleagues and friends who made my stay at the university a memorable and valuable experience.

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

LIST OF SYMBOLS AND ABBREVIATIONS

ABBREVIATION

BP	Base pair
CE	Capillary electrophoresis
CODIS	Combined DNA Index System
Cq	Cycle of quantification
DNA	Deoxyribonucleic acid
HRM	High Resolution Melting
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphisms
SSR	Small sequence repeats
St. Dev.	Standard deviation
STR	Small tandem repeats
SWAGDAM	Scientific Working Group on DNA Analysis Methods
Tm	Melt temperature
VNTR	Variable tandem repeats
YHRD	Y-STR Haplotype Reference Database
Y-STR	Y chromosomal STR

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Eukaryotic genomes are full of repeated sequences which vary in size and sequence (Ellegren 2004). These repeated sequences both differ in sequence or length, and can be used in human identification. In Human identification studies analysis of short tandem repeats (STRs) has become a well-established technology. STRs, also known as microsatellites or simple sequence repeats (SSRs), are repetitive regions of DNA that contain unique core repeat units of 2–6 nucleotides in length (Daniel M. Bornman 2012).

STR genotyping is highly discriminating when compared to other methods based upon antigenic (i.e., ABO typing) and protein markers (i.e.,PG), since they are highly polymorphic, genetically unlinked and reside in noncoding regions (Nguyen 2012). In addition to autosomal STR loci, the polymorphic Y-chromosomal sequences (Y-STRs) has been characterized and used in forensic science to detect and characterize male DNA. Y-STR haplotyping is particularly important for sensitive typing of male DNA in mixed stains as well as for rapid assortment of biological crime scene evidence (Roewer L. 2009).

Both autosomal and Y-STR alleles are routinely analyzed by multiplexed PCR followed by capillary electrophoresis (CE)-based separation. Much research is going on optimization of these multiplex reactions to save time and reagents used. STR genotyping, which utilizes electrophoresis to measure size differences in alleles possessing different numbers of full or partial repeats, is highly discriminating, but labor intensive and expensive (Corach, Filgueira Risso et al. 2001; Butler 2002; Vallone 2008). However, the dissociation temperature or melting temperature (Tm) of a double-

strand DNA (dsDNA) molecule also varies as a function of its nucleotide sequence, its length, and the degree to which its strands are perfectly complementary. This difference in the melting behavior of STR alleles can also be exploited to genotype dsDNA molecules by a process known as melt curve analysis (Nguyen 2012).

High resolution melting (HRM) goes beyond the power of classical melting curve analysis by allowing study of the thermal denaturation of a double-stranded DNA in much more detail and with more information. PCR products can be differentiated based on length, sequence or complementarity; single base changes (single-nucleotide polymorphisms) can be genotyped or single base mutations can be detected(Nicklas, Noreault-Conti et al. 2012).

Applying HRM analysis as screening assay for STRs have been done on autosomal STRs, no Y-STR application has been documented yet. In these studies various simple and complex STR loci; CSF1PO, vWA, D18S51, THO1, TPOX, D3S1358 could be correctly differentiated based upon their respective melt profiles (Halpern and Ballantyne 2009; Nguyen 2012; Nicklas, Noreault-Conti et al. 2012). In this study we aim to develop Y-STR HRM assays which can be used in for forensic case studies and any other Y-STR genotyping studies, resulting in saving money time and resources spent on the analysis of male specific cases.

1.2 DNA POLYMORPHISMS AND SHORT TANDEM REPEATS (STRS)

The location of a gene or a DNA marker on a chromosome is termed a locus. Length polymorphisms are a type of variation which can be found non-coding regions of the human genome and may differ among individuals. Variable number tandem repeats (VNTR), “minisatellite” and short tandem repeat (STR), “microsatellite” markers are examples of length polymorphisms, which vary based on the number of nucleotide repetitions within a particular locus. VNTRs consist of sets of tandemly repeated base pair sequences that can vary in length from approximately 10 to 100 base pairs. (Butler 2005). An example of a VNTR is the forensic DNA marker D1S80. The D1S80 marker has a 16bp repeat unit and containing alleles in the range of 16-41 repeats. (Butler 2005).

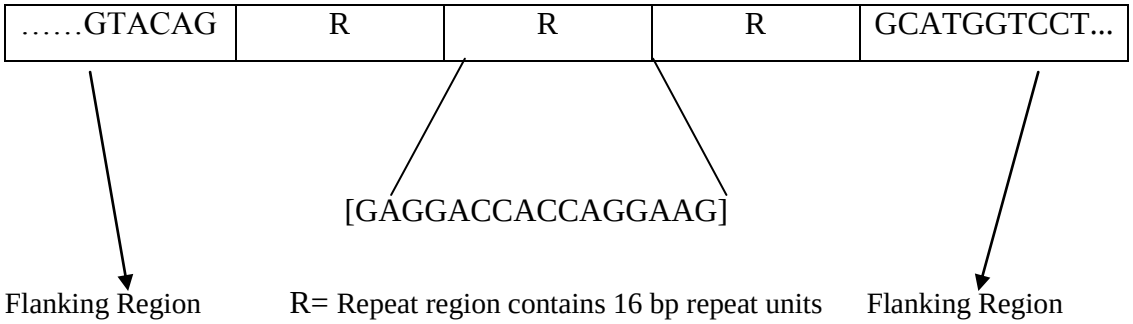

Figure 1.1 VNTR marker (D1S80).

STRs are also known as simple sequence repeats (SSRs), first reported in the late 1980s that contain unique core repeat units of 2–6 nucleotides in length (Butler 2005). A schematic of a STR is given in Figure 1-2. STRs are categorized by the length of the repeat unit. For example, tetranucleotide repeats have four nucleotides repeated next to one another over and over again. An example of a STR commonly used in the forensic community is D5S818. D5S818 is a tetranucleotide AGAT repeat with an allele range of 7-16 (Ellegren 2004). Thousands of these polymorphic microsatellites have been identified in human DNA. It is estimated that STR makers in the human genome occur every 10,000 nucleotides (Primrose 1998). Since STRs are abundant, highly polymorphic and easily scored, they are being very widely used in varied fields such as: genetic mapping, forensic investigations, and evolutionary studies (Britten and Kohne 1968).

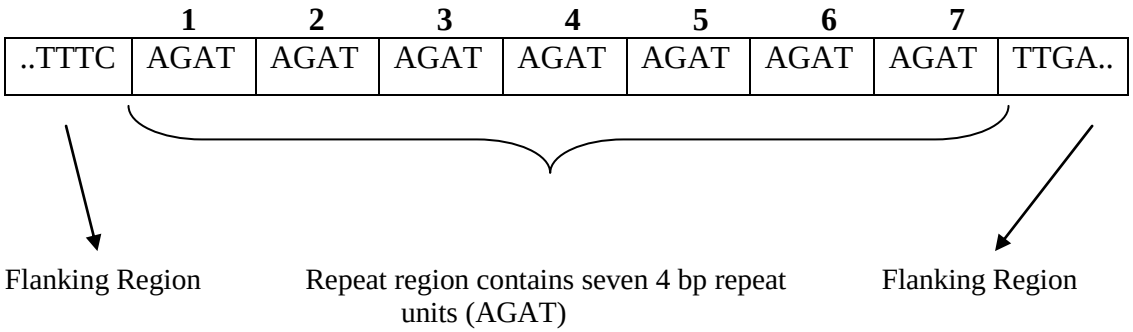

Figure 1.2 Short Tandem Repeat (STR) Marker (D5S818).

In addition to the variety of lenght in the repeat unit and the number of the repeats STRs also vary in their repeat pattern. STRs with units of the same length and sequence fit in the simple repeats group. Compound repeats group include adjacent simple repeats of two or more and complex repeats may contain many repeat blocks of variying unit lenght in addition to intervening sequences.The last repeats group is complex hypervariable repeats containing non-consensus alleles differing in both size and sequence. (Kong, Gudbjartsson et al. 2002; Kopelman, Stone et al. 2009).

Several STR database systems have been established, including the Combined DNA Index System (CODIS) utilized in the United States. This system currently uses a standard set of 13 STR loci, which are highly polymorphic, genetically unlinked and reside in noncoding regions. These STR alleles are routinely analyzed by multiplexed PCR followed by capillary electrophoresis (CE)-based separation. Although the CE-based technique for STR typing is both time and cost-effective, it does not allow for full sequence determination of STR loci and is only semiquantitative (Chambers and MacAvoy 2000).

As the use of STRs for genetic mapping, forensic identification and population studies, new alleles are being discovered additional STR markers are being developed and population data increases with each month of published journals (Kasai, Nakamura et al. 1990; Tautz 1993). Thus, the need of common dynamic information source unavoidably arose and an internet accessable informational database was created in early 1997. STRBase which was officially launched in July 1997, is maintained by the DNA Technologies Group of the National Institute of Standards and Technology. Now over 3600 references STRBase may be reached using the following URL: http://www.cstl.nist.gov/strbase.

1.2.1 Lineage Markers

In contrast to autosomal DNA markers lineage DNA markers are DNA markers on either the Y chromosome or the mitochondrial DNA. Because both the Y chromosome and the mitochondrion is inherited as a unit from the father and mother, respectively, lineage markers constitute a DNA profile that is called a haplotype after the Greek word for one fold (Litt and Luty 1989). This is in contrast to traditional DNA

markers on the autosomes the 22 pairs of non sex chromosomes, which are inherited from both mother and the father (Ellegren 2004).

Lineage DNA markers on the Y chromosome or mitochondrial DNA (mtDNA) are of great interest to both forensic and population genetics due to the patrilineal inheritance of the Y chromosome and matrilineal inheritance of the mtDNA (Butler 2005)

In forensic genetics, Y chromosomal markers become very useful for analyzing male DNA that is masked by large amounts of female DNA (Sibille, Duverneuil et al. 2002). In some forensic settings, the biological material is in poor condition such that no or only a few cell nuclei are present making the DNA impossible to extract. This is e.g. the case with very old samples and hair shaft samples. In such cases, mtDNA can sometimes be extracted as described by Sullivan et al. and sequenced (Sullivan, Hopgood et al. 1991). Hence, lineage DNA markers are important in forensic genetics as they help to solve cases that are otherwise difficult or even impossible to investigate using traditional methods.

Because lineage DNA markers have unique inheritance properties, these are also very interesting in population genetics because Y chromosome and mtDNA reflect male and female inheritance, respectively. Cann et. al. (1987) demonstrated how mtDNA could be used for population genetic studies and Roewer et al (2005) demonstrated how Y-STR markers could be used to infer recent historical events in the European Y-STR haplotype distribution.

1.2.2 Y Chromosome Polymorphisms

1.2.2.1 Application of Y chromosome analysis

Over the last decade, the Y chromosome has become firmly established as a powerful system in forensic analysis, showing particular utility in male–female DNA mixtures (Chambers and MacAvoy 2000). Addition to that Y chromosome markers are used in paternity tests (Edwards, Civitello et al. 1991; Butler 2005), missing persons investigations (Urquhart, Kimpton et al. 1994), human migration patterns addressing historical (Butler 2005), particularly in the SNP arena (Puers, Hammond et al. 1993;

Hammond, Jin et al. 1994; Fan and Chu 2007) and genealogical studies(Lee, Ladd et al. 1994; Collins, Stephens et al. 2003).

Table 1.1 Areas of application in Y-chromosome testing (Adapted from (Butler 2003)).

Use	Advantage
Forensic casework on sexual assault evidence	Male-specific amplification (can avoid differential extraction to separate sperm and epithelial cells)
Paternity testing	Male children can be tied to fathers in motherless paternity cases
Missing persons investigations	Patrilineal male relatives may be used for reference samples
Human migration and evolutionary studies	Lack of recombination enables comparison of male individuals separated by large periods of time
Historical and genealogical research	Surnames usually retained by males; can make links where paper trail is limited

1.2.2.2 Y STRs

Y-STRs are Short Tandem Repeats found on the male specific region of Y chromosome. Y-STRs are polymorphic among unrelated males and inherited through the paternal line without any change.

The first polymorphic Y-chromosome marker Y-27H39 — now defined as the DYS19 was discovered by Lutz Roewer and his colleagues in 1992 (Roewer and Epplen 1992). Later the discovery of Y-chromosomal STRs continued slowly and reached 30 available markers in 2002. The discovery of the new STR markers on Y chromosome speeded up in 2002 with improvement of bioinformatics tools and the ease of availability of sequence information on Human Genome Project (Ayub, Mohyuddin et al. 2000). Till February 2003 more than 200 markers' information has been uploaded to the Genome Database (GDB; http://www.gdb.org).

The European forensic community selected a set of Y-STR markers which include DYS19, DYS389I/II, DYS390, DYS391, DYS392, DYS393, and DYS385 a/b called as "minimal haplotype" in 1997. Since then several Y chromosome population data have been published using these loci. The Y Reference Haplotype Database (http://www.yhrd.org); is an online database that allows searching haplotypes' population-specific frequencies and also gives some geographical information of the individuals based on the "minimal haplotype" set. (de Knijff, Kayser et al. 1997; Kayser, Caglia et al. 1997).

In early 2003, the U.S. Scientific Working Group on DNA Analysis Methods (SWGDAM) selected a core set of 11 markers by adding DYS438, DYS439 to the minimal haplotype. To perform Y-STR analysis private companies has prepared kits including these loci. The most commonly used kit is AmpF*l*STR® Yfiler™ PCR Amplification Kit, which includes 17 specific Y-STR markers. Table 1.2 shows minimal, SWAGDAM extended and Y-Filer Extended loci.

Table 1.2 Minimal, SWAGDAM extended and Y-Filer Extended haplotypes (adapted from Butler 2005 Forensic DNA typing).

	Y-STR Markers	Repeat Motif	Allele Range	Length(bp)
Minimal Haplotypes	DYS19	TAGA	10-19	176-211
	DYS385a/b	GAAA	7-25	242-318
	DYS389I/II	TCTA TCTG	10-15 24-34	142-164 253-293
	DYS390	TCTA TCTG	18-27	192-227
	DYS391	TCTA	7-13	150-176
	DYS392	TAT	7-18	291-326
	DYS393	AGAT	8-16	130-131
SWAGDAM Extended loci	DYS438	TTTTC	8-13	223-248
	DYS439	AGAT	8-15	197-225
Y-Filer Extended loci	DYS437	TCTA	13-17	182-198
	DYS448	AGAGAT	17-24	280-324
	DYS456	AGAT	13-18	104-123
	DYS458	GAAA	14-20	130-155
	Y-GATA-H4	TAGA	8-13	122-142
	DYS635 (Y-GATA-C4)	TSTA COMPOUND	20-26	246-270

1.3 MELTING ANALYSIS AND HIGH RESOLUTION MELT ANALYSIS

1.3.1 Melting Analysis (MA)

Melt curve analysis (MA) is first used in 1997, to determine whether the product of interest is amplified after real-time PCR (Wittwer, Herrmann et al. 1997). By this approach the PCR products are evaluated by monitoring the fluorescence of an intercalating dye (or dye probe / primer combination) associated with double-stranded DNA (dsDNA). During PCR amplification the dye intercalates to the copies of the double-stranded product then, as a result the fluorescence increases. The fluorescence is

in its highest after the amplification, right before the melting. After the amplification has finished the temperature is gradually increased and as the temperature rises, fluorescence decreases. Until the temperature at which the two strands begin to separate is reached. Once complete denaturation is accomplished, the fluorescence decreases to background as intercalation of the probe to dsDNA is required to observe fluorescence. The data is plotted as the change in fluorescence with respect to the change in temperature (-df/dt) which results in a maximum at the melting temperature where the change in fluorescence is greatest. It is this melting profile that may be used to assess the specificity of the generated PCR product or detect differences between products (shown in Figure 1.3.).

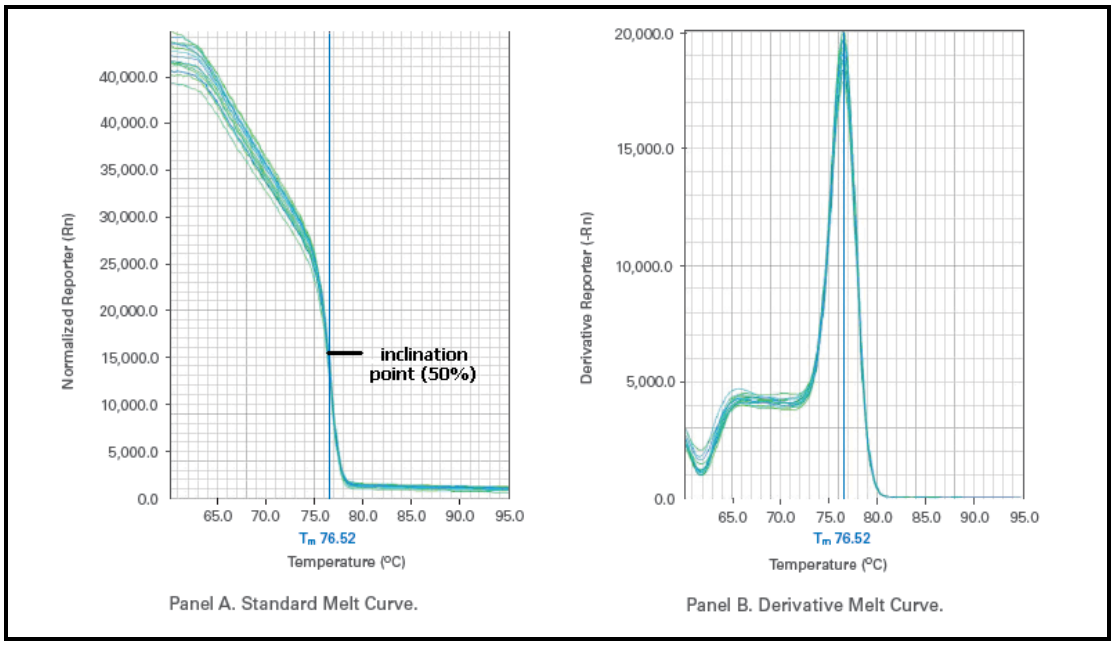

Figure 1.3 Melting analysis (adapted from Life (Edwards, Civitello et al. 1991).

1.3.2 High Resolution Melting Analysis (HRM)

High resolution melting which is a new method for DNA analysis introduced in 2002 by collaboration between academics (University of Utah, UT, USA) and industry (Idaho Technology, UT, USA) goes beyond the power of classical melting curve analysis. It allows study of the thermal denaturation of a double-stranded DNA in much more detail and with more information. PCR products can be differentiated based on

length, sequence or complementarity; single base changes (single-nucleotide polymorphisms) can be genotyped or single base mutations can be detected.

Since it is the simplest method for mutation scanning, genotyping and sequence matching, its popularity is growing. After PCR amplification without any needs of separation or processing of the samples, melting curves are generated by monitoring the fluorescence of a saturating dye that does not inhibit PCR(Reed, Kent et al. 2007).

Table 1.3 Dye types used for Melt Analysis (Karabulut 2012).

Dyes	**Examples**	**Properties**
1st Generation	Ethidium Bromide, Propidium Iodide, DAPI, Hoechst	mutagenic carcinogenic non-specific bindings
2nd generation	SYBR Green	at high concentrations-PCR inhibition at low concentrations-relocation-wrong measurement
3rd Generation	LC Green, Eva Green ve SYTO9	low PCR inhibition effect-can be used at high concentrations high concentration-increasing sensitivity of Tm measurement

The HRM method requires the two amplification primers, a PCR mastermix containing a saturating, intercalating dye (shown in Table 1.3) that binds specifically to dsDNA, but negligibly to ssDNA (such as Eva Green_; Biotium, Hayward, CA) and a proper instrument (such as the Qiagen Rotorgene Q; Qiagen, Valencia, CA) utilizing specific melting parameters and dedicated software to interpret the results.

Eva Green_ is often used for these studies because it has excitation and emission spectra very close to those of fluorescein (FAM), so it is compatible with real-time instruments. It is stable, nonmutagenic, noncytotoxic, and nonfluorescent when not bound to dsDNA and it generates much less PCR inhibition than SYBR_ Green at saturating concentrations (Brookes 1999). SYBR_ Green also performs ‘‘dye jumping,’’

where dye from a melted duplex may get reincorporated into regions of dsDNA which had not yet melted causing inaccuracies in the resulting melt curves.

HRM is a closed tube homogenous assay which is laborsaving and efficient (Li, Chu et al. 2010). Compared to probe-based assays, HRM is simpler and less expensive (Corbett 2006). For HRM there is not a requirement of separation or processing of the samples as in CE-based separation. Generation of the melting curves is done by monitoring the fluorescence of the ds-binding dye, following PCR amplification (Reed, Kent et al. 2007).

CHAPTER 2

MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

Samples for this study were collected from 12 healthy male individuals whose Y-STR allele numbers were previously collected by AmpF*l*STR® Yfiler® PCR kit followed by capillary electrophoresis (Yücebilgili 2011). Buccal swab and blood samples were collected with informed consent from individuals (see Appendix B).This research was supported by Fatih University Scientific Research Projects Fund (BAP), under the project number P50051401_B.

Table 2.1 Information of the samples' Y-STR loci.

	Sample Id Number & Allele Repeat Numbers												
Y-STR locus	**4**	**5**	**6**	**13**	**19**	**27**	**52**	**65**	**66**	**90**	**108**	**112**	**113**
DYS19	16	13	15	14	14	16	14	14	14	16	14	17	13
DYS390	26	24	22	24	24	23	24	23	23	25	23	22	24
DYS391	10	10	10	10	10	11	10	11	11	11	11	10	9
DYS392	11	11	11	11	11	11	14	14	11	11	14	11	11
DYS393	13	13	14	12	12	13	12	14	12	13	12	13	13
DYS437	14	14	15	15	15	14	15	14	16	14	14	16	14
DYS438	11	10	10	10	9	11	11	10	9	11	12	10	10
DYS439	10	12	11	11	12	10	12	10	11	10	13	12	10
DYS448	20	19	21	19	19	20	19	19	22	20	19	21	20

2.2 EXPERIMENTAL PROCESS

2.2.1 DNA isolation and quantitation

Buccal cells were collected with buccal swabs by rubbing the swab inside of the individual's mouth for at least 30 seconds. And the DNA was isolated with the described protocol (Wang, Fan et al. 1998), the swabs were separated from the sticks and the cotton tip section of the swab was placed in- side a 1.5-mL microcentrifuge tube, and 600 microliters of NaOH 50 mM were added to each tube. The tubes were closed and vortexed for 10 sec. They were placed in a drybath for 5 min at 95^0C removed and discarded. Thirty microliters of 1 M Tris HCl, pH = 8.0 were added to each tube. The tubes were centrifuged at 13,000 rpm for 2 min. The supernatant containing the isolated DNA in each tube was used for analysis. Isolated DNA samples were stored at -20^0C. The isolated DNA quantity was measured using a Nanodrop® ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

The whole blood samples were obtained by venipuncture and collected into EDTA vacutainer tubes. The DNA was isolated from blood either using Thermoscientific MagJET Genomic DNA Kit according to the manifacturer's protocol or isolated by the company BURÇ GENETİK. Isolated DNA samples were stored at -20^0C. The isolated DNA quantity was measured using a Nanodrop® ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

2.2.2 Amplification of DNA with BioRadSsoFAst ™ EvaGreen® Supermix for Real-Time PCR

The amplification of the Y-STR loci was performed with specific primers (shown in table 2.2) for each locus using the BioRadSsoFAst ™ EvaGreen® Supermix for Real-Time PCR. The supermix is a 2X concentrated ready to use mixture containing optimized buffer, EvaGreen dye and Sso7-fusion polymerase. For each reaction tube 10µl (1x) BioRadSsoFAst ™ EvaGreen® Supermix, 0,8 µl of each forward and reverse primers resulting of final concentration of 500nM and 1-5µl of template DNA samples and finally up to 20 µl ddH_2O were added to reach a total volume of 20 µl reaction volume as recommended by the manufacturer. (Bio-Rad Laboratories, Inc.) For the

optimization of final DNA concentration used in the reaction serial dilutions were made from 0,5ng/µl to 10ng/µl and 6ng/µl was chosen as optimum for HRM analysis.

For amplification of each Y-STR loci the previously designed primers were used (Andreasson, Asp et al. 2002) and the designed primers shown in table 2.2 were synthetized at Bio Basic Inc. Canada. For each primer set to find the optimum annealing temperatures gradient PCR was performed by using the DNA samples id66 and id 112 with conventional PCR device TECHNE - FTC51H2D and BioRad CFX96 TouchTM Real-Time PCR Detection System. Amplification was performed using the following cycling parameters: 98^0C for 2 minutes, 40 cycles of 98^0C C for 5 seconds optimum annealing temperature for 5 seconds. Following gradient PCR, products were run on an agarose gel to confirm the product sizes. HRM melting was subsequently performed first from 65^0C to 95^0C, $0{,}1^0$C /2 seconds to determine the melting range of each primer. Then the optimum melting ranges were used for HRM of each locus amplified.

Table 2.2 Primers for Y-STR Real-Time PCR prior to HRM analysis.

Y-STR locus	**PCR forward primer [F]**	**PCR reverse primer [R]**
DYS19	GACTACTGAGTTTCTGTTATAGTG	ATCTCTGCACCTGGAAATAG
DYS390	TATATTTTACACATTTTTGGGCC	TGACAGTAAAATGAACACATTGC
DYS391	CTATTCATTCAATCATACACCCA	GATTCTTTGTGGTGGGTCTG
DYS392	TCATTAATCTAGCTTTTAAAAACAA	AGACCCAGTTGATGCAATGT
DYS393	GTGGTCTTCTACTTGTGTCAATAC	AACTCAAGTCCAAAAAATGAGG
DYS437	GACTATGGGCGTGAGTGCAT	AGACCCTGTCATTCACAGATGA
DYS438	TGGGGAATAGTTGAACGGTAA	GTGGCAGACGCCTATAATCC
DYS439	TCCTGAATGGTACTTCCTAGGTTT	GCCTGGCTTGGAATTCTTTT
DYS448	TCTTCCTTAACGTGAATTTCCTC	TGTCAAAGAGCTTCAATGGAGA

2.2.3 HRM analysis

The PCR products generated with the BioRadSsoFAst ™ EvaGreen® Supermix for Real-Time PCR were subjected to HRM right after the reaction with the same instrument BioRad CFX96 Touch™ Real-Time PCR Detection System. HRM data were analyzed using Precision Melt Analysis™ software (BioRad). After HRM was done with BioRad CFX96 Touch™ Real-Time PCR the PCR reactions were transferred to single tubes and then again subjected to HRM with second instrument QIAGEN Rotor Gene 6000 and HRM data were analyzed with Rotor Gene Q series software (QIAGEN). And melting profiles of the samples were compared.

2.2.4 Sequencing

For further confirmation of genotyping by HRM of Y-STRs automated sequencing for selected PCR products were done in BGI Tech Solutions Co., Ltd. (BGI Tech) (http://bgitechsolutions.com/). The results were obtained online as ABI extension documents and analyzed by the SnapGene Version 2.6, chromatograph analyzing program.

CHAPTER 3

RESULTS

In this study 9 Y-STR loci were amplified with specific primers by real-time PCR and the amplified PCR products were subjected to HRM to discriminate male haplotypes. The DNA samples isolated from male donors were listed in table 3.1. The DNA's used in this study were isolated from buccal and blood cells with three different isolation methods. Comparison of these methods' purity is given in table 3.2.

3.1 ISOLATED DNA'S CONCENTRATION AND PURITY COMPARISON

Of the three methods used for DNA isolation Burç Genetik Company's isolated samples gave clearer results than MagJET Genomic DNA Kit that gave the second and NaOH method (Lareu, Puente et al. 2001) gave the least clean results. And the experiments continued with the cleaner samples from Burç Genetik and Magjet isolated DNAs.

Table 3.1 Sample names (ID stands for identifier), material DNA was isolated from and DNA concentrations

DNA Samples	DNA concentration (ng/µl)(blood)	DNA concentration (ng/µl)(buccal)
ID 66	-	49,5
ID 112	126,2	136,6
ID 4	79,7	75,8
ID 19	-	26,9
ID 52	-	48,3
ID 90	-	102,5
ID 113	-	75,5
ID 65	-	88,9
ID 108	-	82,9
ID 13	165,2	123,7
ID 5	145,1	34,5
ID 27	-	23,6
ID 6	-	88,1
ID 106	-	175
ID 92	85,1	-
ID 65	-	25
ID 48	-	55,4

Table 3.2 Purity comparisons (OD stands for optical density) of three different isolation methods , used in this thesis: NaOH, MagJET Genomic DNA Kit and isolation by Burç Genetik Company.

ID	OD 260/280			OD 260/230		
	NaOH	Burç Genetik	Magjet	NaOH	Burç Genetik	Magjet
4	1,01	1,85	1,95	0,22	2,01	1,97
5	1,15	1,84	1,87	0,26	1,88	1,46
13	1,14	1,82	1,96	0,25	1,76	1,21
112	1,27	1,86	1,82	0,28	1,73	1,45
OD Mean	1.14	1,84	1,9	0,25	1,84	1,52

3.2 GRADIENT PCR AND HRM OPTIMIZATION FOR EACH Y-STR SPECIFIC PRIMERS

After the gradient PCR, products were subjected to HRM, starting from 65^0C to 95^0C melt temperature range. The temperature which resulted with most clear sharpest peak was chosen as the optimum annealing temperature of the specific primer and the optimum melt temperature range was determined. Loci which gave multiple peaks in HRM graphs were excluded due to the complexity of comparison.

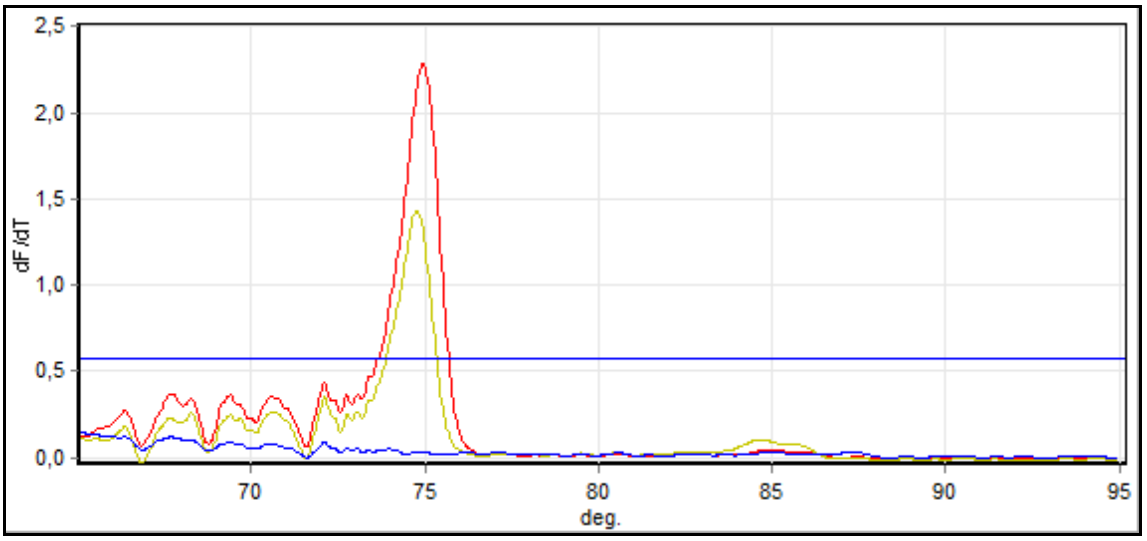

Figure 3.1 A HRM result performed after gradient PCR of the primers for Y-STR locus DYS 437:Starting from 65^0C to 95^0C. The Gradient PCR was performed with three different annealing temperatures $60,1^0$C (shown as red), $63,2^0$C (shown as yellow), $67,2^0$C (shown as blue); below, similar and above temperatures of the calculated Tm of the primers, respectively.

Table 3.3 Optimum annealing temperatures, number of peaks generated by HRM and HRM temperature ranges of Y-STR primers.

Y-STR primers Loci	**Optimum Annealing Temperature (^{0}C)**	**No of Peaks generated by HRM**	**HRM Temperature(^{0}C) Range**
DYS19	61,1	3	65-90
DYS390	55,7	1	67-83
DYS391	60,3	2	65-95
DYS392	58	2	67-83
DYS393	58,5	1	67-78
DYS437	60,1	1	65-85
DYS438	59,3	2	65-90
DYS439	61,4	1	67-82
DYS448	63,3	1	67-85

3.3 HRM PATTERNS OF DIFFERENT SAMPLES CARING DIFFERENT Y-STR HAPLOYTPES

For each Y-STR loci samples caring different alleles were chosen to subject HRM. After amplification step replicates or triplicates who gave closer quantification cycle (Cq: cycle number at which fluorescence has increased above background) values were chosen to further analyze for HRM patterns. Then HRM graphs were analyzed and Tm values were detected. Thirdly difference curves of HRM graphs were constructed with Precision Melt Analysis™ software (Bio-Rad).

3.3.1 DYS 437

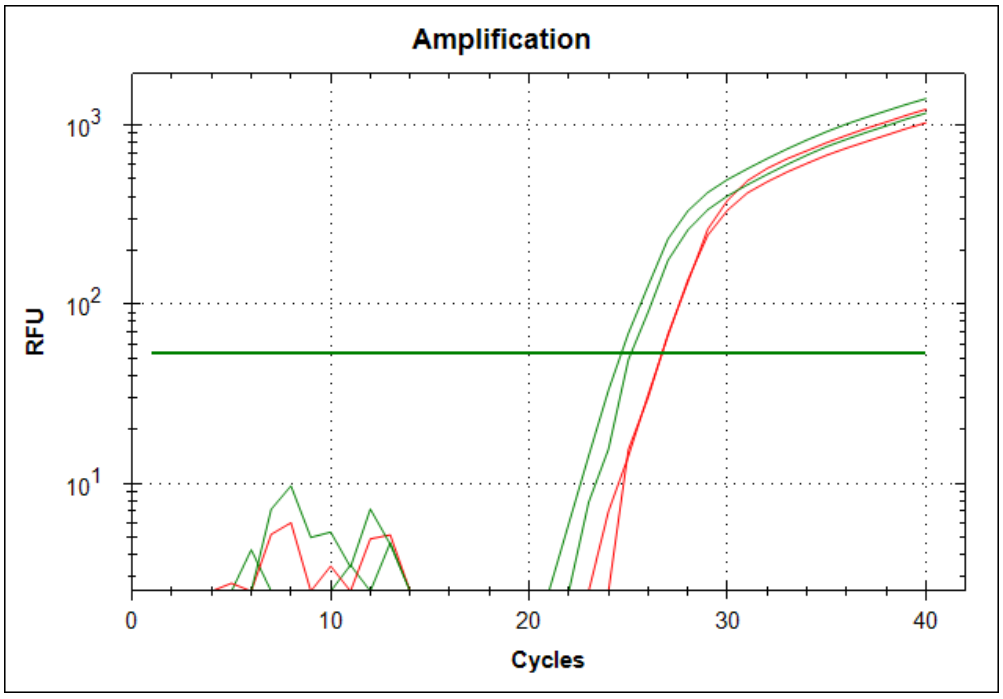

Figure 3.2 Amplification graph of Y-STR locus DYS 437 of alleles 15 (shown as green) and 16(shown as red) with replicates.

Table 3.4 The quantification summary of Y-STR locus DYS 437. (Cq stands for quantification cycle).

Allele	Cq	Cq Mean	Cq Std. Dev
15	24,56	24,83	0,382
15	25,10		
16	26,60	26,61	0,008
16	26,61		

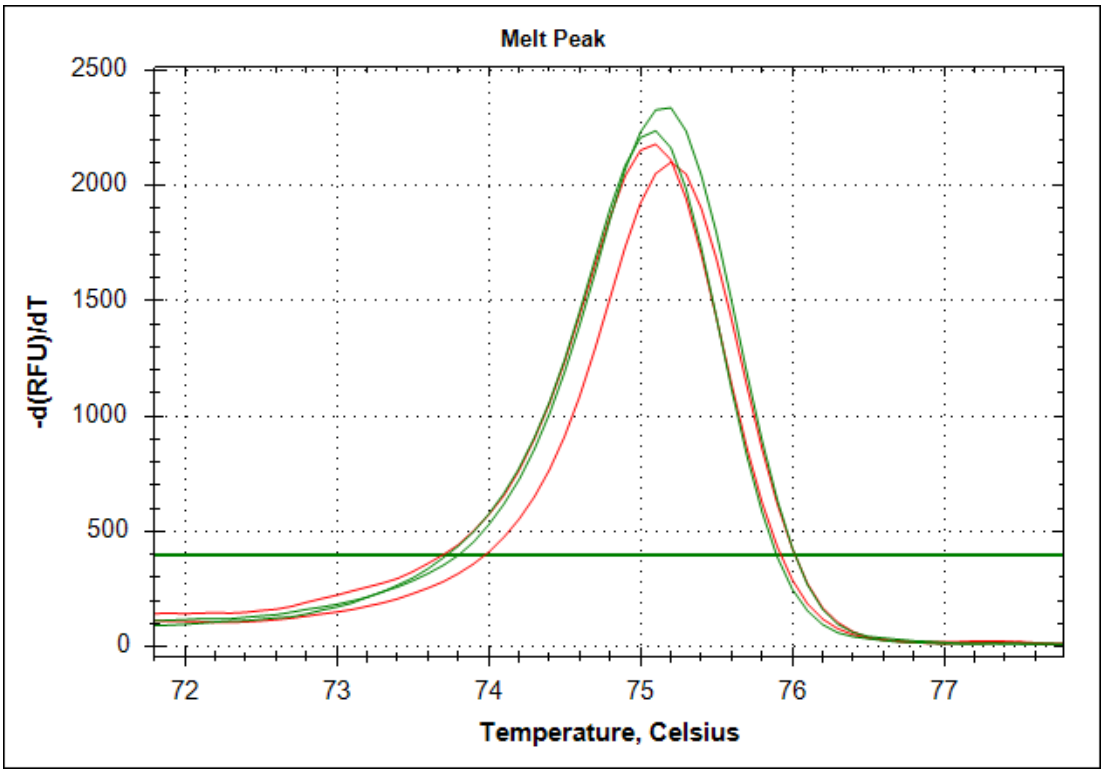

Figure 3.3 High resolution melting profile for Y-STR locus DYS437 of alleles 15 (shown as red) and 16(shown as green) with replicates. One of the replicates of each allele behaved same as the other allele.

Table 3.5 The HRM Curve summary of Y-STR locus DYS 437 (Tm stands for melt temperature).

Allele	Tm	Tm Mean	Tm St Dev.
15	75,20	75,15	0,0707107
15	75,10		
16	75,10	75,15	0,0707107
16	75,20		

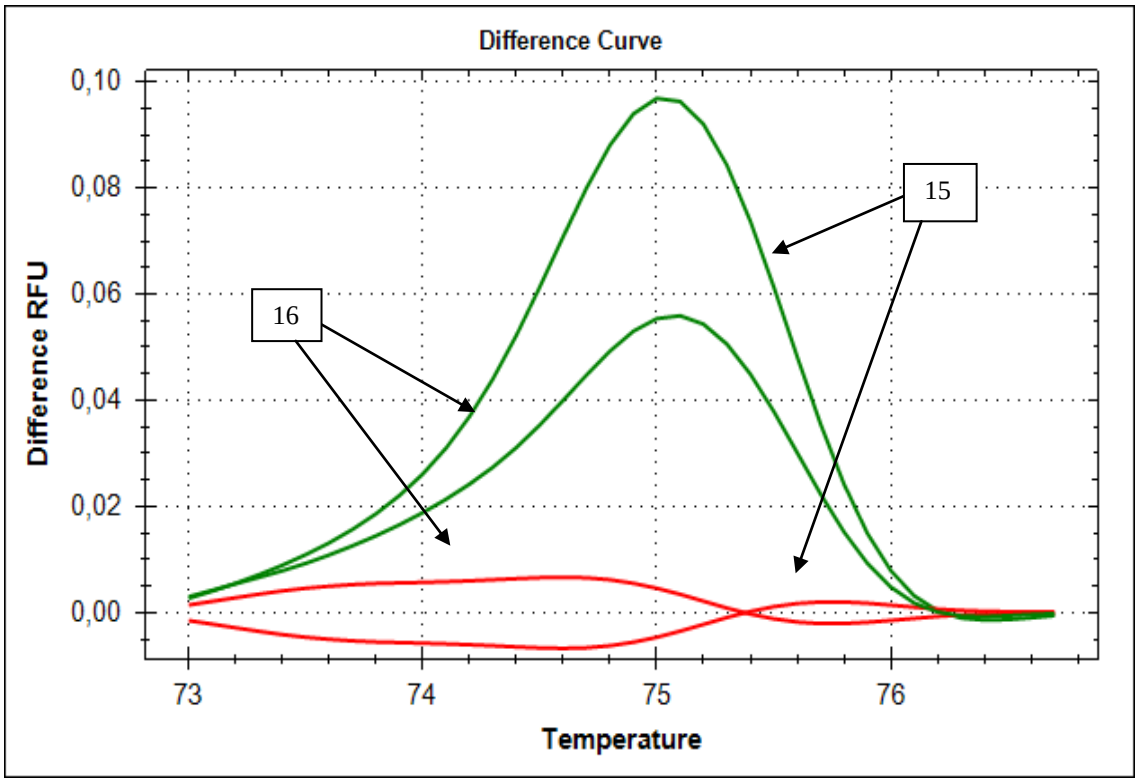

Figure 3.4 Difference curves of HRM profiles for Y-STR locus DYS437 constructed with precision melt analysis software. Allele clusters gave consistent results as in melt profiles; one of the replicates of allele 15 and 16 fit in the same cluster, 1 (red) and the other 2 (green). Reference cluster 1 (shown as red).

Table 3.6 Precision Melt results of Y-STR locus DYS 437.

Allele	Cluster	Cluster color	Percent Confidence
15	Cluster 1		96,8
16	Cluster 1		96,2
15	Cluster 2		86,6
16	Cluster 2		97,2

3.3.2 DYS 393

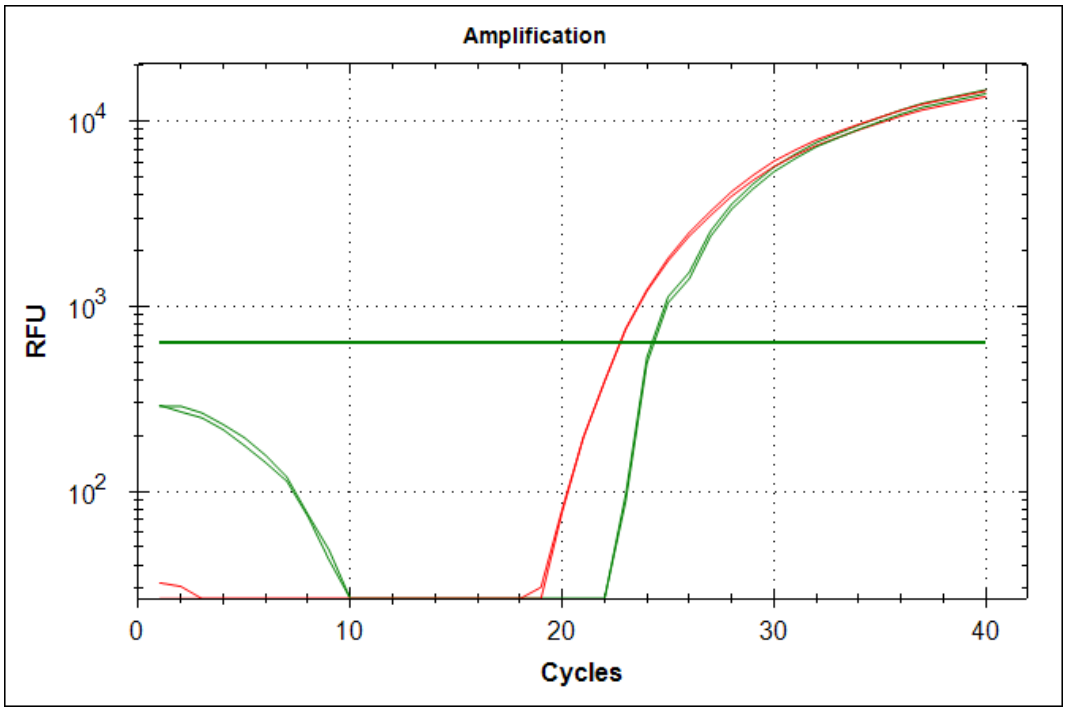

Figure 3.5 Amplification graph of Y-STR locus DYS 393 of alleles 12 (shown as green), 13 (shown as red) with replicates.

Table 3.7 The quantification summary of Y-STR locus DYS 393. (Cq stands for quantification cycle)

Allele	Cq	Cq Mean	Cq Std. Dev
12	24,26	24,22	0,0580365
12	24,18		
13	22,66	22,67	0,0120294
13	22,68		

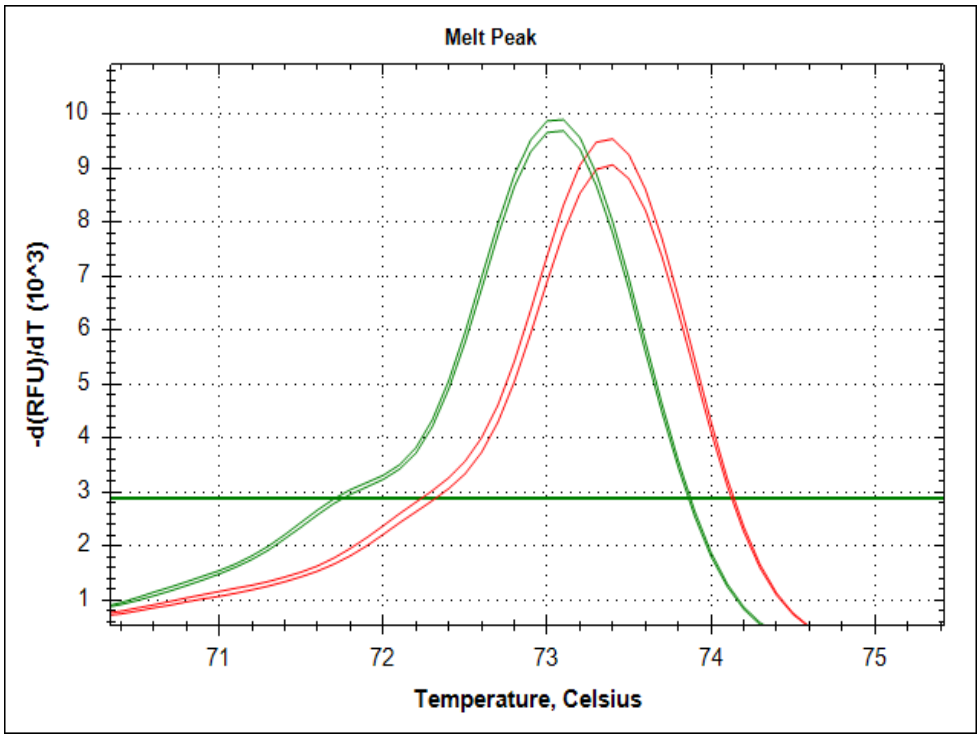

Figure 3.6 High resolution melting profile for Y-STR locus DYS 393 of alleles 12 (shown as green), 13 (shown as red) with replicates.

Table 3.8 The HRM Curve summary of Y-STR locus DYS 393. (Tm stands for melt temperature)

Allele	Tm	Tm Mean	Tm St. Dev.
12	73,10	73,10	0
12	73,10		
13	73,40	73,40	0
13	73,40		

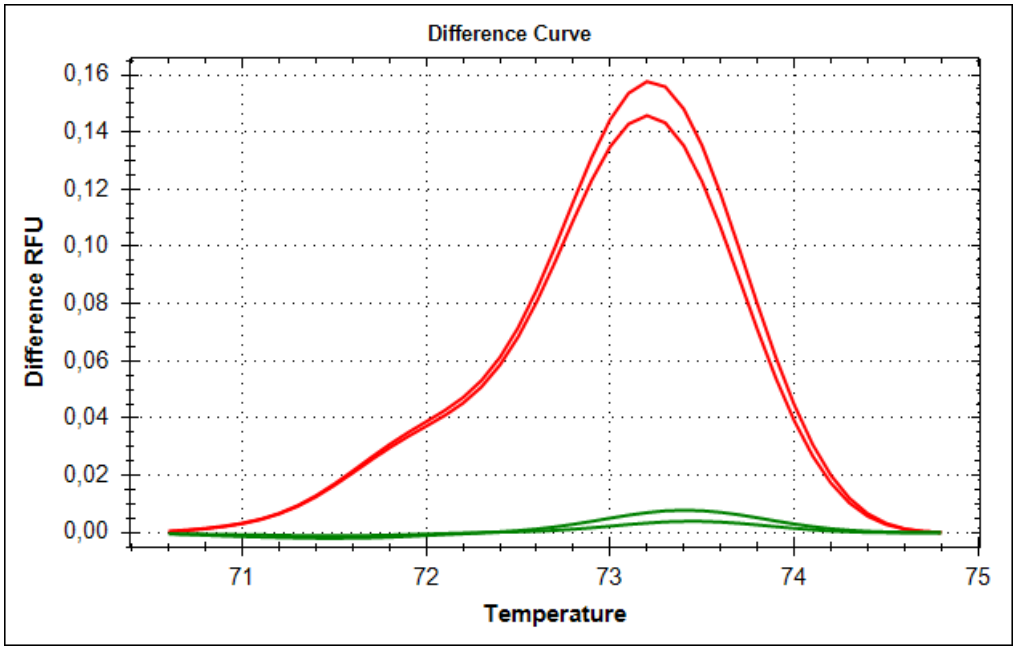

Figure 3.7 Difference curves of HRM profiles for Y-STR locus DYS 393 constructed with precision melt analysis software: Alleles were distinguished by their curve differences and replicates of the same alleles fit in the same cluster; 12 green, 13 red. Reference cluster 1 (shown as green).

Table 3.9 Precision Melt results of Y-STR locus DYS 393.

Allele	Cluster	Cluster Color	Percent Confidence
12	Cluster 1		99,3
12	Cluster 1		99,0
13	Cluster 2		99,8
13	Cluster 2		99,6

3.3.3 DYS 390

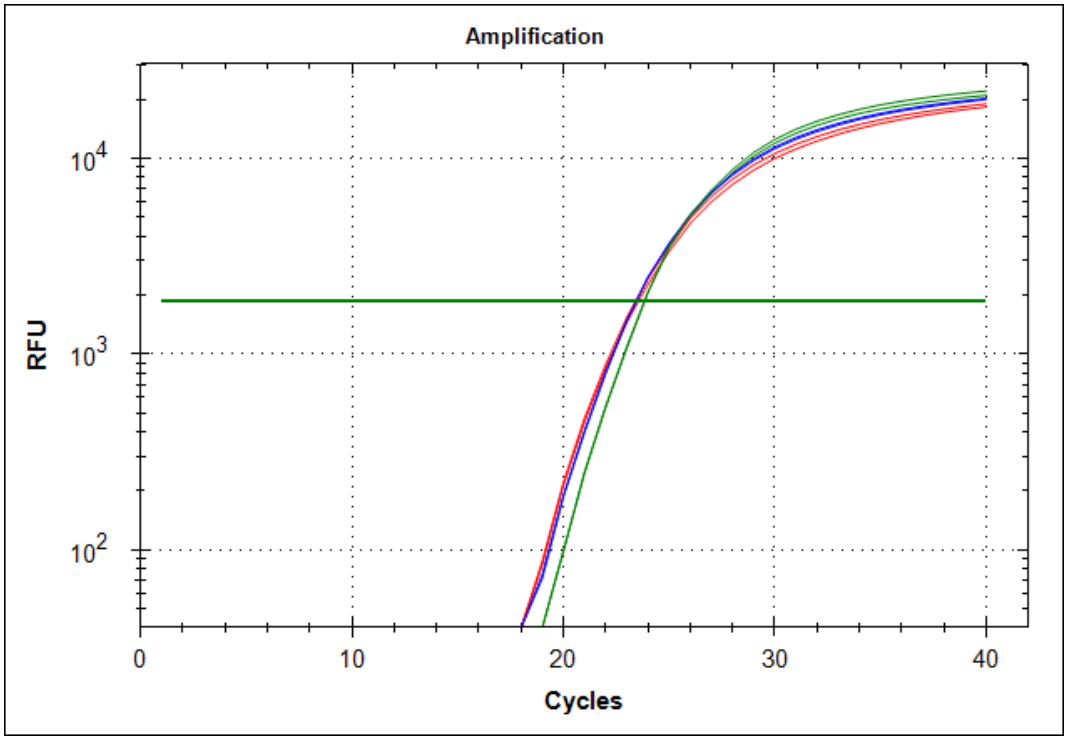

Figure 3.8 Amplification graph of Y-STR locus DYS 390 of alleles 22 (shown as red), 24 (green) and 26 (blue).

Table 3.10 The quantification summary of Y-STR locus DYS 390. (Cq stands for quantification cycle)

Allele	Cq	Cq Mean	Cq Std. Dev
24	23,79	23,79	0,000
24	23,79		
22	23,36	23,43	0,097
22	23,50		
26	23,39	23,385	0,004
26	23,38		

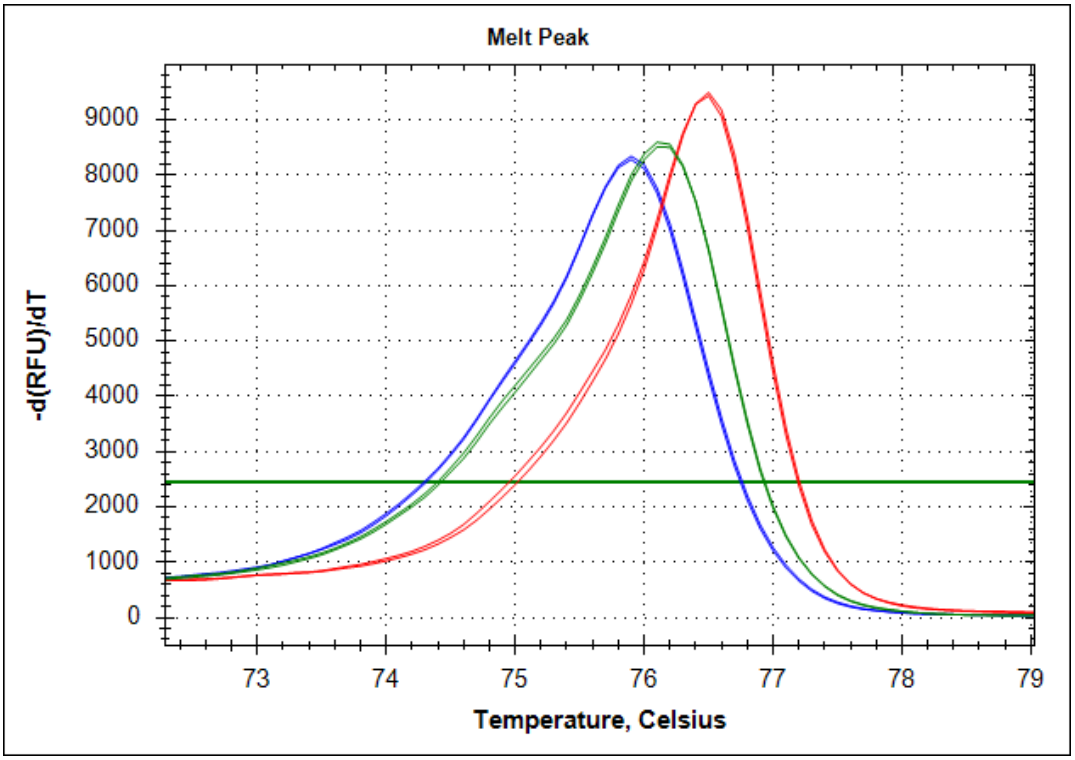

Figure 3.9 High resolution melting profile for Y-STR locus DYS390. Melt profiles of 3 genotypes with duplicates with 3 different melting patterns. Allele repeat numbers are 22 (shown as red), 24 (green) and 26 (blue).

Table 3.11 The HRM Curve summary of Y-STR locus DYS 390. (Tm stands for melt temperature)

Allele	Tm	Tm Mean	Tm St. Dev.
24	76,10	76,10	0
24	76,10		
22	76,50	76,50	0
22	76,50		
26	75,90	75,90	0
26	75,90		

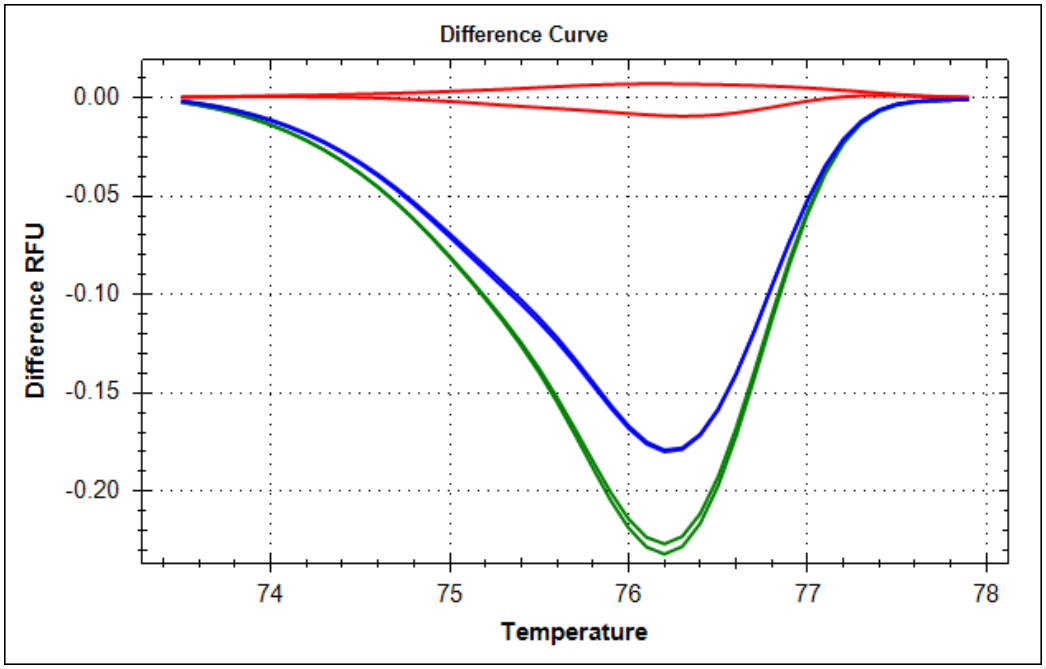

Figure 3.10 Difference curves of HRM profiles for Y-STR locus DYS 390 constructed with precision melt analysis software. Alleles could be distinguished by their curve differences and replicates of the same alleles fit in the same cluster; 22 red, 24 green and 26 blue. Reference cluster 1 (shown as red).

Table 3.12 Precision Melt results of Y-STR locus DYS 390.

Allele	Cluster	Cluster color	Percent Confidence
22	Cluster 1		92,0
22	Cluster 1		96,9
26	Cluster 2		90,7
26	Cluster 2		94,4
24	Cluster 3		72,1
24	Cluster 3		71,6

3.3.4 DYS 439

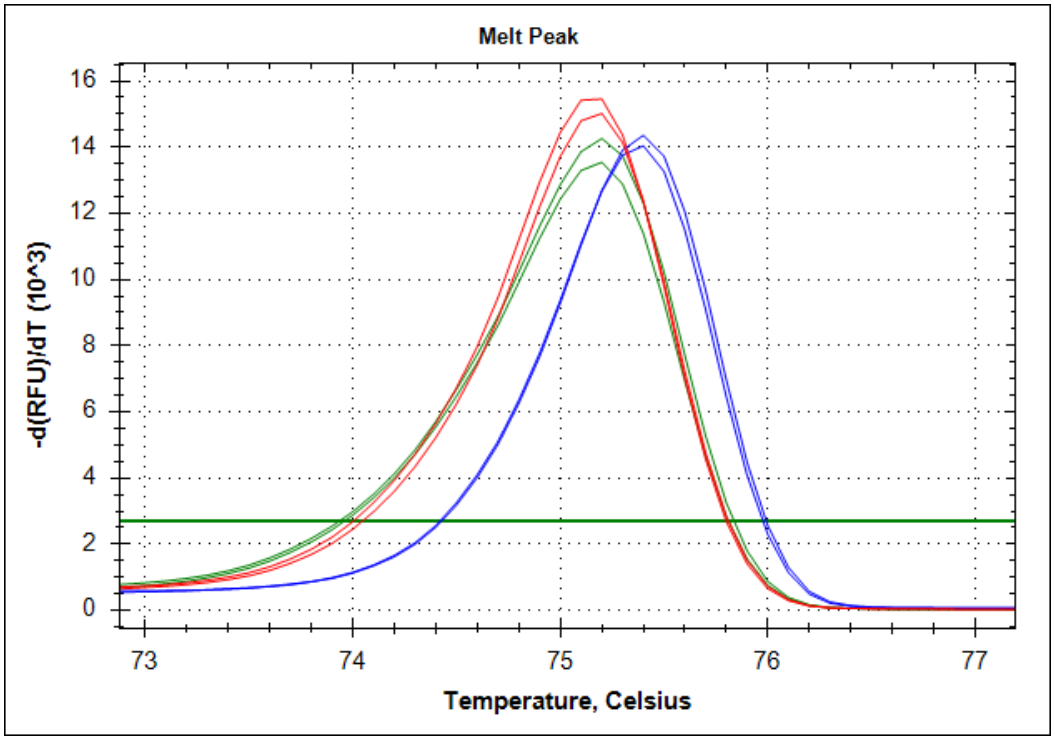

Figure 3.11 High resolution melting profile for Y-STR locus DYS439. Melt profiles of 3 genotypes with duplicates with 2 different melting patterns for locus DYS439. Allele repeat numbers 10 (shown as red), 11 (green) gave similar melting patterns whereas 12 (blue) gave different.

Table 3.13 The HRM Curve summary of Y-STR locus DYS 439. (Tm stands for melt temperature).

Allele	Melt Temp	Tm Mean	Tm St. Dev.
10	75,20	75,20	0
10	75,20		
11	75,20	75,20	0
11	75,20		
12	75,40	75,40	0
12	75,40		

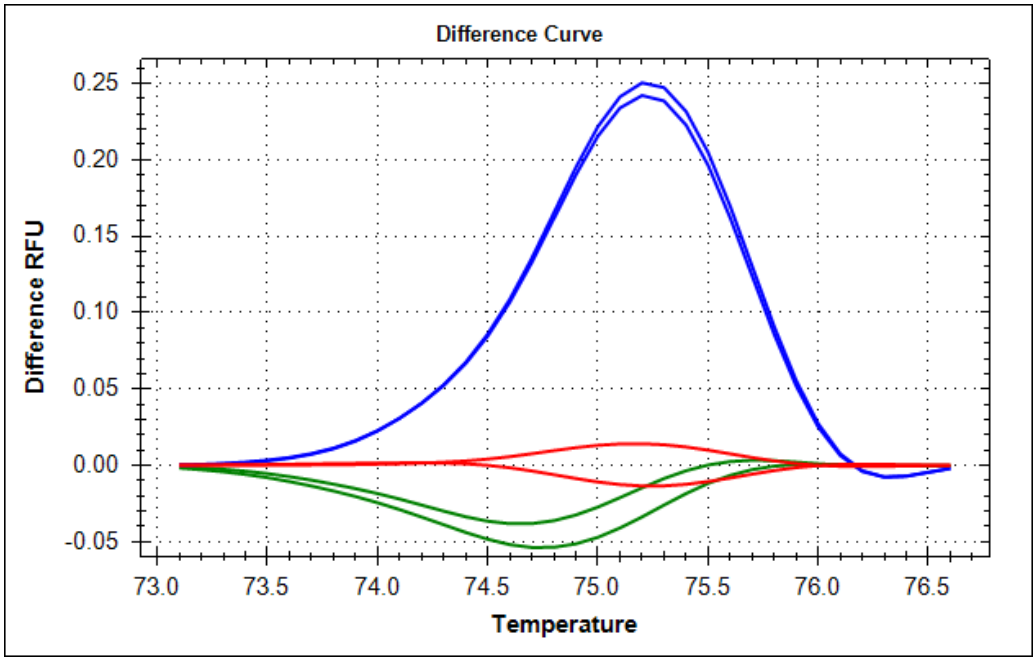

Figure 3.12 Difference curves of HRM profiles for Y-STR locus DYS 439 constructed with precision melt analysis software and all 3 alleles could be distinguished by their curve differences. And replicates of the same alleles fit in the same cluster; 10 red, 11 green and 12 blue. Reference cluster 1 (shown as red). Sequence results were consistent with fragment length results 4:10,13:11,112:12 repeats.

Table 3.14 Precision Melt results of Y-STR locus DYS 439.

Allele	Cluster	Cluster color	Percent Confidence
10	Cluster 1		90,5
10	Cluster 1		99,3
11	Cluster 2		99,9
11	Cluster 2		99,6
12	Cluster 3		99,3
12	Cluster 3		99,1

3.3.5 DYS 448

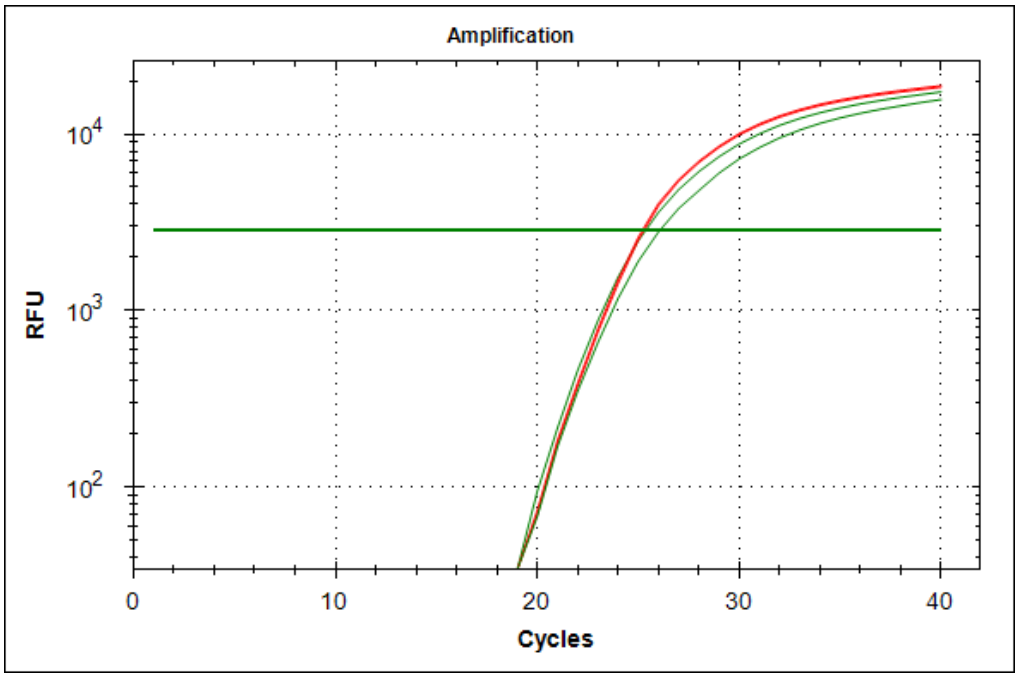

Figure 3.13 Amplification graph of Y-STR locus DYS 448 of alleles 19 (shown as red), 20 (green) with replicates.

Table 3.15 The quantification summary of Y-STR locus DYS 448 (Cq stands for quantification cycle).

Allele	Cq	Cq mean	Cq St. Dev.
19	25,18	25,21	0,052047209
19	25,25		
20	25,33	25,70	0,519292669
20	26,07		

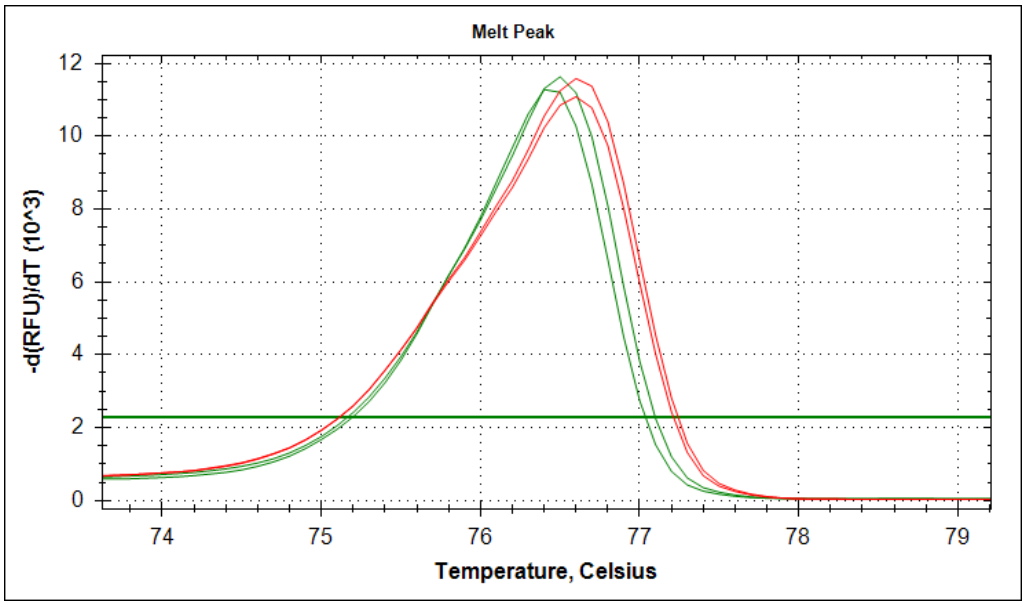

Figure 3.14 High resolution melting profile for Y-STR locus DYS448 Melt profiles of 2 genotypes with duplicates with 2 different melting patterns for locus DYS448. Allele repeat numbers are 19 (shown as red), 20 (green) very close melting patterns.

Table 3.16 The HRM Curve summary of Y-STR locus DYS 448 (Tm stands for melt temperature).

Allele	Tm	Tm Mean	Tm St. Dev.
19	76,60	76,6	0
19	76,60		
20	76,50	76,45	0,0707107
20	76,40		

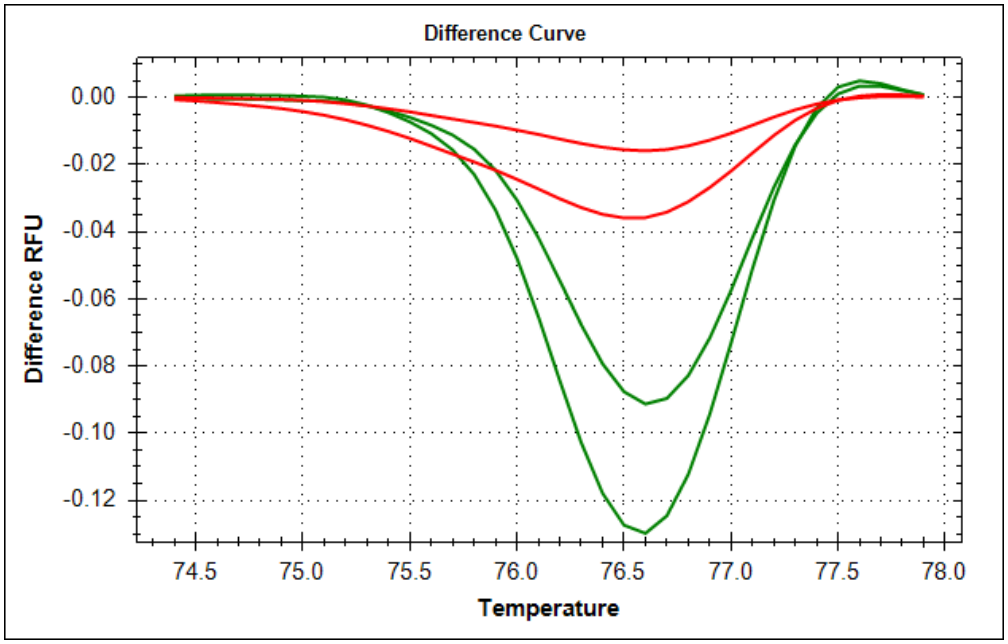

Figure 3.15 Difference curves of HRM profiles for Y-STR locus 448 constructed with precision melt analysis software and all 2 alleles could be distinguished by their curve differences and and replicates of the same alleles fit in the same cluster; 19 red, 20 green. Reference cluster 1(shown as red).

Table 3.17 Precision Melt results of Y-STR locus DYS 448.

Allele	Cluster	Cluster color	Percent Confidence
19	Cluster 1		98,2
19	Cluster 1		97,9
20	Cluster 2		98,2
20	Cluster 2		98,5

3.4 INSTRUMENT AND SOFTWARE COMPARISON

The HRM experiments performed with the device BIORAD CFX96 were also repeated with Rotor-Gene 6000. Figures 3.15 and 3.16 and tables 3.18 and 3.19 shows the results obtained from the instrument Rotor-Gene 6000. To generate difference curves with Rotor-Gene Q software first one sample has to be defined as a reference genotype. In figure 3.16 the reference genotypes were set as the first of the replicates of the alleles 15 and 16. Then the software calls the samples according to their curve differences to one genotype.

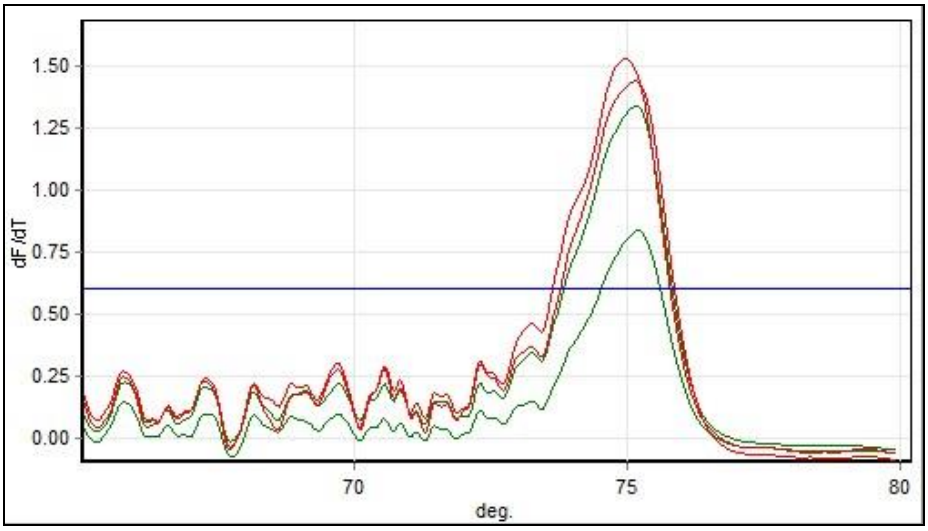

Figure 3.16 High resolution melting profile for Y-STR locus DYS437 of alleles 15 (shown as green) and 16(shown as red) with replicates.

Table 3.18 The HRM Curve summary of Y-STR locus DYS 437(Tm stands for melt temperature).

Allele	Tm	Tm Mean	Tm St. Dev.
16	75,22	75,20	0.028284
16	75,18		
15	74,98	75,07	0.13435
15	75,17		

Table 3.19 The comparison of Tm's of Y-STR locus DYS 437 alleles 15 and 16 generated by BIORAD CFX96 and Rotor-Gene 6000 instruments. (Tm stands for melt temperature ^{0}C).

Allele	Tm (BioRad)	Tm (Rotor Gene)	Tm Difference	Tm St. Dev.
16	75.20	75.22	0.02	0.014
16	75.10	75.18	0.08	0.056
15	75.10	74.98	0.12	0.084
15	75.20	75.17	0.03	0.021

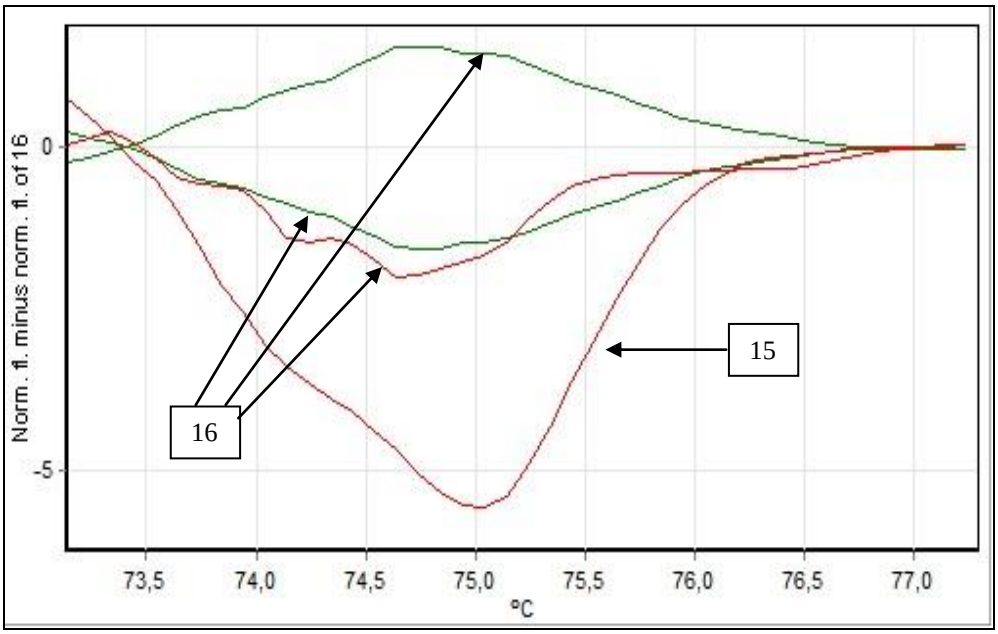

Figure 3.17 Difference curves of HRM profiles for Y-STR locus 448 constructed with Rotor-Gene Q series software and one of the replicates of allele 15 (shown as red) was called as allele 16(shown as green) by the software according to the curve differences.

Table 3.20 Precision Melt results of Y-STR locus DYS 439.

Genotype	Color	Confidence %
16		97,2
15		96,01
16		97,2
16		96,29

3.5 SEQUENCING

HRM results of DYS 437 and 439 Y-STR loci were further confirmed by sequencing. The PCR products of samples carrying 15 and 16 repeats for DYS 437 and samples carrying 10, 11 and 12 repeats for DYS 439 were sequenced by BGI Tech Solutions Co., Ltd. (BGI Tech) (http://bgitechsolutions.com/). The results were obtained online as ABI extension documents and analyzed by the SnapGene Version 2.6, chromatograph analyzing program.

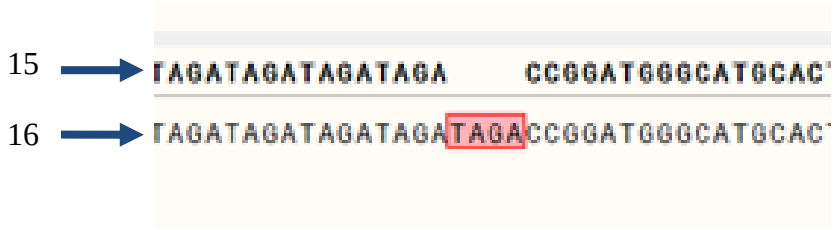

Figure 3.18 Sequence comparison of the samples carrying 15 and 16 allele repeats of the Y-STR locus DYS 437. The red boxes showing the one more TAGA repeat in allele 16.

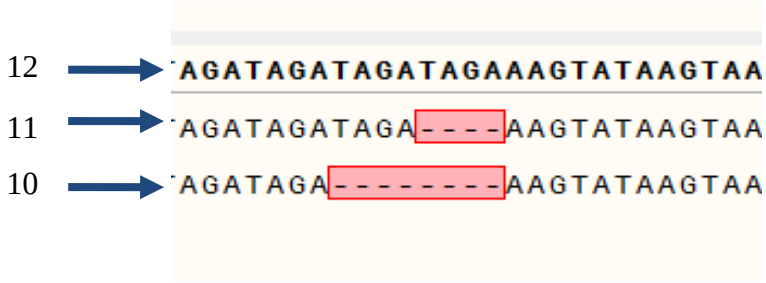

Figure 3.19 Sequence comparison of the samples carrying 10, 11 and 12 allele repeats of the Y-STR locus DYS 439. The red boxes showing the lack of TAGA repeats in alleles 11 and 10 each short line representing one nucleotide.

CHAPTER 4

DISCUSSION & CONCLUSION

4.1 DISCUSSION

In this study it is aimed to develop Y-STR HRM assays which can be used as a screening tool. Similar researches have been done on autosomal STRs and successful results were obtained with the loci: CSF1PO, vWA, D18S51, THO1, TPOX, and D3S1358. (Halpern and Ballantyne 2009; Nguyen 2012; Nicklas, Noreault-Conti et al. 2012). Therefore specific Y-STRs primers were selected from an outstanding article (Goedbloed, Vermeulen et al. 2009) and their HRM analyses were done following real-time PCR amplification. After the HRM analysis the Y-STR loci DYS437, DYS 393, DYS 390, DYS 439 and DYS 448, whose PCR products gave single melt peaks were selected for further screening.

For collection of the samples buccal cells and whole blood were used and DNA isolation was done with three different methods. The NaOH method (Wang, Fan et al. 1998), gave relatively impure results with mean OD 260/280 1.14, indicating RNA and protein contamination and OD 260/230 0.25, indicating phenol, salt, protein, and polysaccharide contamination. The other two isolation methods gave similar results in OD 260/280 1.84 and 1.9, Burç Genetik Company and Thermoscientific MagJET Genomic DNA Kit respectively. For OD 260/230 Burç Genetik isolation results were clearer with average ratio 1,.4 within the accepted range of 1.8-2.2 whereas Thermoscientific MagJET Genomic DNA Kit resulted 1.5 (Glasel 1995).

Reed et. al. recommends that all the samples should be prepared with the same method and added to the reaction with the same amount to obtain a better result in HRM analysis (Reed, Kent et al. 2007). In this study all the samples were added to the reaction with the same amount but isolated with different methods as described above.

The relatively impure samples (isolated by the NaOH method) resulted with more than 0.5 cycle difference between Cq values of the two replicates used in a reaction. The cause of this difference can be due to the impurities in the isolated sample DNAs which makes it harder to add the same amount of DNA in every replicate. When other relatively pure samples isolated by Thermoscientific MagJET Genomic DNA Kit or by the company BURÇ GENETİK were used, this much difference in Cq values of the replicates were not observed. Therefore the experiments were continued with the sample DNAs isolated by both Burç Genetik Company and Thermoscientific MagJET Genomic DNA Kit.

According to Reed and Wittwer, the number of the repeat in the allele is positively proportional to the Tm value. Since every additional repeat means more hydrogen bonds, higher energy is needed to separate an allele with more repeats (Reed and Wittwer 2004).

Two genotypes for locus DYS437 allele with repeat number 15 and 16 were screened. Alleles 15 and 16 could not be distinguished properly since one replicate of allele 15 gave the same pattern with allele 16 and the other replicates behaved the same in melt peak curve analysis. When constructed the difference curves of samples with the help of precision melt analysis software all two alleles clusters gave consistent results as in melt profiles. After obtaining this unexpected result the samples were also sequenced by means Sanger DNA Sequencing Technique in a private company (BGI) to see if the samples chosen to screen their HRM profiles as having repeat number 15 and 16 from the fragment analysis results done before were true 15 and 16. Sequence results were consistent with the fragment analysis results and concluded that HRM cannot distinguish all samples sufficiently for this particular locus DYS 437. Further analysis should be done by increasing the sample numbers carrying other repeat number seen on this locus, such as; 13, 14 and 17. The same experiment repeated in another instrument Rotor-Gene™ 6000 gave more distinguished patterns of all the alleles but when the results comes to allele differentiation they were consistent with the device Bio-Rad CFX96 Touch™ Real-Time PCR Detection System.

In the locus DYS393, High resolution melting profile of 2 genotypes with repeat numbers 12 and 13 were screened. Both the melt curves and the difference curves were discriminative in this locus. Thus this locus can be used for HRM screening assays of

individuals but these results may be supported by additional samples with different repeat numbers, since this locus has an allele range of 8-16.

The third locus (DYS 390) analysed gave the most distinguishable melting profiles for the different alleles, 22, 24 and 26 and the difference curves were consistent with the melt profiles. The easily separated and clear results gave insight that this locus can be good to be used in HRM screening but these clear results could be because of the two repeat-number long differences between the samples. The power of discernment of the analysis for this locus can be increased by using alleles which have one repeat difference between each other, which in this study was not available.

3 haplotypes (10, 11 and 12 repeat numbers) of HRM profile for Y-STR locus DYS 439 were screened. Although melting curve results didn't give clear distinguishable results for the alleles of 10 and 11, the difference curves of samples constructed with *precision melt analysis software* clustered all three alleles separately. Even though the alleles 10 and 11 were appeared to be as the same allele in melt curve results, the sequence results verified that they are different alleles with 10 and 11 repeat numbers.

The last locus analysed was DYS 448 with 2 haplotype with 19 and 20 allele repeat numbers. According to melt profiles, these two alleles could be distinguished with both Tm and melt profile difference. And the difference curves of those alleles could distinguish them and replicates of the same alleles fit in the same cluster. In 2012, Nguyen (Nguyen 2012) and Nicklas et. al. (Nicklas, Noreault-Conti et al. 2012) gained successful results for discriminating autosomal STRs by HRM analysis. According to these results different genotypes of the STRs CSF1PO, THO1, vWA and D18S51, could be successfully differentiated. Therefore, also in our research we obtained supporting data to these previous studies. As mentioned above, especially in the loci DYS 390, DYS 393 and DYS 448 quite successful data has been observed.

For product sizes it is advised to use small sizes of PCR products to be able to separate heterozygous single nucleotide polymorphisms, but for homozygous alleles it was found that long products give better differentiating melt curves (Sajantila 1998). For Y-STRs no difference in melting profiles with differing product lengths were reported. Here in this study the Y-STR loci amplified were lower than 250bp. To have a

conclusion that the length affects the distinguishing properties of melting patters for Y-STRs, additional Y-STR loci with longer sizes should also be tested. But longer product size in HRM analysis has the restriction that longer product sequences might comprise other polymorphisms around the repeat region which affects the melt character and results in more than one melting domain (Corbett 2006).

In this study, real-time PCR and following HRM experiments were performed mostly by Bio-Rad CFX96 device. To test reproducibility of this assay some of the HRM experiments were repeated with Rotor-Gene 6000 by transferring the PCR products of the experiments done with Bio-Rad CFX96 and HRM were repeated with the same conditions. The results were consistent between the two instruments, except some minor differences such as shown in figure 3.15 for DYS 437 locus, one of the replicates of allele 15 which had the highest difference between melt temperatures generated; 0.12^0C with 0.084 st.dev. And for the difference curve Rotor-Gene Q series software called one of the allele 16 as of allele 15. This difference might be due to the volume loss during the transfer of the PCR products to the tubes suitable for the Rotor-Gene 6000 instrument from the Bio-Rad CFX96 plates. There is no data reported showing that volume differences can cause melting shifts but the impurities of the sample affect the melt characteristics such that increased salt concentration shifts melt peaks to the right and increased ethanol concentration shifts the peaks to the left (Corach, Filgueira Risso et al. 2001; Rolf H.A.M. Vossen 2009; QIAGEN 2013-2015). Since this experiment was performed with samples isolated by the relatively dirty NaOH method the samples' DNA homogeneity was questionable and salt contamination was high.

In addition to the consistent results of the two instruments used in this study, they were different in various aspects such as; speed, resolution and thermal gradient function. Rotor-Gene 6000 could measure even the smallest amplification of the samples with very little amount of template DNA added such as 0,5ng/µl final concentration. Compared to Rotor-Gene 6000, for Bio-Rad CFX96 to capture any change in amplification higher amounts of DNA template was needed. In overall speeds of the devices comprising PCR and HRM reactions totally there weren't any significant difference. But according to the duration of the annealing, Bio-Rad CFX96 is faster than Rotor-Gene 6000, with duration times 5 and 30 seconds, respectively. And the duration

of HRM with Rotor-Gene 6000 (20 minutes) is faster than that of Bio-Rad CFX96 (40 minutes). One other advantage of Bio-Rad CFX96 over Rotor-Gene 6000 and many other devices of other brands is it has thermal gradient function. On the other hand, Bio-Rad CFX96 has thermal gradient function whereas Rotor-Gene 6000 and many other devices of other brands do not. Lastly when the two softwares of these two devices are compared, Bio-Rad Precision Melt Analysis™ can cluster the unknown samples and call automatically the haplotype/genotypes but Rotor-Gene Q series system does cluster the samples manually by determining a genotype as the control and if there are samples with same names, the sample with smaller number in the list should be manually selected as controls, shows that Bio-Rad Precision Melt Analysis is much more user friendly than that of Rotor-Gene Q.

4.2 CONCLUSION

In conclusion, of the 5 Y-STR loci screened; except DYS 437, different alleles of DYS393, 390,439, 448 could be distinguished by HRM analysis. From these 4 loci DYS 393 gives the most sufficient power to make a useful screening assay.

In the beginning of this study, nine commonly used Y-STR loci were selected for HRM screening and Y-STR loci DYS 19, 391, 392 and 438 were excluded from the study, because their HRM graphs resulted with more than one melt peak. Multiple melting peaks indicated that they carry multiple melting domains which generate complexity of allele determination.

To conclude, the four loci: DYS393, 390,439 and 448, can be added to the list of STRs that had been studied for HRM screening: vWA, D18S51, THO1 and CSF1PO, as sex determining loci.

REFERENCES

Andreasson, H., Asp, A., et al.,"Mitochondrial sequence analysis for forensic identification using pyrosequencing technology", Biotechniques. Vol. 32 pp. 124-126, 128, 130-123, 2002.

Ayub, Q., Mohyuddin, A., et al.,"Identification and characterisation of novel human Y-chromosomal microsatellites from sequence database information", Nucleic Acids Res. Vol. 28 pp. e8, 2000.

Britten, R. J. and Kohne, D. E.,"Repeated sequences in DNA. Hundreds of thousands of copies of DNA sequences have been incorporated into the genomes of higher organisms", Science. Vol. 161 pp. 529-540, 1968.

Brookes, A. J.,"The essence of SNPs", Gene. Vol. 234 pp. 177-186, 1999.

Butler, J. M.,"Recent developments in Y-single tandem repeat and Y-single nucleotide polymorphism analysis", Forensic Sci Rev Vol. 15 pp. 2003.

Butler, J. M. Forensic DNA typing : biology, technology, and genetics of STR markers. Amsterdam ; Boston, Elsevier Academic Press.(2005)

Butler, J. M., Schoske, R., Vallone, P. M., Kline, M. C., Redd, A. J., & Hammer, M. F.,"A novel multiplex for simultaneous amplification of 20 Y chromosome STR markers", Forensic Science International. Vol. 129 pp. 10-24, 2002.

Cann, R. L., Stoneking, M., et al.,"Mitochondrial-DNA and Human-Evolution", Nature. Vol. 325 pp. 31-36, 1987.

Chambers, G. K. and MacAvoy, E. S.,"Microsatellites: consensus and controversy", Comp Biochem Physiol B Biochem Mol Biol. Vol. 126 pp. 455-476, 2000.

Collins, J. R., Stephens, R. M., et al.,"An exhaustive DNA micro-satellite map of the human genome using high performance computing", Genomics. Vol. 82 pp. 10-19, 2003.

Corach, D., Filgueira Risso, L., et al.,"Routine Y-STR typing in forensic casework", Forensic Sci Int. Vol. 118 pp. 131-135, 2001.

Corbett, R.,"HRM Assay Design and Analysis Corprotocol 6000". Vol. pp. 1-24, 2006.

Daniel M. Bornman, M. E. H., Jared M. Schuetter,"Short-read, high-throughput sequencing technology for STR genotyping", BioTechniques, The International Journal of Lifescience Methods. Vol. pp. 1-6, 2012.

de Knijff, P., Kayser, M., et al.,"Chromosome Y microsatellites: population genetic and evolutionary aspects", Int J Legal Med. Vol. 110 pp. 134-149, 1997.

Edwards, A., Civitello, A., et al.,"DNA typing and genetic mapping with trimeric and tetrameric tandem repeats", Am J Hum Genet. Vol. 49 pp. 746-756, 1991.

Ellegren, H.,"Microsatellites: simple sequences with complex evolution", Nat Rev Genet. Vol. 5 pp. 435-445, 2004.

Fan, H. and Chu, J. Y.,"A brief review of short tandem repeat mutation", Genomics Proteomics Bioinformatics. Vol. 5 pp. 7-14, 2007.

Glasel, J. A.,"Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios", Biotechniques. Vol. 18 pp. 62-63, 1995.

Goedbloed, M., Vermeulen, M., et al.,"Comprehensive mutation analysis of 17 Y-chromosomal short tandem repeat polymorphisms included in the AmpFlSTR Yfiler PCR amplification kit", Int J Legal Med. Vol. 123 pp. 471-482, 2009.

Halpern, M. D. and Ballantyne, J.,"A single nucleotide polymorphism melt curve assay employing an intercalating dye probe fluorescence resonance energy transfer for forensic analysis", Anal Biochem. Vol. 391 pp. 1-10, 2009.

Hammond, H. A., Jin, L., et al.,"Evaluation of 13 short tandem repeat loci for use in personal identification applications", Am J Hum Genet. Vol. 55 pp. 175-189, 1994.

Karabulut, D. S., Optimizations in SNP Detection Using Real-Time PCR High Resolution Melting Analysis, M.S. Thesis, Fatih University, 2012.

Kasai, K., Nakamura, Y., et al.,"Amplification of a variable number of tandem repeats (VNTR) locus (pMCT118) by the polymerase chain reaction (PCR) and its application to forensic science", J Forensic Sci. Vol. 35 pp. 1196-1200, 1990.

Kayser, M., Caglia, A., et al.,"Evaluation of Y-chromosomal STRs: a multicenter study", Int J Legal Med. Vol. 110 pp. 125-133, 141-129, 1997.

Kong, A., Gudbjartsson, D. F., et al.,"A high-resolution recombination map of the human genome", Nat Genet. Vol. 31 pp. 241-247, 2002.

Kopelman, N. M., Stone, L., et al.,"Genomic microsatellites identify shared Jewish ancestry intermediate between Middle Eastern and European populations", BMC Genet. Vol. 10 pp. 80, 2009.

Lareu, M., Puente, J., et al.,"The use of the LightCycler for the detection of Y chromosome SNPs", Forensic Sci Int. Vol. 118 pp. 163-168, 2001.

Lee, H. C., Ladd, C., et al.,"DNA typing in forensic science. I. Theory and background", Am J Forensic Med Pathol. Vol. 15 pp. 269-282, 1994.

Li, Y. D., Chu, Z. Z., et al.,"A cost-effective high-resolution melting approach using the EvaGreen dye for DNA polymorphism detection and genotyping in plants", J Integr Plant Biol. Vol. 52 pp. 1036-1042, 2010.

Litt, M. and Luty, J. A.,"A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene", Am J Hum Genet. Vol. 44 pp. 397-401, 1989..

Nguyen, Q., McKinney, J., Johnson, D. J., Roberts, K. A. and Hardy, W. R. ,"STR Melting Curve Analysis as a Genetic Screening Tool for Crime Scene Samples.", Journal of Forensic Sciences. Vol. 57 pp. 887–899, 2012.

Nicklas, J. A., Noreault-Conti, T., et al.,"Development of a fast, simple profiling method for sample screening using high resolution melting (HRM) of STRs", J Forensic Sci. Vol. 57 pp. 478-488, 2012.

Primrose, S. B. Principles of genome analysis : a guide to mapping and sequencing DNA from different organisms. Oxford ; Malden, MA, Blackwell Science.(1998)

Puers, C., Hammond, H. A., et al.,"Identification of repeat sequence heterogeneity at the polymorphic short tandem repeat locus HUMTH01[AATG]n and reassignment of alleles in population analysis by using a locus-specific allelic ladder", Am J Hum Genet. Vol. 53 pp. 953-958, 1993.

QIAGEN "Critical Success Factors for HRM Performance.". from ttp://www.qiagen.com/tr/resources/technologies/hrm/critical%20success%20factors%20for%20hrm%20performance/.(2013-2015).

Reed, G. H., Kent, J. O., et al.,"High-resolution DNA melting analysis for simple and efficient molecular diagnostics", Pharmacogenomics. Vol. 8 pp. 597-608, 2007.

Reed, G. H. and Wittwer, C. T.,"Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis", Clin Chem. Vol. 50 pp. 1748-1754, 2004.

Roewer, L., Croucher, P. J. P., et al.,"Signature of recent historical events in the European Y-chromosomal STR haplotype distribution", Human Genetics. Vol. 116 pp. 279-291, 2005.

Roewer, L. and Epplen, J. T.,"Rapid and sensitive typing of forensic stains by PCR amplification of polymorphic simple repeat sequences in case work", Forensic Sci Int. Vol. 53 pp. 163-171, 1992.

Roewer L.,"Y chromosome STR typing in crime casework", Forensic Science, Medicine, and Pathology. Vol. 5 pp. 77-84, 2009.

Rolf H.A.M. Vossen, E. A., Anja Roos, Johan T. den Dunnen,"High-Resolution Melting Analysis (HRMA)—More Than Just Sequence Variant Screening", Human Mutation. Vol. 30 pp. 860-866, 2009.

Sajantila, A. Second European Symposium on Human Identification. Madison, Wisconsin, Promega Corporation.(1998)

Sibille, I., Duverneuil, C., et al.,"Y-STR DNA amplification as biological evidence in sexually assaulted female victims with no cytological detection of spermatozoa", Forensic Science International. Vol. 125 pp. 212-216, 2002.

Sullivan, K. M., Hopgood, R., et al.,"Automated Amplification and Sequencing of Human Mitochondrial-DNA", Electrophoresis. Vol. 12 pp. 17-21, 1991.

Tautz, D.,"Notes on the definition and nomenclature of tandemly repetitive DNA sequences", EXS. Vol. 67 pp. 21-28, 1993.

Urquhart, A., Kimpton, C. P., et al.,"Variation in short tandem repeat sequences--a survey of twelve microsatellite loci for use as forensic identification markers", Int J Legal Med. Vol. 107 pp. 13-20, 1994.

Vallone, P. M., Hill, C. R., & Butler, J. M.,,"Demonstration of rapid multiplex PCR amplification involving 16 genetic loci", Forensic Science International: Genetics. Vol. 3 pp. 42-45, 2008.

Wang, D. G., Fan, J. B., et al.,"Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome", Science. Vol. 280 pp. 1077-1082, 1998.

Wittwer, C. T., Herrmann, M. G., et al.,"Continuous fluorescence monitoring of rapid cycle DNA amplification", Biotechniques. Vol. 22 pp. 130-131, 134-138, 1997.

Yücebilgili, K., The allele and haplotype analysis of 17 Y-STR loci on human populatıon of Turkey, M.S. Thesis, Fatih University, 2011.

APPENDIX A

GACTATGGGCGTGAGTGCATGCCCATCCGGTCTATCTATCTATCTATCTATC
TATCTATCTATCTATCTATCTGTCTGTCTATCTATCTATCTATCATCTGTGAATGAT
GTCTATCTACTTATCTATGAATGATATTTATCTGTGGTTATCTATCTATCTAT
ATCATCTGTGAATGACAGGGTCTC

15 repeats 181 bp (TCTA) 9 (TCTG)2 (TCTA)4

a)

GACTATGGGCGTGAGTGCATGCCCATCCGGTCTATCTATCTATCTATCTATC
TATCTATCTATCTATCTATCTGTCTGTCTATCTATCTATCTATCATCTGTGAA
TGATGTCTATCTACTTATCTATGAATGATATTTATCTGTGGTTATCTATCTAT
CTATATCATCTGTGAATGACAGGGTCTC

16 repeats 185 bp (TCTA) 10 (TCTG)2 (TCTA)4

b)

Figure A.1: Sequence result of Y-STR locus DYS 437: samples a) id52 and b) id66 carrying 15 and 16 repeats respectively. (Green – Forward and reverse primer sequence, Blue, Pink – Repeat motif(s), Black – Non-repeating sequence.)

TCCTGAATGGTACTTCCTAGGTTTTCTTCTCGAGTTGTTATGGTTTTAGGTCT
AACATTTAAGTCTTTAATCTATCTTGAATTAATAGATTCAAGGTGATAGATA
TACAGATAGATAGATACATAGGTGGAGACAGATAGATGATAAATAGAAGA
TAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGAAAGTATAAGTA
AAGAGATGATGGGTAAAAGAATTCCAAGCCAGGC

10 repeats 240 bp (AGAT) 10

a)

TCCTGAATGGTACTTCCTAGGTTTTCTTCTCGAGTTGTTATGGTTTTAGGTCT
AACATTTAAGTCTTTAATCTATCTTGAATTAATAGATTCAAGGTGATAGATA
TACAGATAGATAGATACATAGGTGGAGACAGATAGATGATAAATAGAAGA
TAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGAAAGTATA
AGTAAAGAGATGATGGGTAAAAGAATTCCAAGCCAGGC

11 repeats 244 bp (AGAT) 11

b)

TCCTGAATGGTACTTCCTAGGTTTTCTTCTCGAGTTGTTATGGTTTTAGGTCT
AACATTTAAGTCTTTAATCTATCTTGAATTAATAGATTCAAGGTGATAGATA
TACAGATAGATAGATACATAGGTGGAGACAGATAGATGATAAATAGAAGA
TAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGAAA
GTATAAGTAAAGAGATGATGGGTAAAAGAATTCCAAGCCAGGC

12 repeats 248 bp (AGAT) 12

c)

Figure A.2: Sequence result of Y-STR locus DYS 439: samples a) id4, b) id13 and c) id112 carrying 10, 11 and 12 repeats respectively. (Green – Forward and reverse primer sequence, Blue – Repeat motif(s), Black – Non-repeating sequence.)

APPENDIX B

TÜRKİYE POPÜLASYONUNUN Y-STR ANALIZI

ANKET FORMU

1. Adı Soyadı:

2. Telefon:

3. E-mail:

4. Cinsiyet:

5. Memleket:

6. Doğum yeri:

7. Etnik köken:

8. Doğum yılı:

9. Ailede genetik hastalık var mı?

10. Akraba evliliği durumu?

11. Baba tarafından dedeleri

	Adı	Yaşadığı Yer	Yaklaşık Doğumyılı
Baba			
Dede 1			
Dede 2			
Dede 3			
Dede 4			
Dede 5			

Alınan numunenin sadece bu araştırmada kullanılmasına müsaade ediyorum.

İmza Tarih: