Ormos, Andrea. Cell line authentication and contamination assessment for human cell cultures. Master of Science (Forensic Genetics), May 2015, 82 pp., 7 tables, 25 figures, 33 references.

Cell line authentication is an essential step in ensuring the integrity and reproducibility of biomedical research. The major contaminants in cell cultures are fungi, viruses, bacteria and contamination from other cell lines of the same or different species. Contaminants alter the physiology and properties of cells, compromising the results of experiments.

In this study, an improved multiplex assay was developed, detecting mycoplasma and mouse cell line contamination, while performing DNA typing.

The assay was tested on cell cultures, the reproducibility of the assay was verified, sample collection and procedures were optimized and limit of detection for contaminants were determined.

A survey was conducted to assess the interest in an in-house cell line authentication and contamination assessment service.

CELL LINE AUTHENTICATION AND CONTAMINATION ASSESSMENT FOR HUMAN CELL CULTURES

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Andrea Ormos, B.S

Fort Worth, TX

May 2015

ACKNOWLEDGEMENTS

I would first like to thank my major professors and advisory committee members for their time, support and guidance: Dr. Rhonda Roby, Dr. Arthur Eisenberg, Dr. John Planz, Dr. Abbot Clark and Dr. Kunlin Jin. Special thanks goes to Dr. Kim Wise at the J. Craig Venter Institute for providing mycoplasma specimens and Dr. Nathalie Sumien at the University of North Texas Health Science Center for the mouse blood sample, which were essential positive controls for my experiments. I thank Helena Halasz in Dr. Ferenc Gallyas, Dr. Nirupama Sabnis, Marlyn Panchoo and Jessica Johnson in Dr. Andras Lacko's laboratory, Smrithi Rajendiran in Dr. Jamboor Vishwanatha's laboratory, Jaclyn Bermudez and Tara Tovar in Dr. Abbot Clark's laboratory as well as Leslie Tabor-Simecka in Dr. Jerry Simecka's and Dr. Riyaz Basha's laboratory for the cell culture samples. I also must recognize the invaluable help of Casandra Setser in editing, Linda Larose, Marc Sprouse, Alessandra Alicea-Centeno in the laboratory and Michele Whitehead in the library.

Finally, I would like to thank my fellow graduate students, especially the FGEN class of 2015, my friends and family for their continuous support and belief in me throughout my Master's program.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Cell lines are invaluable tools in research. Since the early 1900's, cell cultures have made it possible for scientists to gain knowledge of normal and abnormal cell function, biochemical pathways, and cellular processes. In 1952, Dr. George Gey established the first immortal human cell line, HeLa, which provided a laboratory model for cancer cells and still continues to play an essential role in cancer and biomedical research (1). Since the establishment of the HeLa cell line, over 6,000 human and hundreds of non-human cell lines from over 150 species are available from cell banks such as ATCC (American Type Culture Collection), ECACC (European Collection of Cell Cultures), RIKEN (Rikagaku Kenkyusho, Institute of Physical and Chemical Research, Japan), JCRB (Japanese Collection of Research Bioresources), COG (Children's Oncology Group Cell Culture and Xenograft Repository) and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, German Collection of Microorganisms and Cell Cultures). These cell lines provide models of human disease and offer greater understanding of developmental biology and genetic evolution (2).

Cell cultures are established from tissues of humans, animals and plants as primary cell cultures. Primary cultures can be immortalized to obtain a continuous cell line. Continuous cultures can be established by viral or chemical transformation; seldom, primary cultures can spontaneously become continuous (3). Primary cultures divide approximately 55 times before reaching senescence where they stop proliferating. This is also known as the Hayflick limit.

Continuous cell lines and cell cultures derived from tumor tissues can divide indefinitely, acquiring infinite lifespan, which is referred to as immortal (4). In fact, the HeLa cells have been growing in flasks in laboratories for 63 years, over twice as long, as Henrietta Lacks, the donor of this cell line, lived in her life (5, 6).

The primary cells may lose heterogeneity during cell culturing relative to the original tissue after a couple of passages. Cells grown under selective pressure produce a homogeneous cell culture of the most vital cell type (7). Subsequent cultures have cells that retain many of the properties of the original tissue of origin (8). After a significant amount of time and constant proliferation, genetic instability can occur (7). Animal tissues and primary cell cultures are diploid, while continuous cell lines, especially cancerous cell lines are commonly heteroploid (4). Heteroploidy is a phenomenon when individual cells in a culture contain various number of chromosomes, and often, half of the cells in a culture do not have the same number of chromosomes. Immortalized cells are also characterized by aneuploidy, a condition in which the cells have additional or missing chromosomes. Chromosomal aberrations such as inversions, deletions and chromosomal translocations are also common and provide unique markers for the characterization of cell lines (7). The longer the cell line is in culture, the more genetic changes can occur. Microsatellite instability has also been observed, and gives rise to the loss of heterozygosity in polymorphic regions of the genome, such as short tandem repeat (STR) loci (9).

As with any laboratory procedure, contamination can occur, and the sooner it is detected the less time and resources may be lost. The key is to detect and eliminate contamination as early as possible. Cell cultures are handled in laminar flow hoods under sterile conditions, but a single airborne spore or cell can cause contamination in cell cultures. Contamination is a serious concern since it compromises the integrity and reproducibility of experimental results. The most common

biological contaminants are bacteria, fungi, viruses, mycoplasma and cross-contamination with other cell lines (10). When bacteria and fungi contaminate a cell culture, the contamination can be visually detected since the culture becomes turbid and the microorganisms can be observed under the microscope (10, 11).

Mycoplasma is a small bacterium without a cell wall. Due to its very small size, it can contaminate cell cultures without obvious signs. They are an order of magnitude smaller than other bacteria and do not form colonies in cell cultures and, as a result, they cannot be detected with light microscopy (10, 11). The optical density of the medium does not change until the contamination is substantial and reaches high concentration in the cell culture. During mycoplasma contamination, the bacteria attach to the membrane of the host cell through attachment organelles (12). Mycoplasmas modify the properties and physiology of the cells: inhibiting cell metabolism; hindering DNA and RNA synthesis; introducing chromosomal abnormalities; and slowing the growth rate of cells. Consequently, the results and conclusions of the research experiments are compromised. There are over 100 species of mycoplasma, with 95% of laboratory contamination caused by four species: *Mycoplasma hyorhinis*, *Mycoplasma orale*, *Mycoplasma arginini* and *Acholeplasma laidlawii*. Mycoplasma is small enough to pass through bacterial filters. They do not respond to antibiotics, which adds to the risk of spreading in the laboratory once contamination occurred. It is estimated that up to 35% of cell lines can have mycoplasma contamination (13). There are several tests available to detect mycoplasma contamination which include immuno-staining, fluorescent staining, ELISA and PCR (10). Many laboratories handle and grow multiple cell cultures simultaneously. The practice can lead to cross-contamination, when one cell line contaminates another cell line. The contaminant can overgrow or coexist with the original cell

culture. This can occur with a cell line from the same species or a different species. Contamination with other cell lines often exists without any obvious signs and it can compromise experiments.

Researchers started to recognize the problem of cell line cross-contamination in the early 1960's. However, even today, many laboratories are still conducting experiments without confirming the authenticity of their cell lines (14, 15). Numerous research papers reported misidentified cell lines and the persistent problem has resulted in publications in today's most prestigious journals (9). In 2011, the ATCC Standard Development Organization published a document for human cell line authentication: "Authentication of Human Cell Lines: Standardization of STR Profiling" (ANSI/ATCC ASN-0002-2011) (16). The International Cell Line Authentication Committee (ICLAC) was formed in 2012 to provide guidance and to raise awareness of cell line authentication testing (17). The group maintains an up to date list of misidentified cell lines based on previous publications in the "Database of Cross-Contaminated or Misidentified Cell Lines". Version 7.2, updated in October 2014, contains 473 cell lines confirmed as misidentified, 438 of which have no existing authentic stocks available (18). Cell lines become contaminated in laboratories every day; therefore, regular testing and authentication is a crucial quality assurance measure to ensure the integrity of research. It is recommended that cell lines are tested on arrival and periodically during use, especially before and after cryopreservation, and experimental use. It is best to obtain cell lines from reputable cell line repositories and cell banks (8).

There are several methods available to authenticate cell lines such as isoenzyme analysis, karyotyping and HLA typing. However, the best method currently for human cell line authentication is the STR profiling (16). It is the simplest, least expensive, reliable method, which provides identification to the level of the individual cell line. Human identification based on short

tandem repeat (STR) polymorphisms have been used in forensic science since the early 1990's (19). The same method can be implemented for the verification of cell line identity (20). DNA typing is a procedure, in which several STR loci are amplified by PCR in a multiplex reaction from DNA template using fluorescently labeled, locus specific primers. The amplified fragments are separated via capillary electrophoresis using a genetic analyzer. The electropherograms are further analyzed using software applications such as GeneMapper® ID-X Version 1.4 (Applied Biosystems by Life Technologies, Carlsbad, CA) to obtain the DNA profiles.

The standards for human cell line authentication published in 2011 recommend the use of eight loci for uniquely identifying human cells (16). The recommended STR loci are CSF1PO, D5S818, D7S820, D13S317, D16S539, TH01, TPOX and vWA. These eight markers provide the DNA profile, a unique numerical pattern for each cell line. Most of the STR markers are on different chromosomes or far distance apart on a chromosome allowing free recombination. The STR markers are in linkage equilibrium and as a result, they provide high discrimination power between cell lines. The estimated probability of two cell lines matching with the use of the eight STR loci is 1 in $2x10^8$ (16). The DNA profiles stored in databases serve as references for cell line authentication. The DNA profile of the tested cell line is compared to the reference profile. Based on the results of DNA typing, the cell line can be considered "authenticated", "misidentified", "cross-contaminated", "unique" or the results can be "inconclusive" based on the classification of the ATCC standards (16).

Many validated STR PCR amplification kits for cell line verification are commercially available from several companies. GenePrint® 10 System (Promega Corporation, Madison, WI) is the simplest and least expensive kit which amplifies the recommended eight STR loci plus an additional STR locus D21S11 and the gender-determining locus Amelogenin (21). DNA markers

are shown in (Table 1) including the 13 core CODIS (Combined DNA Index System) STR loci and Amelogenin, the recommended eight STR loci for human cell line authentication by the standards (ANSI/ATCC ASN-0002-2011), the ATCC and DSMZ database STR loci and Amelogenin and the GenePrint® 10 System STR loci and Amelogenin.

CODIS LOCI	ANSI/ATCC ASN-0002-2011	GenePrint® 10 System	ATCC and DSMZ markers
Amelogenin	N/A	Amelogenin	Amelogenin
CSF1PO	CSF1PO	CSF1PO	CSF1PO
D3S1358	N/A	N/A	N/A
D5S818	D5S818	D5S818	D5S818
D7S820	D7S820	D7S820	D7S820
D13S317	D13S317	D13S317	D13S317
D16S539	D16S539	D16S539	D16S539
D18S51	N/A	N/A	N/A
D19S433	N/A	N/A	N/A
D21S11	N/A	D21S11	N/A
FGA	N/A	N/A	N/A
TH01	TH01	TH01	TH01
TPOX	TPOX	TPOX	TPOX
vWA	vWA	vWA	vWA

Table 1. List of markers used in human identification and human cell line authentication

Cell line authentication is a requirement for the submission of manuscripts to many journals, such as Nature, International Journal of Cancer, Cancer Research, Cancer Discovery, Cancer Prevention Research, Clinical Cancer Research, Cancer Epidemiology Biomarkers and Prevention, Molecular Cancer Therapeutics, Molecular Cancer Research, BioTechniques, Cell Biochemistry and Biophysics, In Vitro Cellular and Developmental Biology and Molecular Vision. Other journals such as Science and PNAS do not require, but highly encourage, cell line authentication as good scientific practice. Many grant-funding agencies, such as the NIH suggest the use of cell line authentication (22). Small research bodies require the proof of cell line

authentication in their grant application review process. The NIH published guidelines for reporting pre-clinical research on November 7, 2014; which includes the requirement to report source, authentication and mycoplasma contamination status for each cell line used in the research (23).

The National Public Radio (NPR) aired a series on the topic of cell line authentication in research on December 9 and 10, 2014 (24, 25). The Global Biological Standards Institute (GBSI) held a BioPolicy Summit in December of 2014, discussing the importance of cell line identity (26). Recently, a quantitative estimate was published in Science about the damage cell line contamination has caused to research already, "We are looking at tens of thousands of publications, millions of journal citations, and potentially hundreds of millions of research dollars." says Dr. Korch, the author (27).

Cell line authentication and contamination testing are essential quality control steps in research to ensure the reproducibility and reliability of results and consequently the conclusions of research experiments. It is not only a best scientific practice but also often a requirement for grant applications and for publications in numerous scientific journals. In the recent years, a solution has emerged with the establishment of the ANSI/ATCC ASN-0002-2011 standard for human cell line authentication and the formation of International Cell Line Authentication Committee providing continuous guidance for the research community with growing online resources. Several reputable cell line repositories sell authenticated cell lines and companies are emerging every day with more and more affordable prices to provide this valuable service for the scientific community.

CHAPTER II

MATERIALS AND METHODS

Cell line collection

Forty-two cell lines were collected from five laboratories. Four laboratories were located at the University of North Texas Health Science Center, Fort Worth, Texas and one at the University of Pecs Medical School in Hungary. The collected cell lines are shown in (Table 2).

	Name of cell line	Species		Name of cell line	Species		Name of cell line	Species
1	CHLA-9	human	15	Panc-1	human	29	H9c2	rat
2	CHLA-10	human	16	T24/83	human	30	SMS_KCNR	human
3	TC-71	human	17	T24/83	human	31	HepG2	human
4	LA1-55n	human	18	T-47D	human	32	MDA-MB-231	human
5	SMS_KCNR	human	19	U251	human	33	LNCaP/SWMC	human
6	BTM 73N	bovine	20	A549	human	34	LDL-A7	hamster
7	BTM 81N	bovine	21	HeLa	human	35	SH-SY-5Y	human
8	Optic Nerve Astrocyte	mouse	22	Hep G2	human	36	SCC-25	human
9	GTM-3	human	23	MCF7	human	37	DOK	human
10	LC66Tr	human	24	Panc-1	human	38	DOK	human
11	A549	human	25	T24/83	human	39	OSC-2	human
12	HeLa	human	26	U251	human	40	SCC-25	human
13	Hep G2	human	27	HEK-293T	human	41	NIH3T3	mouse
14	MCF7	human	28	WLR 68	human	42	DU-145	human

Table 2. List of collected cell line samples for cell line authentication.

The cells were either cultured or frozen at collection. The cells were counted and resuspended either in phosphate buffered saline (PBS) or cell line specific medium. Cell suspension of 50µL of 25,000 cells/µL (total of 1,250,000 cells) were collected in the first half of the project then adjusted to 20µL of 10,000 cells/µL (total of 200,000 cells) to follow to the recommendations in the literature (28). The cells were blotted onto labelled Whatman® BFC180 Human ID Bloodstain Card (GE Healthcare, Life Sciences, Piscataway, NJ) as shown on (Figure 1) then dried on the bench and stored at room temperature for further downstream analysis (Appendix A).

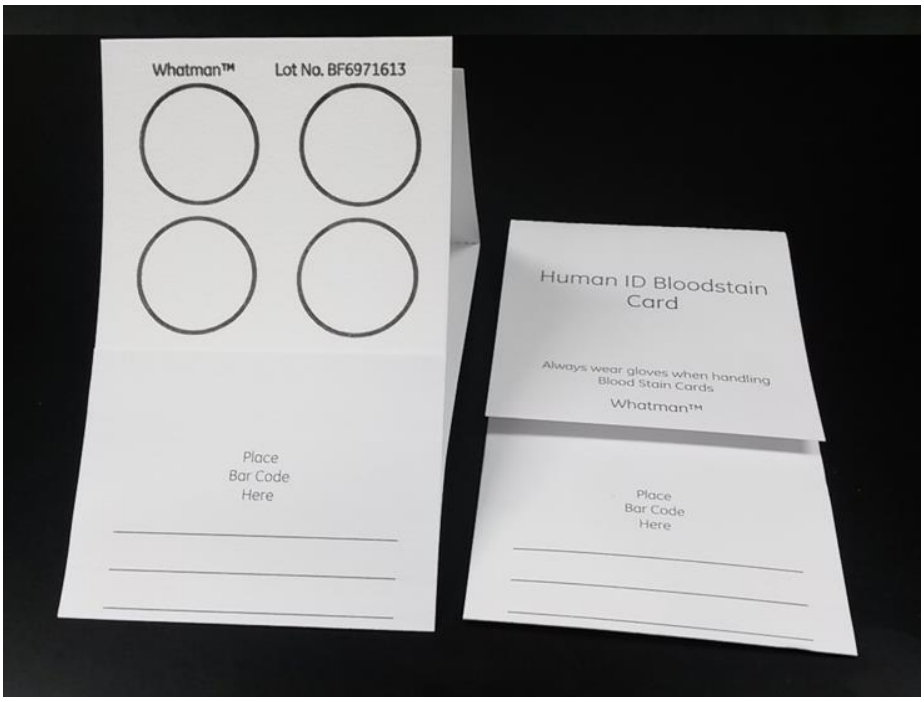


Figure 1. Whatman® BFC180 Human ID Bloodstain Card, the storage medium for the collection of cell lines

Mycoplasma collection

Mycoplasma arginini was grown at the J. Craig Venter Institute in liquid SP4 medium containing fetal bovine serum and supplemented with 0.25% (w/v) arginine. The culture was grown at 37°C until turbidity was observed and pH became basic as evidenced by phenolphthalein red indicator. The cells were centrifuged for 10 minutes at 15°C at 5000x g. The cells were washed, resuspended in PBS and kept frozen overnight. The mycoplasma pellet was thawed and incubated at 56°C for 45 minutes to render non-viable. The pellet was re-frozen for storage and transported to UNTHSC. *Mycoplasma hominis* strain 1620, *Mycoplasma penetrans* and *Acholeplasma laidlawii* strain PG8 were also grown (Appendix B).

Mouse blood collection

Whole mouse blood was collected by UNTHSC researchers from *Mus musculus* by tail incision. The fresh blood sample was placed into a 2mL collection tube containing Ethylenediaminetetraacetic acid (EDTA) solution and stored at 4°C.

Extraction of mouse and mycoplasma DNA

DNA extraction of mouse blood was performed with QIAamp® DNA Mini Kit (Qiagen Corporation, Germantown, MD). DNA was extracted from 200µl mouse blood as a positive control, following the manufacturer recommendations and protocol: DNA Purification from Blood or Body Fluids (spin protocol) (29). DNA was eluted in 150µL AE buffer and stored in -20°C (Appendix C).

DNA extraction of the mycoplasma pellet was performed with the QIAamp® DNA Mini Kit. Visible inactivated mycoplasma pellet was extracted to obtain positive control mycoplasma DNA

following the manufacturer recommendations and protocol: DNA Purification from Tissues (spin protocol) (29). DNA was eluted in 200µL AE buffer and stored in freezer (Appendix D).

Quantification of mycoplasma and mouse DNA

The extracted mouse and mycoplasma DNA were quantified with Qubit® dsDNA BR Assay Kit (Invitrogen, Life Technologies, Carlsbad, CA), according to the manufacturer's recommended protocol using Qubit® 2.0 Fluorometer (30). The fluorometer was calibrated with two pre-diluted DNA standards. The assay reagents, buffer and 2µL DNA extract were incubated for two minutes before DNA concentration was read using the fluorometer (Appendix E).

Amplification of human STR markers

The collected cell line samples were sampled with 1.2mm Harris MicroPunch device (Ted Pella, Inc., Redding, CA). Each punch was incubated with 10µL UNTHSC Incubation Buffer for 30 minutes at 70°C or until the buffer evaporated. The direct amplification of human STR loci was performed with the manufacturer validated primers and reagents from GenePrint® 10 System amplification kit. The amplification of the 25µL PCR reactions were carried out on a GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA) with the manufacturer recommended cycling parameters for GenePrint® 10 System: initial denaturation step at 96°C for 1 minute; 26 cycles of 94°C for 10 seconds, 59°C for 1 minutes and 72°C for 30 seconds; followed by the final extension at 60°C for 10 minutes and hold at 4°C indefinitely (Appendix F).

Amplification of mouse amplicon

The amplification of the mouse specific amplicon was set up with the GenePrint® 5X Mouse Primer Pair Mix (Promega Corporation, Madison, WI) and the manufacturer validated reagents from GenePrint® 10 System amplification kit (31). The primers were labeled with fluorescein, a

blue fluorescent dye. The amplification of 10ng mouse control DNA in a 25µL PCR reaction was carried out on a GeneAmp® PCR System 9700 Thermal Cycler with the manufacturer recommended cycling parameters for GenePrint® 10 System: initial denaturation step at 96°C for 1 minute; 26 cycles of 94°C for 10 seconds, 59°C for 1 minutes and 72°C for 30 seconds; followed by the final extension at 60°C for 10 minutes and hold at 4°C indefinitely. The collected mouse cell line sample (cell line sample 8 in Table 2) was sampled with 1.2mm Harris MicroPunch device, incubated with 10µL UNTHSC Incubation Buffer for 30 minutes at 70°C or until the buffer evaporated. The sample was amplified with GenePrint® 10 System reagents and under the same conditions as described above (Appendix G).

Amplification of mycoplasma amplicon

The amplification of the mycoplasma amplicon was set up with published mycoplasma specific primer pair GPO-3 and MGSO from Masters *et al*. (13). The primers were ordered from IDT. The reverse primer was labeled with fluorescein (6-FAM), a blue fluorescent dye for the detection via capillary electrophoresis on the Applied Biosystems 3130xL Genetic Analyzer (Applied Biosystems, Foster City, CA). The primer pair GPO-3 and FL-MGSO are shown in (Table 3). The primers were ordered from IDT (IDT, Coralville, IA).

Primer name	Primer sequence (5'-3')	Tm*
GPO-3	GGGAGCAAACAGGATTAGATACCCT	58.1°C
FL-MGSO	6-FAM/TGCACCATCTGTCACTCTGTTAACCTC	60.1°C

Table 3. List of mycoplasma primers
*Tm represents melting temperature

HotStarTaq Plus DNA polymerase kit (Qiagen Corporation, Germantown, MD) was utilized to set up the amplification of 1µL mycoplasma control DNA in the total volume of 50µL reaction. The PCR reaction was carried out on a GeneAmp® PCR System 9700 Thermal Cycler with the

published cycling parameters: initial denaturation step at 95°C for 5 minute; 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute; followed by the final extension at 72°C for 10 minutes and hold at 4°C indefinitely (13).

The amplification was also tested with the GenePrint® 10 System amplification kit. The amplification of 1µL mycoplasma control DNA, 5µL GenePrint®10 5x Master Mix, 0.5µL 10mM GPO-3 and 0.5µL 10mM FL-MGSO primers in the total volume of 25µL PCR reaction was carried out on a GeneAmp® PCR System 9700 Thermal Cycler with the manufacturer recommended cycling parameters for GenePrint® 10 System: initial denaturation step at 96°C for 1 minute; 26 cycles of 94°C for 10 seconds, 59°C for 1 minutes and 72°C for 30 seconds; followed by the final extension at 60°C for 10 minutes and hold at 4°C indefinitely.

Multiplex assay

The human cell line authentication and contamination assessment multiplex assay was set up with the manufacturer validated GenePrint® 10 System amplification kit with the addition of mycoplasma specific primer pair and GenePrint® 5X Mouse Primer Pair Mix. The 25µL PCR reaction, which is the full reaction, was carried out on a GeneAmp® PCR System 9700 Thermal Cycler with the manufacturer recommended cycling parameters for GenePrint® 10 System (Appendix H). The reaction amplified nine human STR loci, Amelogenin as well one mouse and one mycoplasma specific amplicons as shown in (Table 4). Colors in the table represent the color of the fluorescent dye tag (TMR: yellow, JOE: green, FL: blue) for each fragment, which were detected via electrophoresis and visualized on the electropherograms.

GenePrint® 10 System	Chromosomal location of STR marker	Repeat sequence GenBank top strand	Number of alleles	Allele range
Amelogenin	Xp22.1-22.3	AMELX 106bp AMELY 112bp	2	X, Y
TPOX	2q25.3	AATG	17	4-16
vWA	12p13.31	[TCTA][TCTG][TCCA] complex	29	10-25
CSF1PO	5q33.1	AGAT	23	5-16
D5S818	5q23.2	AGAT	16	6-18
D7S820	7q21.11	GATA	30	5-16
D13S317	13q31.1	TATC	20	5-17
D16S539	16q24.1	GATA	21	6-16
D21S11	21q21.1	[TCTA][TCTG] complex	70	24-38.2
TH01	11p15.5	TCAT	21	3-14
Mouse	Chromosome 4 Locus near D4Mit113	84 base pair	1	N/A
Mycoplasma 16S	Bacterial chromosome	243 and 265 base pair	1	N/A

Table 4. List and genetic information of the amplicons generated in the human cell line authentication and contamination assay (21, 32).

The reaction was also carried out in half reaction, 12.5µl volume (Appendix I).

Capillary electrophoresis

Applied Biosystems 3130xl Genetic Analyzer was used to separate the DNA fragments for the samples. The capillary electrophoresis (CE) was performed with 9µL HiDi Formamide (Applied Biosystems, Foster City, CA) and 1µL GenePrint®10 ILS 600 Size Standard (Applied Biosystems, Foster City, CA) for each sample in addition to 1µL of STR PCR product. The samples were set

up in a 96 well PCR plate and heated for 3 min at 95C° then snap cooled for 3 minutes on ice and loaded in the sixteen capillary 3130xl instrument using POP-6 polymer (Appendix F).

Data analysis

Raw data files (.fsa) from the Genetic Analyzer were uploaded to GeneMapper® ID-X Version 1.4 software and analyzed using the recommended GenePrint_10_IDX_v1_1 bins, panels and stutter filters (Promega Corporation, Madison, WI). Internal lane standard (ILS) was placed in each sample to determine the proper size of the fragments. The amplicons sizes were calculated by the Local Southern method. One allelic ladder was added to each electrophoresis injection run to enable the genotyping software to assign the most common alleles to the sample peaks in each STR locus. The maximum detection threshold was determined as 6000RFU. The analytical threshold for blue, green and yellow dye was set to 75RFU and 40RFU for the red dye. The peak height ratio was established at 0.7 (70%). The analysis method parameters are shown in (Figure 2). Electropherograms were used to visualize the amplification products by graphing the length of the amplicons in base pairs (bp) versus the detected fluorescence in relative fluorescent (RFU) units. Positive and negative controls were tested in each run of injections for proper quality control.

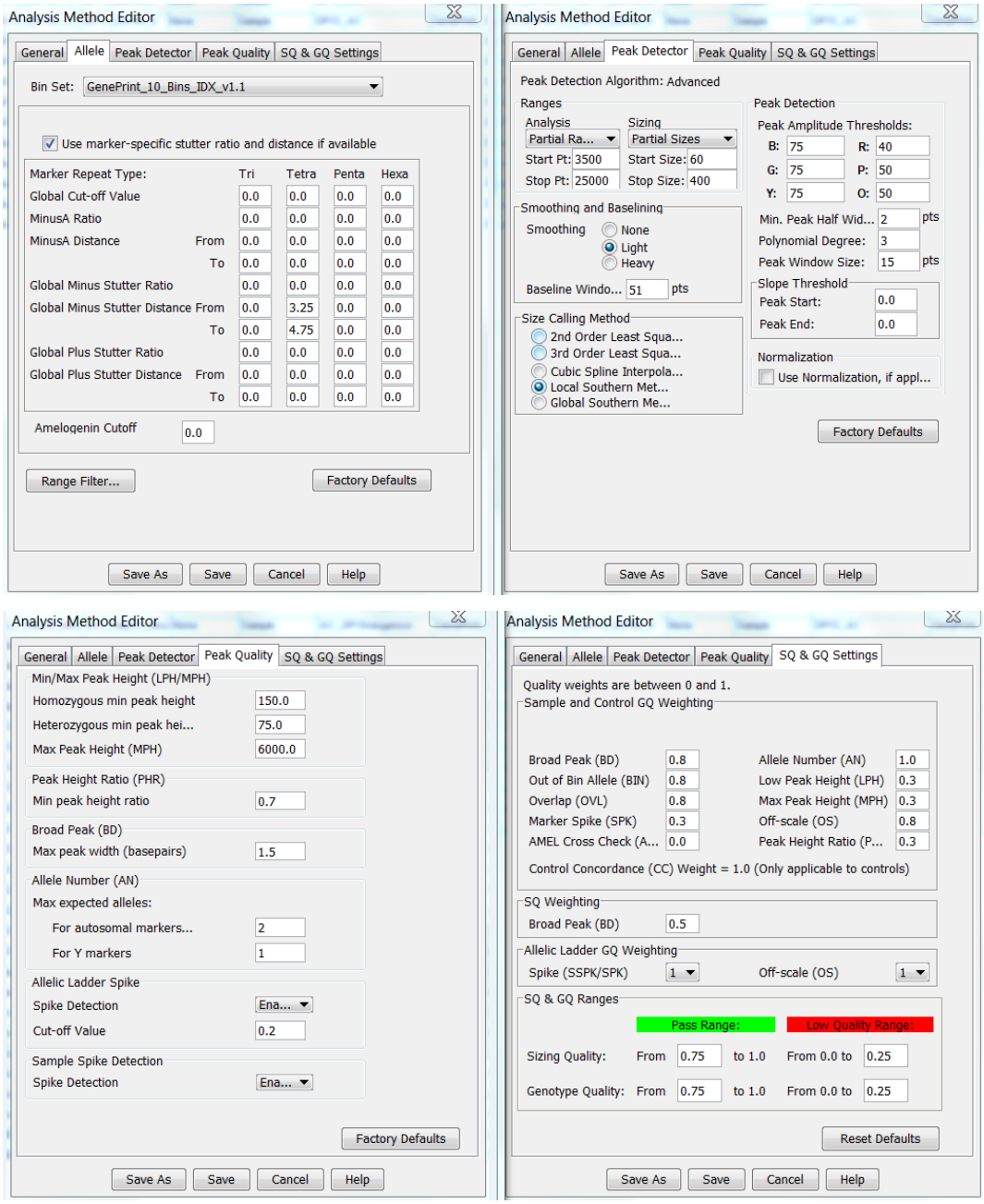


Figure 2. Analysis method in GeneMapper® ID-X

The raw data was analyzed, artifacts were evaluated with GeneMapper® ID-X Version 1.4 software and the DNA profile of the tested cell line was generated. Contamination with mouse cell line and mycoplasma was assessed based on the presence or absence of the mouse and mycoplasma

specific peaks in the electropherogram. A reference DNA profile (CHLA-9 cell line) obtained from Children Oncology Group Cell Culture and Xenograft Repository is shown in (Table 5).

Cell Line Name	Marker	Allele 1	Allele 2
CHLA-9	TH01	7	9.3
CHLA-9	D21S11	28	29
CHLA-9	D5S818	12	
CHLA-9	D13S317	11	12
CHLA-9	D7S820	9	10
CHLA-9	D16S539	11	
CHLA-9	CSF1PO	12	
CHLA-9	AMEL	X	
CHLA-9	vWA	16	17
CHLA-9	TPOX	8	11

Table 5. DNA profile of CHLA-9 cell line

The DNA profile of the tested cell line was compared to the reference sample acquired from cell culture databases. Calculations were performed manually using the recommended Matching Algorithm by the ANSI/ATCC ASN-0002-2011 standards to determine the percent match between the profiles of the tested cell line and the corresponding reference profile. The "percent % match between two cell lines equal = the number of shared alleles in both STR profiles divided ÷ by the total number of alleles in the questioned profile (homozygous alleles are counted as one allele)" (16). Conclusions were drawn on the degree of authenticity and a Certificate of Authentication or a Certificate of Testing was drafted to report results back to the researchers providing the cell line for authentication.

Cell Line Authentication and Contamination Assessment workflow

The initial steps in the cell line authentication and contamination assessment are shown in (Figure 3), from cell line sampling to obtaining raw data.

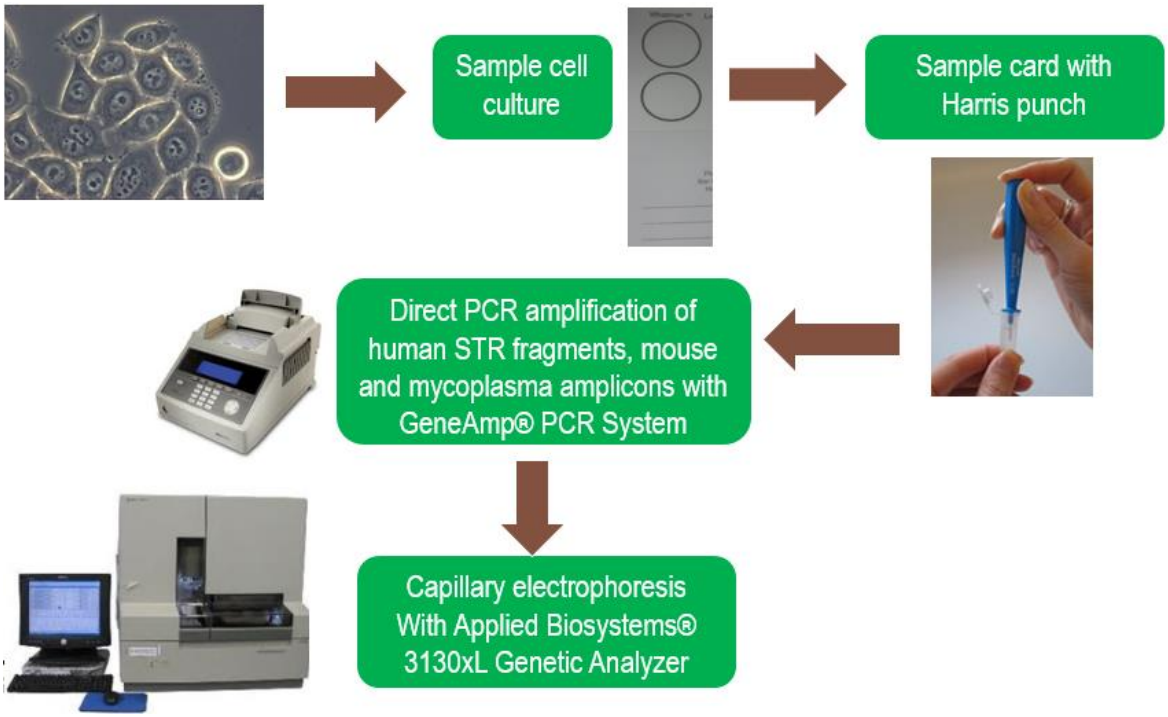


Figure 3. Initial steps in cell line authentication and contamination assessment

The DNA profile of the cell line and the determination of the presence or absence of mouse and mycoplasma amplicons were obtained during data analysis as shown in (Figure 4). The DNA profiles were compared, percent match was determined and results were reported to the submitting research laboratory as shown in (Figure 5). Certificate of Authentication was issued to report the official results (Figure 6).

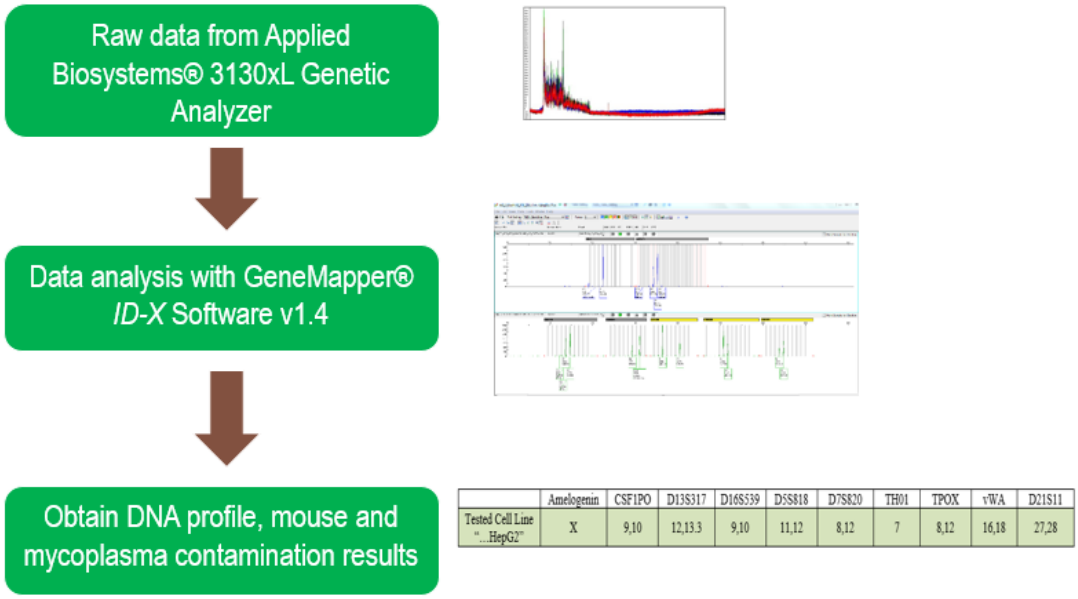


Figure 4. Data analysis steps in cell line authentication and contamination assessment

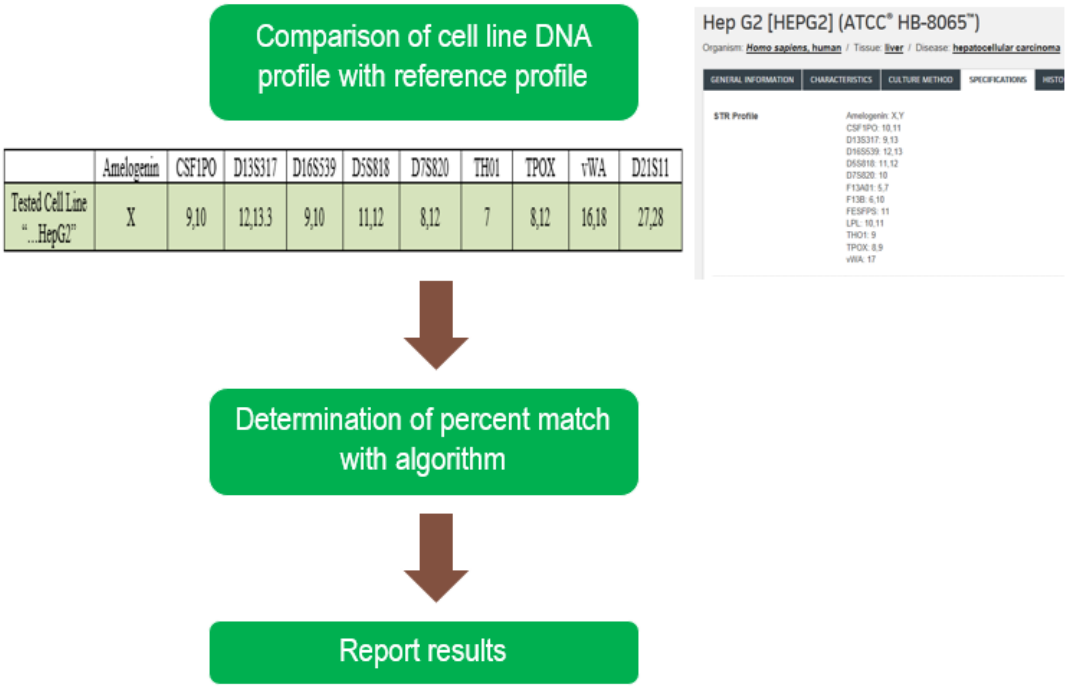


Figure 5. Final steps of cell line authentication and contamination assessment

UNT HEALTH SCIENCE CENTER

Certificate of Authentication

April 7, 2015

	Amelogenin	CSF1PO	D13S317	D16S539	D5S818	D7S820	TH01	TPOX	vWA	D21S11
Reference CHLA-9 Children Oncology Group CCXR	X	12	11, 12	11	12	9, 10	7, 9.3	8, 11	16, 17	28, 29
Tested Cell Line "…CHLA-9..." Received MMDDYYYY from Dr. Name	X	12	11, 12	11	12	9, 10	7, 9.3	8, 11	16, 17	28, 29

The tested cell line (highlighted green in the table above) was DNA typed with GenePrint® 10 System (Promega Corporation) amplification kit and electrophoresed on the 3130*xl* Genetic Analyzer (Applied Biosystems). The profile of the tested cell line is compared to the profile of the reference (shown in the table above) published by Children Oncology Group Cell Culture and Xenograft Repository (COG CCXR), (www.cogcell.org). Based on the "Authentication of Human Cell Lines: Standardization of STR Profiling (ANSI/ATCC ASN-0002-2011)", a minimum of eight core STR (short tandem repeat) loci are recommended to uniquely identify human cells. The matching criterion is based on an algorithm comparing the number of shared alleles of the reference to the tested cell line and expressed as a percentage. A cell line with ≥80% match to the reference profile is considered to be authenticated; this allows for a small degree of profile variation which can occur in cell cultures due to genetic instability. The cell line is considered misidentified if the DNA profile fails to match ≥80% to the reference profile or if it matches ≥80% to another cell line in the database. A small number of cell lines have a great degree of genetic instability resulting in a lower than 80% match to the reference. In these cases, further data are needed to confirm or refute authenticity. Cell lines with ≤56% match are considered unrelated.
Furthermore, the cell line was tested for mouse cell line and mycoplasma contamination.

The tested cell line, "…CHLA-9…", has a 100% match to the reference CHLA-9 (COG CCXR).
The tested cell line is authenticated.
The tested cell line, "…CHLA-9…", has no evidence of mouse cell line contamination.
The tested cell line, "…CHLA-9…", has no evidence of mycoplasma contamination.

Andrea Ormos, BS
Graduate Student
UNT Health Science Center

Name, PhD
Professor/Laboratory Director
UNT Health Science Center

Texas College of Osteopathic Medicine • Graduate School of Biomedical Sciences • School of Public Health • Institutes for Discovery • Physicians & Surgeons Medical Group
3500 Camp Bowie Boulevard, Fort Worth, Texas 76107-2699 • 817-735-2000

Figure 6. Sample of Certificate of Authentication

Survey

A UNTHSC Institutional Review Board (IRB) and the Office of Strategy and Organizational Excellence in conjunction with the Marketing and Communication Department approved survey was distributed via email to UNTHSC research faculty and staff to gauge their interest in an in-house cell line authentication and contamination assessment service. The survey was powered by Qualtrics (Appendix J).

CHAPTER III

RESULTS

Preliminary data

Nine cell lines were collected (50µL of 25,000 cells/µL) on Whatman® BFC180 Human ID Bloodstain Card. The cell lines were DNA typed using the GenePrint® 10 System. Based on the genetic data obtained and analyzed, three cell lines were misidentified, while six were authentic. There was inconsistency in the quantity of DNA from the samples taken from the cards and consequently variability in the quality of the electropherograms, which suggested the need for the optimization of cell collection and downstream protocols.

Improvement in capillary electrophoresis set up

Four GenePrint® 10 System PCR amplification of four cell line samples were set up in duplicates for electrophoresis with 1ul PCR product, HiDi Formamide and Internal Lane Standard (ILS). (Appendix F). The first set of samples were placed directly on the 3130xl DNA analyzer for capillary electrophoresis to separate and detect the STR fragments. A second set of samples were incubated for three minutes at 95C° followed by three minutes cooling on ice prior to capillary electrophoresis. The number of artifacts, which were not explained by pull up, raised baseline, minusA and stutter, were recorded with and without the heat denaturation and snap cooling treatment.

The samples without treatment had an average of six artifacts compared to the treated samples, which had an average of three artifacts. The improved denaturation of amplified STR fragments prior to capillary electrophoresis provided an approximately 50% improvement in the elimination of artifacts as shown is (Figure 7). Light blue bars represent samples without treatment; dark blue bars represent samples with heat denaturation and snap cooling treatment.

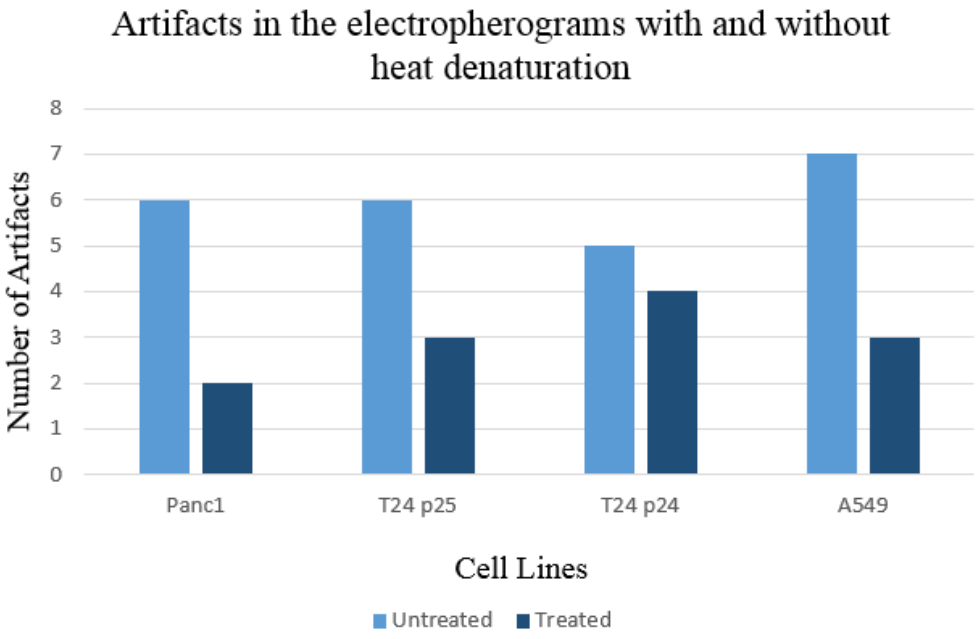


Figure 7. Artifacts in the electropherograms with and without heat denaturation

Artifacts in locus TH01 and TPOX were affected the most. The artifacts in those loci were eliminated in most samples as shown in (Figure 8) and (Figure 9). The red arrows point out the locations of the detected artifacts by the genotyping software, the peaks, which were above the analytical threshold. The electropherograms are scaled to 6000RFU.

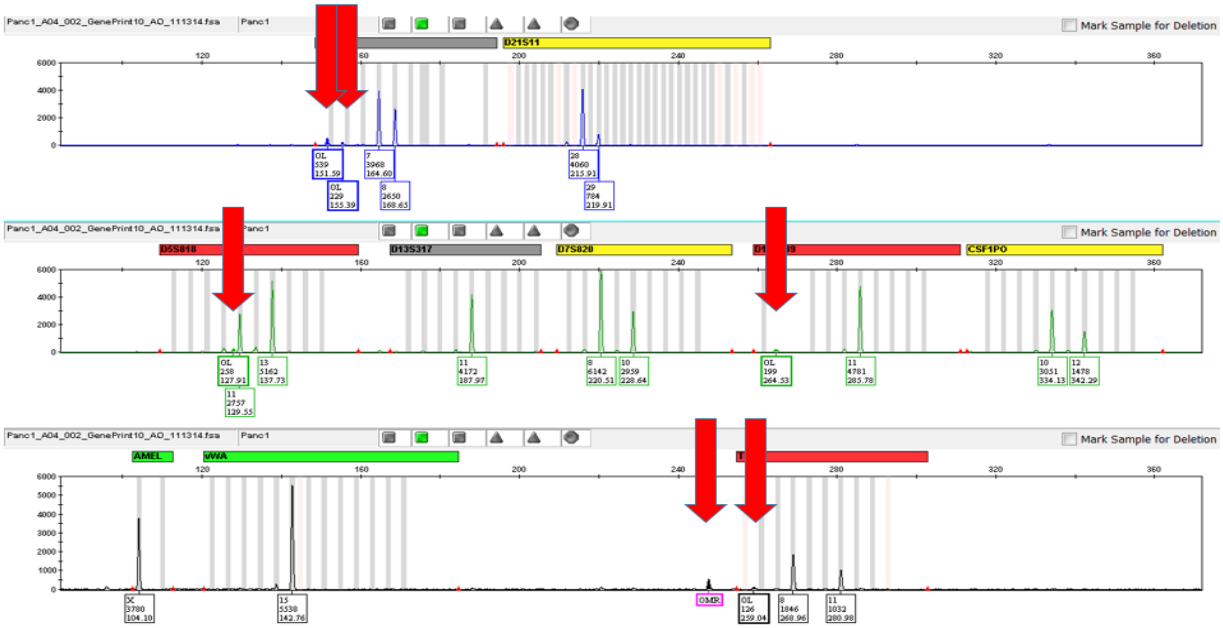


Figure 8. Electropherogram of Panc-1 cell line without additional denaturation step prior to electrophoresis

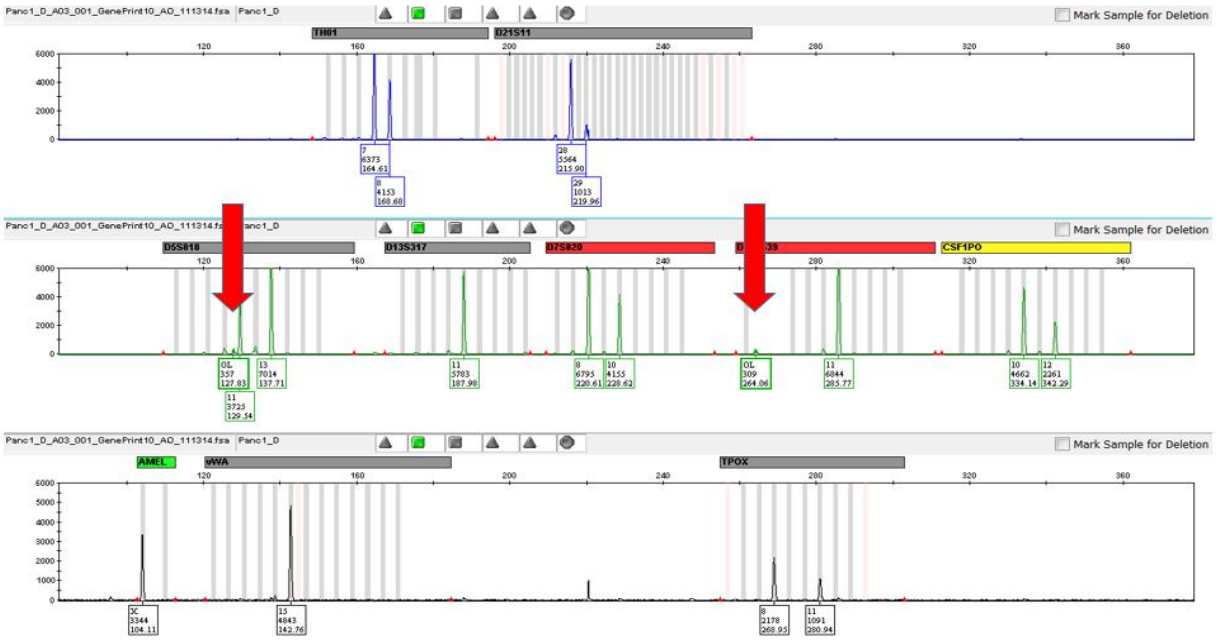


Figure 9. Electropherogram of Panc-1 cell line with additional denaturation step prior to electrophoresis

The improvement in TPOX locus after additional denaturation treatment is illustrated in (Figure 10). The red arrows point at the detected artifacts in the electropherogram. The electropherograms are scaled to 2800RFU.

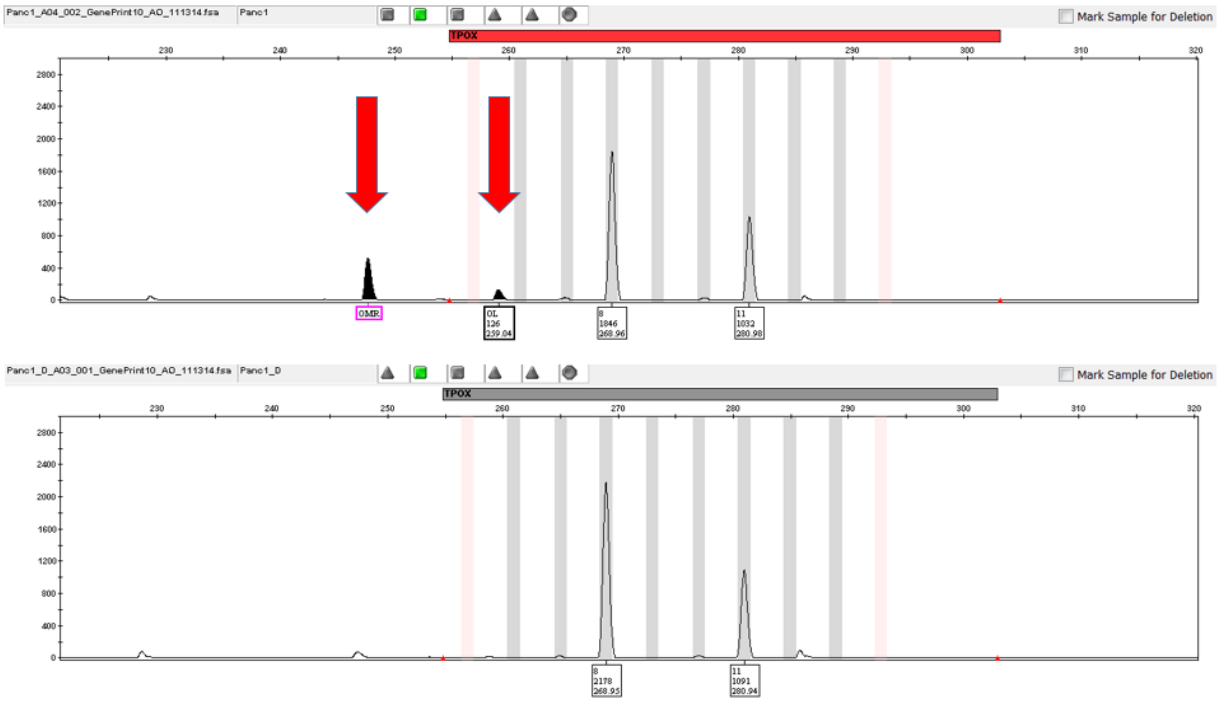


Figure 10. Comparison of artifacts in TPOX locus without heat denaturation step (above) and with heat denaturation step (below)

Optimization of cell line collection

Three cell lines were resuspended in PBS and in medium prior to blotting the cell suspension onto the storage card to determine in which solution would be optimal for cell line authentication. The electropherogram of SCC-25 cell line resuspended in PBS is shown in (Figure 11), resuspended in DMEM medium is shown in (Figure 12).

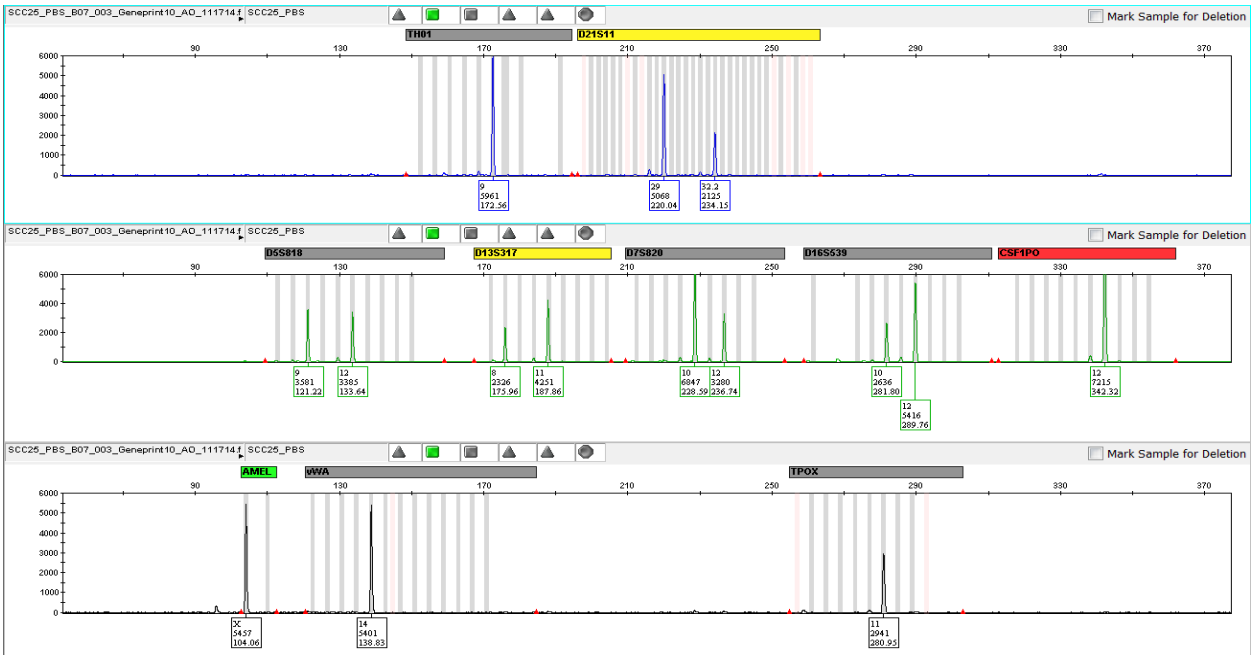


Figure 11. Electropherogram of SCC-25 cell line resuspended in PBS prior to blotting

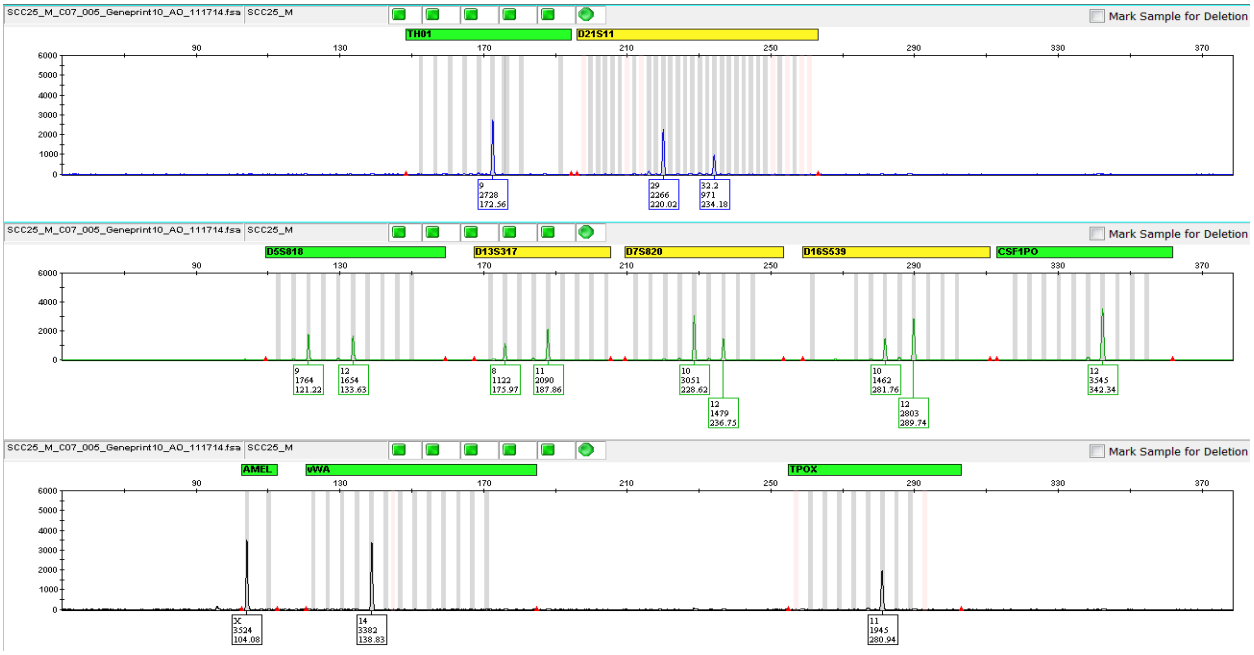


Figure 12. Electropherogram of SCC-25 cell line resuspended in medium prior to blotting

The electropherograms did not show major differences apart from increased peak heights, which was expected due to the use of same quantity of template (1.2mm punch).

Three cell lines were collected from frozen stocks and three cell lines were collected from cultured flasks. The electropherograms of the cell lines were compared. They did not show notable differences.

A series of different quantities of cells of DU-145 cell line were collected in 20µL and blotted on storage cards to determine the ideal cell quantity range for optimal DNA typing results. The quantities were the following: approximately 1,800,000; 600,000; 200,000; 65,000; 20,000 and 10,000 cells in 20µL (cell concentration of 90,000cell/µL; 30,000 cell/µL; 10,000 cell/µL; 3,000 cell/µL; 1,000 cell/µL and 500 cell/µL respectively). The storage cards were sampled with the Harris punch in the middle of the cell line stain taking a subset of the blotted cell quantity. The experiment was repeated three times. The number of artifacts and allele drop outs corresponding to each cell quantity blotted were recorded. Average and standard deviation for artifacts and drop outs were calculated and visualized on a bar graph.

The graph on (Figure 13) depicts the number of artifacts (grey bars) and allele drop outs (yellow bars) in each cell quantity. The bars represent the average of triplicate experiments with standard deviation “whiskers”.

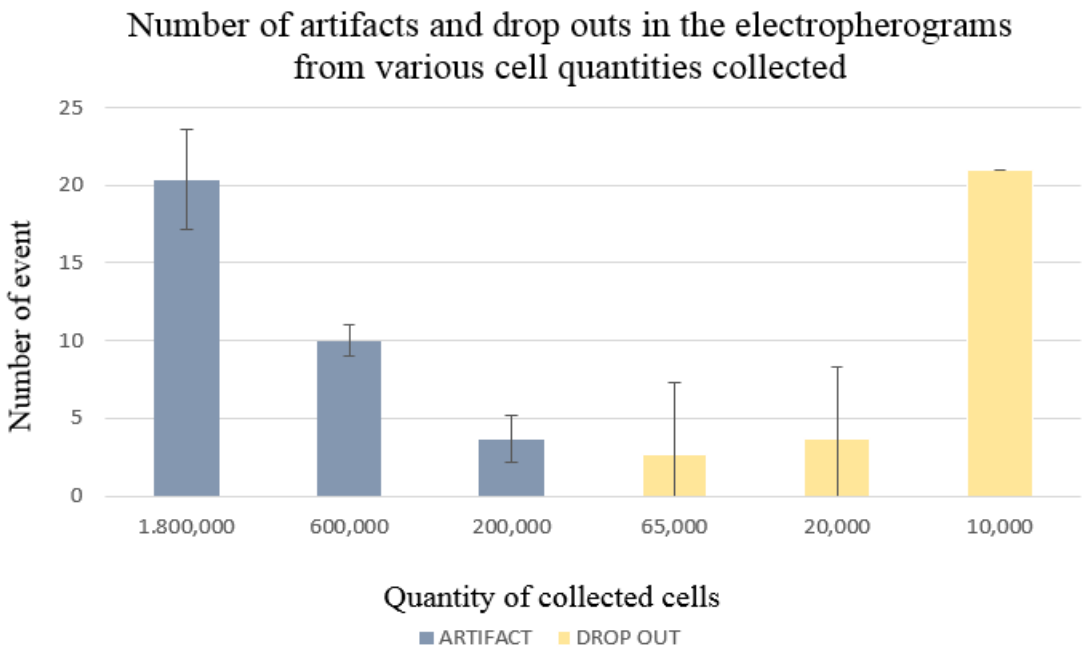


Figure 13. Number of artifacts and allele drop out using different cell quantities for cell line authentication

There were several artifacts in the electropherograms such as pull up, elevated stutter and raised baseline when the number of cells was over 600,000 cells per card as (Figure 14) illustrates in the green (JOE) dye channel. The electropherograms are scaled to 2,000 RFU showing five loci in increasing size: D5S818, D13S317, D7S820, D16S539 and CSF1PO. The black arrows point to the artifact labels. Good quality electropherograms and optimal DNA profile results can be obtained when low number of artifacts and no allele drop out is observed. The electropherograms in (Figure 15) represent the results for cell quantities of 200,000 cells per card and 65,000 cells per card. The electropherograms are scaled to 2000 RFU. The black arrows point to the artifact labels. Allele drop out was observed when the peak height fell below the analytical threshold and the genotyping software did not call the peaks. Allele drop out occurred when the number of cells was lower than 65,000 cells per card. The electropherograms from storage cards containing 20,000 and 10,000 cells per card are illustrated in (Figure 16). The electropherograms are scaled to 2000 RFU. The black arrows point to the drop out peaks.

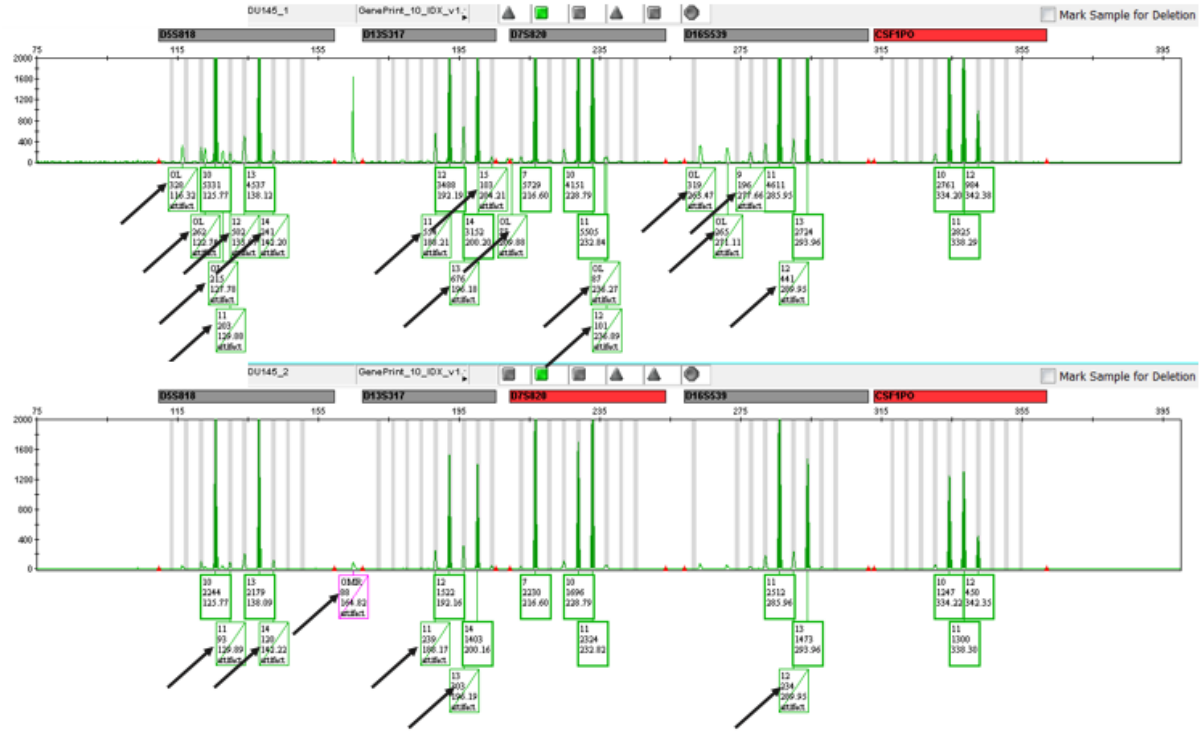


Figure 14. Green dye channels from the electropherograms of storage cards containing 1,800,000 cells per card (top) and 600,000 cells per card (bottom)

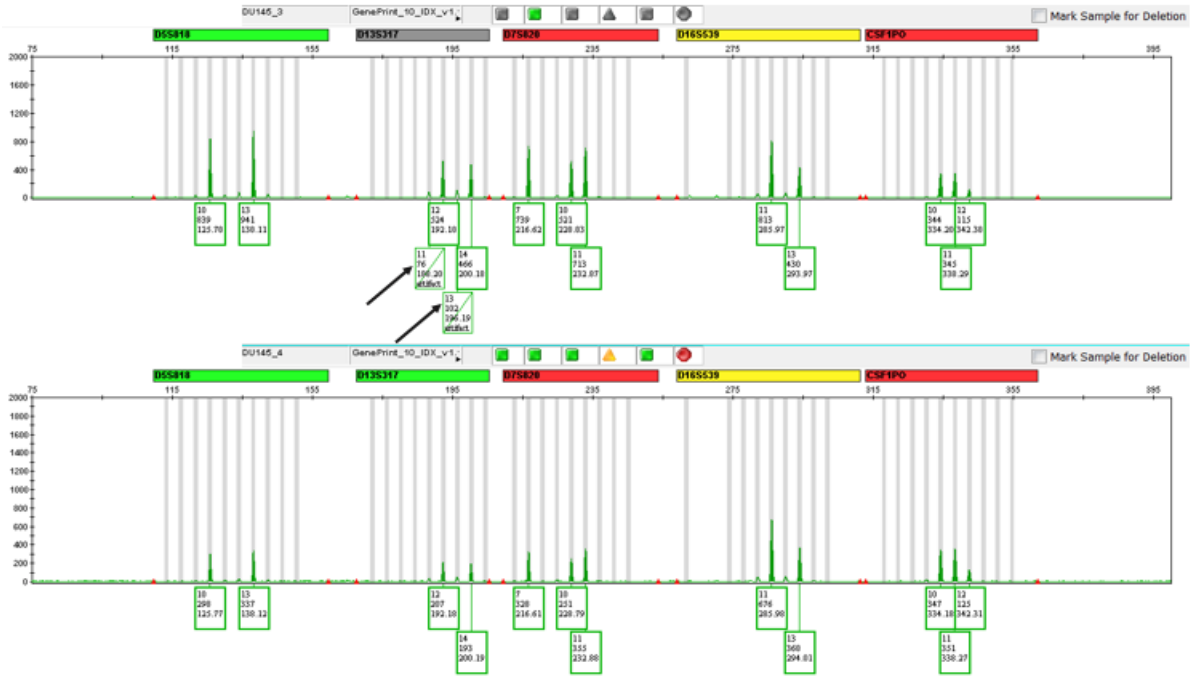


Figure 15. Green dye channels from the electropherograms of storage cards containing 200,000 cells per card (top) and 65,000 cells per card (bottom)

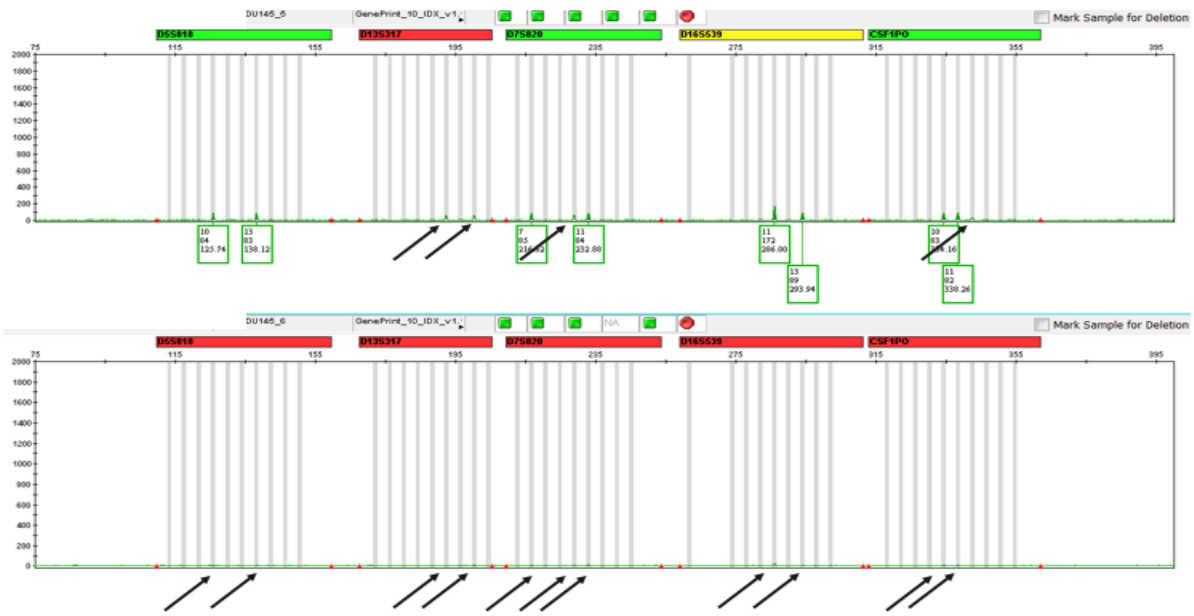


Figure 16. Green dye channels from the electropherograms of storage cards containing 20,000 cells per card (top) and 10,000 cells per card (bottom)

The complete electropherograms corresponding to each cell quantity can be found in (Appendix K). The range of peak heights (PH), average PH and standard deviation corresponding to each cell quantity are shown in (Table 6). The table represents the lowest and highest peaks of triplicate experiments.

Cell Quantity	Lowest Peak Height (RFU)	Highest Peak Height (RFU)	Average Peak Height (RFU)	Standard Deviation
1,800,000	432	7309	2830	1546
600,000	234	3565	1624	659
200,000	115	1805	789	366
65,000	24	676	232	154
20,000	23	523	167	108
10,000	0	61	15	16

Table 6. Peak height range corresponding to each cell quantity

The peak height range corresponding to the cell quantity where low number of artifacts and no drop out was observed was approximately 200 to 1,700 RFU.

Single-Plex PCR reaction for mouse amplicon

The mouse specific primer set was tested in a single-plex reaction with GenePrint® 10 System (Appendix G). DNA from mouse blood was extracted, quantified and 10ng mouse DNA was used as DNA template in the PCR reaction. The mouse specific primer set was labeled with fluorescent dye, fluorescein, which was detected in the blue (FL) dye channel as shown in (Figure 17). The black arrow is pointing to the mouse amplicon in the electropherogram, which is substantially smaller then TH01 locus and such it is easily distinguishable.

Figure 17. Electropherogram of mouse control DNA in single-plex reaction

The size of the mouse amplicon was an average of 83.62 base pair with a standard deviation of 0.36. The primer set was tested on a mouse cell line (Mouse Optic Nerve Astrocyte). The peak detected in the electropherogram for the mouse cell line had strong signal, over 6000RFU.

Single-Plex PCR reaction for mycoplasma amplicon

Mycoplasma specific primer set was tested in a single-plex reaction with HotStarTaq Plus DNA polymerase kit. DNA from a deactivated *Mycoplasma arginini* (*M. arginini*) pellet was extracted, quantified and 1ng mycoplasma DNA was used as DNA template in the PCR reaction. The mycoplasma specific primer set was labeled with fluorescent dye, Fluorescein (6-FAM), which was detected in the blue (FL) dye channel as shown in (Figure 18). The amplification was repeated with GenePrint® 10 System with the same results. The black arrows point to the mycoplasma amplicons in the electropherogram. The mycoplasma peaks had strong signal, saturating the charged-coupled device (CCD) camera of the genetic analyzer causing pull up in the other two dye channels. The size of the amplicons were an average of 243.24 bp with a standard deviation of 0.37 for the shorter fragment and 264.60 bp with a standard deviation of 0.11 for the longer fragment. The shorter fragment is overlapping with the D21S11 locus. Based on the published data, only the longer fragment (270bp) was expected to be observed, which was not overlapping with the D21S11 locus since the longest allele size in D21S11 locus is 261bp (13).

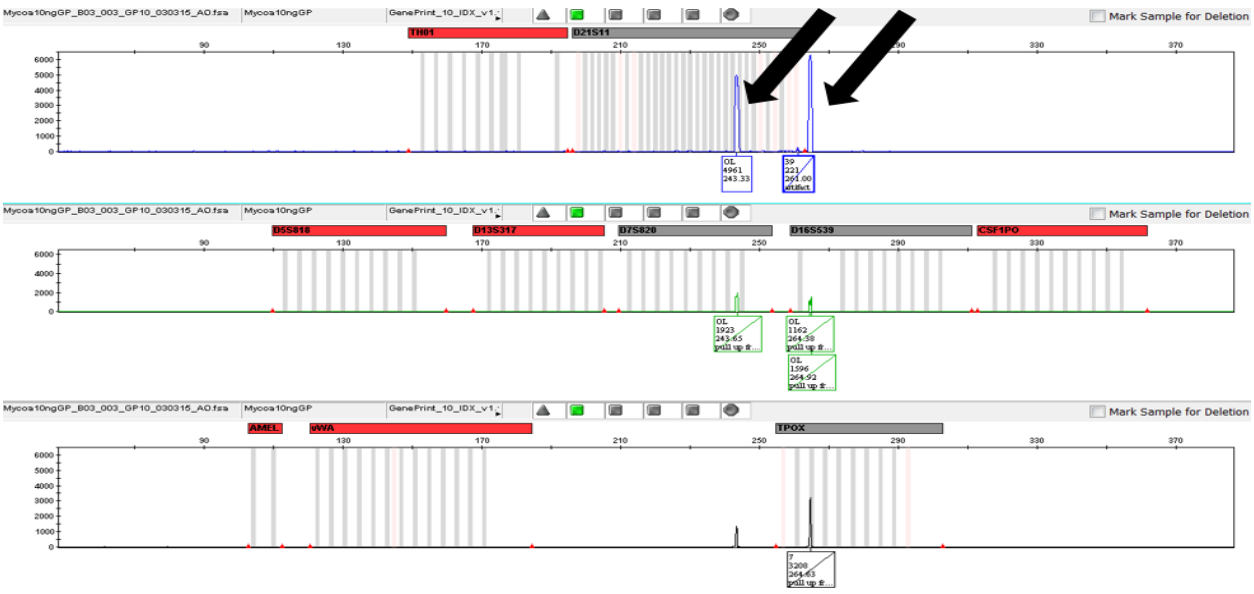


Figure 18. Electropherogram of mycoplasma control DNA in single-plex reaction

Multiplex study

First, the mouse primer set was added to the GenePrint® 10 System PCR reaction with control human DNA (2800M) and control mouse DNA. The reaction amplified all expected human markers and the mouse amplicon.

Second, the mycoplasma primer set was added to the GenePrint® 10 System PCR reaction with control human DNA (2800M) and control mycoplasma DNA (*M. arginini*). The reaction amplified all expected human markers and the mycoplasma amplicons.

In the final step of the multiplex development, both the mycoplasma and mouse primer sets were added to the GenePrint® 10 System PCR reaction with human, mouse and mycoplasma control DNA for simultaneous amplification, which simulates contaminated cell lines. The reaction amplified all expected human markers, mouse and mycoplasma amplicons as shown in (Figure 19). The electropherogram is scaled to 4000RFU. The yellow arrow is pointing to the mouse amplicon, the grey arrows to the human markers and the pink arrows to the mycoplasma amplicons.

The cell line authentication and contamination assessment assay was successfully tested on seven cell lines.

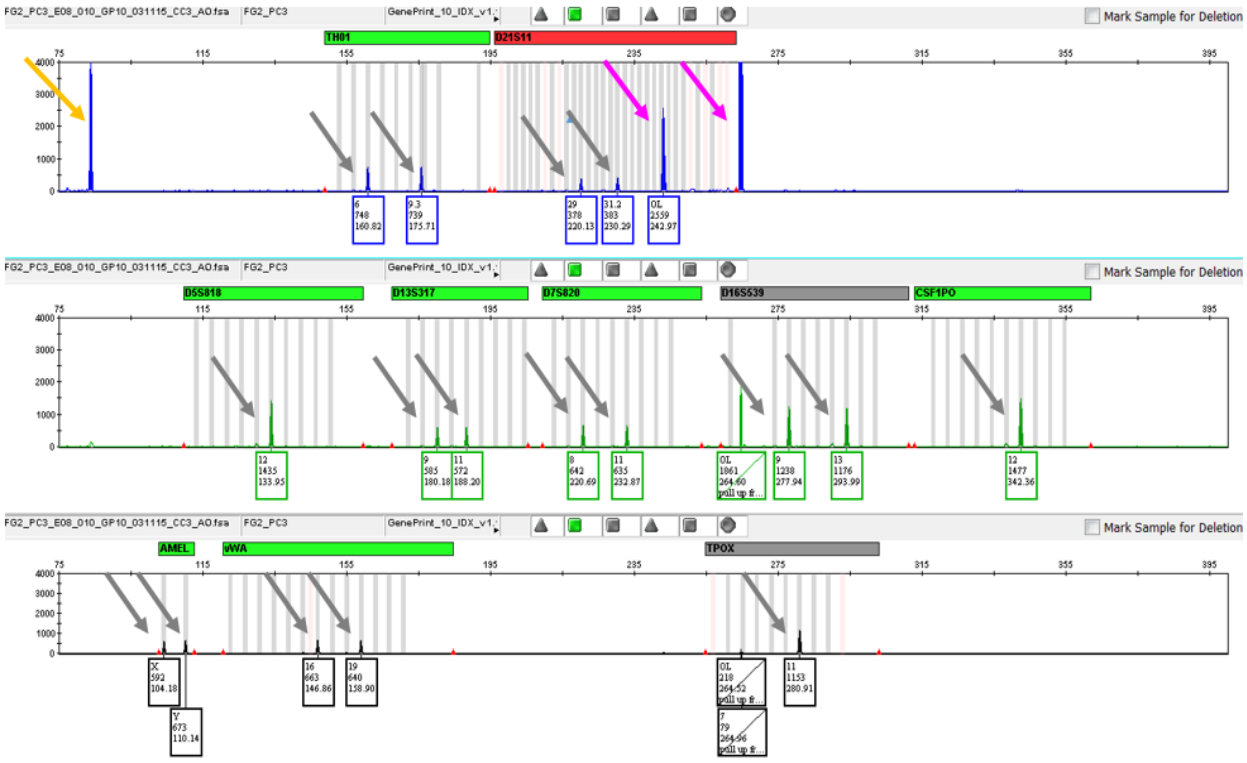


Figure 19. Electropherogram of human, mouse and mycoplasma control DNA in the multiplex reaction with GenePrint® 10 System

Sensitivity study

A sensitivity study was performed to determine the limit of detection for mouse DNA. A dilution series of mouse DNA (10, 1, 0.1 and 0.01ng) was amplified with the GenePrint® 10 System and GenePrint® 5X Mouse Primer Pair Mix (Appendix G). The experiment was performed in triplicates. The electropherograms were analyzed and peak heights of the mouse amplicon in each template quantity were recorded. Average peak heights (PH) and standard deviation was calculated for each DNA quantity and visualized on a bar graph.

The analytical threshold for the Fluorescein, blue dye channel was set to 75RFU. The average detected peak heights of the mouse amplicon fell below the threshold when ≤0.1ng template was amplified as depicted in (Figure 20). The red line represents the analytical threshold.

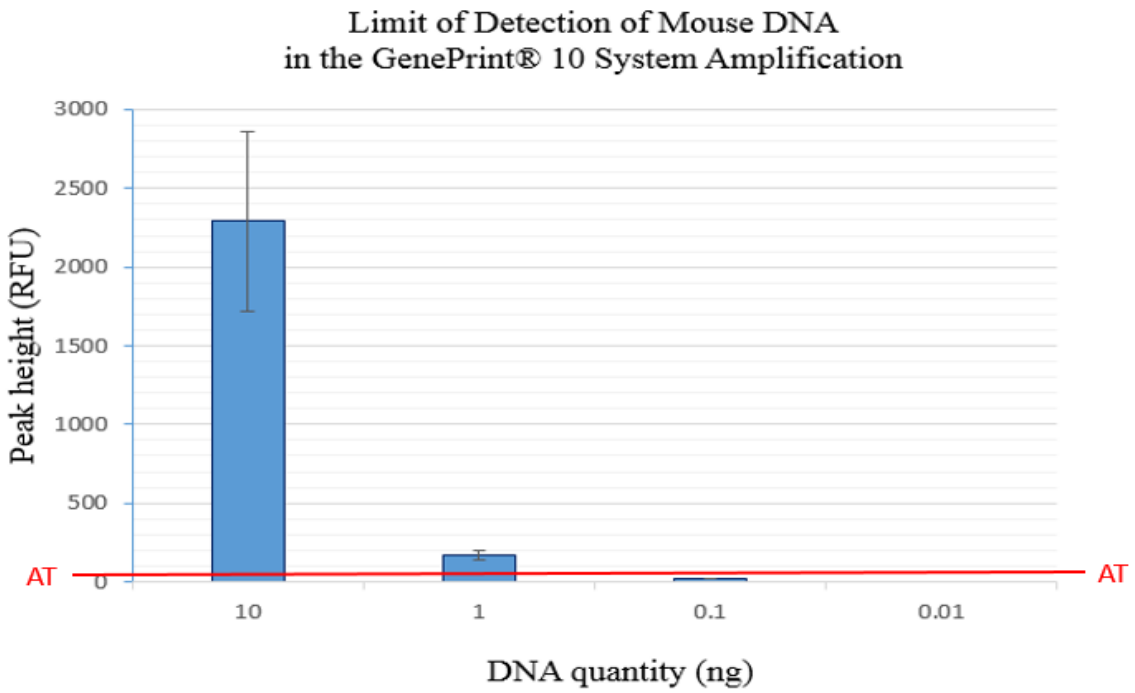


Figure 20. Limit of detection of mouse DNA

A zoomed image of the Fluorescein dye channel of the electropherograms focused on 40-140 bp for each template DNA quantity are shown in (Figure 21). The electropherograms are scaled to 3000RFU. Each line represents a set of experiments. The arrows point to the mouse amplicon.

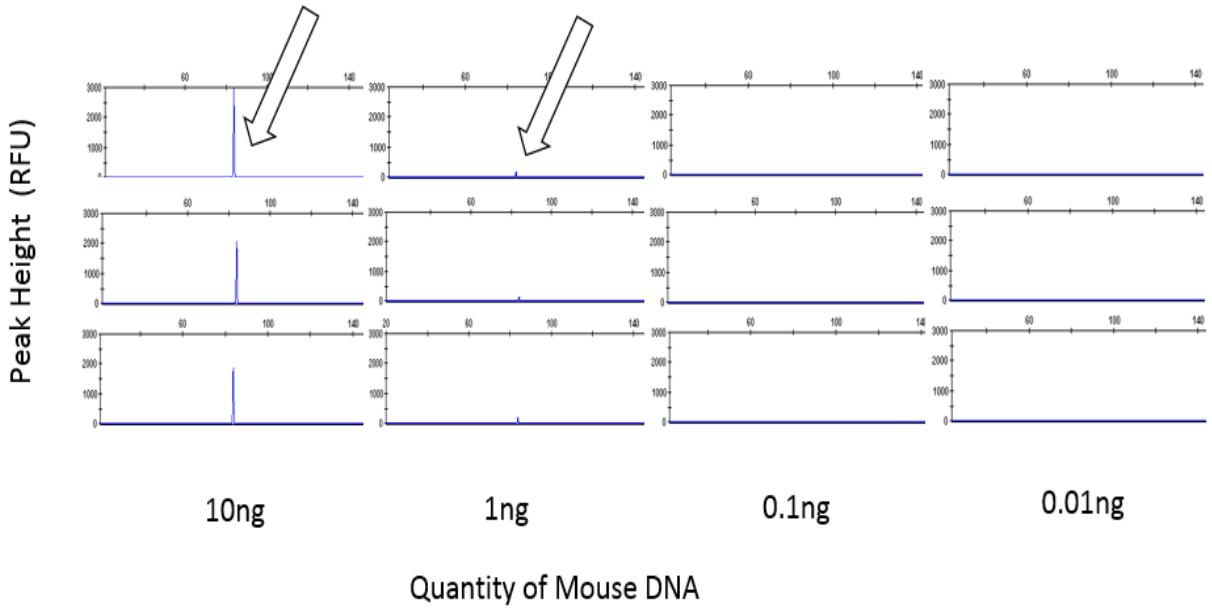


Figure 21. Electropherograms of the mouse amplicons in the Fluorescein dye channel

A sensitivity study was performed to determine the limit of detection for mycoplasma DNA. A dilution series of *M. arginini* DNA (series (1, 0.1, 0.01, 0.001ng = 1pg, 0.0001ng = 0.1pg) was amplified with the GenePrint® 10 System and mycoplasma specific primers. The electropherograms were analyzed and the peak heights of the mycoplasma amplicons in each template quantity were recorded. Average peak heights and standard deviation were calculated for each DNA quantity and visualized on a bar graph. There were two peaks detected in the mycoplasma amplification. The experiment was performed in triplicates.

The analytical threshold for the Fluorescein dye channel was 75RFU. The average detected peak heights of the longer mycoplasma amplicon fall below the threshold when ≤0.001ng template was amplified as depicted in (Figure 22). The light blue bars represent the average peak height of the shorter fragments, the dark blue bars represent the average peak height of the longer fragments. The red line denotes the analytical threshold.

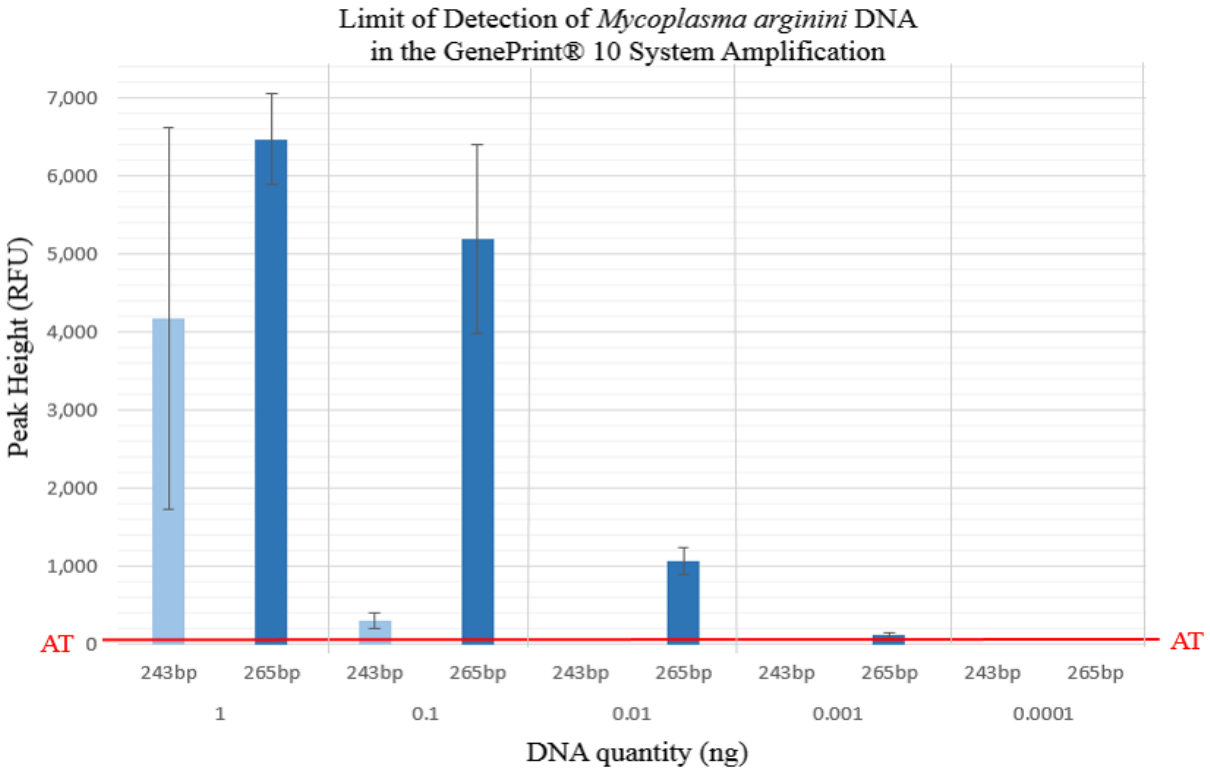


Figure 22. Limit of detection of *Mycoplasma arginini* DNA

At high DNA template concentrations, two mycoplasma amplicons were distinguished. At lower concentrations, only the longer fragment was detected. The peak height ratio of the two fragments decreased from the average of 62.33% with the standard deviation of 34.2 at 1ng template DNA to 5.67% with the standard deviation of 0.58 at 0.1ng template DNA. The shorter fragment was not detected when ≤0.01ng quantity of DNA template was amplified. A zoomed image of the Fluorescein dye channel of the electropherograms focused on 200-280 bp at each template DNA quantity are shown in (Figure 23). The electropherograms are scaled to 6000RFU. Each line represents a set of experiments of the triplicates. The arrows point to the mycoplasma amplicons.

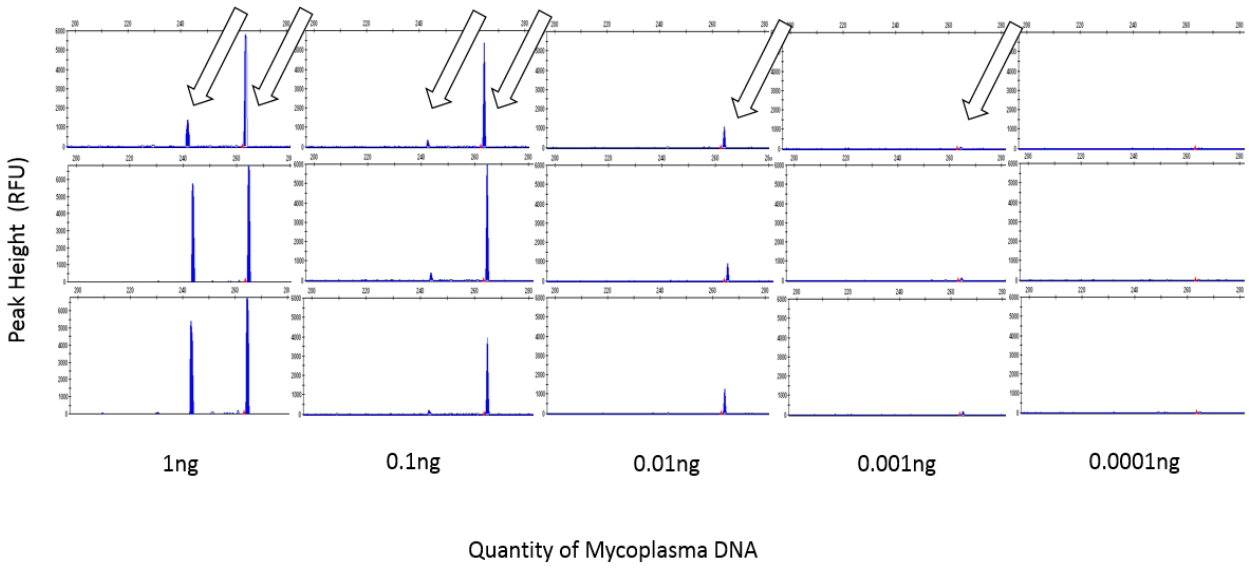


Figure 23. Electropherograms of the mycoplasma amplicons in the Fluorescein dye channel

Half reaction

To make the procedure more cost efficient, a half reaction was tested (Appendix I). Three cell lines were processed as full reactions and half reactions. The electropherograms of the two reactions were compared and provided in (Appendix L). The quality of the electropherograms was similar and the results were concordant.

Mycoplasma locus

In addition to *Mycoplasma arginini*, three other species of mycoplasma, which are known to contaminate cell lines, were tested. Amplification was carried out in half reactions with the GenePrint® 10 System, mycoplasma specific primers and 0.1ng of one of the mycoplasma species *Mycoplasma penetrans*, *Mycoplasma hominis* strain 1620, *Acholeplasma laidlawii* strain PG8 or *Mycoplasma arginini* DNA. The mycoplasma amplicons were different in length among the four species. A zoomed image of the Fluorescein dye channel of the electropherograms focused on 220-295 bp for each species are shown in (Figure 24). Vertical grey lines are used to help distinguish between peaks. The numbers below the peaks represent their amplicon sizes in base pair.

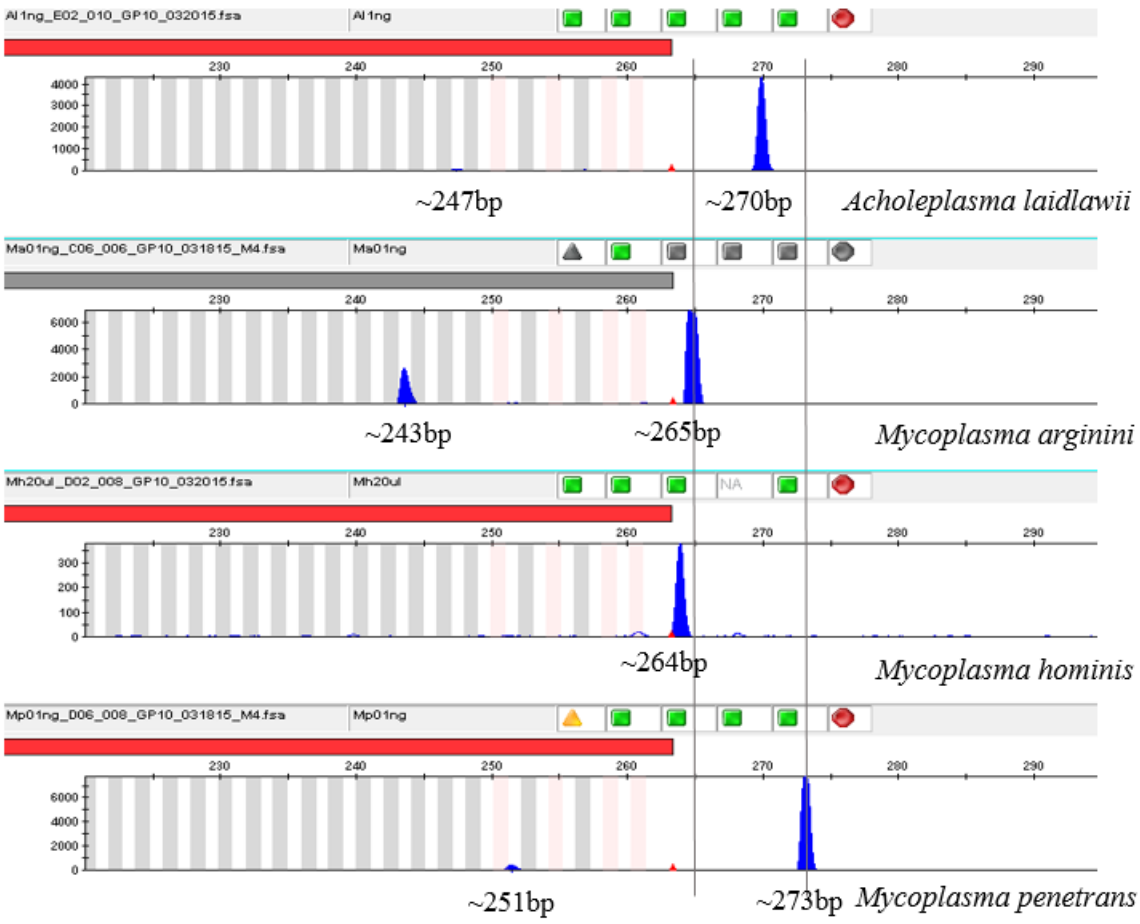


Figure 24. Electropherogram of four different mycoplasma species

Reproducibility

Three human cell line samples were processed in triplicate using two GenePrint® 10 System kits from different lot numbers and two different GeneAmp® PCR System 9700 Thermal Cyclers to determine the reproducibility of the STR based cell line authentication and mouse cell line and mycoplasma contamination assessment process. The reactions were set up with the multiplex half reaction protocol (Appendix I) and the samples were DNA typed and assessed for contamination. The DNA profiles, the presence or absence of mouse cell line and mycoplasma peaks were compared. They were concordant for all three cell line samples.

Cell line authentication

Forty-two cell lines were collected for authentication. Six cell lines originated from other species then human: two from mouse, two from bovine, one from rat and one from hamster.

Eighteen cell lines, listed 1 to 18 in (Table 7) were tested for human markers only. Seven cell lines, listed 19 to 25 in (Table 7) were tested for human and mouse (Mus) markers. Samples 26 to 28 in (Table 7) were not tested. Seven cell lines, listed from 29 to 35 in (Table 7) were tested for human and mycoplasma (Myco) markers. An additional seven cell lines, listed 36 to 42 in (Table 7) were tested using the human, mycoplasma and mouse marker multiplex. Cell line designation in (Table 7) does not correspond to cell line designation in (Table 2) to maintain the anonymity of the results. Researchers were notified of the authentication and contamination result of their cell lines. Twenty-one of the thirty-six tested human cell lines were authentic. Two cell lines did not have available reference DNA profile and thirteen were misidentified from which four did not have human origin. A substantial portion of the tested cell lines (36%) was not authentic. None of thirteen cell lines tested for mouse cell line contamination tested positive.

	Species	CLA	Mus	Myco		Species	CLA	Mus	Myco		Species	CLA	Mus	Myco
1	human	Mis	N/A	N/A	15	human	Auth	N/A	N/A	29	human	Mis	N/A	Pos
2	human	Auth	N/A	N/A	16	human	Auth	N/A	N/A	30	human	Auth	N/A	Pos
3	human	Mis	N/A	N/A	17	human	Auth	N/A	N/A	31	human	N/A	N/A	Neg
4	human	Mis	N/A	N/A	18	hamster	N/A	N/A	N/A	32	human	Mis	N/A	Pos
5	human	Mis	N/A	N/A	19	human	Mis	Neg	N/A	33	rat	N/A	N/A	Neg
6	human	Auth	N/A	N/A	20	human	Mis	Neg	N/A	34	human	N/A	N/A	Pos
7	human	Auth	N/A	N/A	21	human	Auth	Neg	N/A	35	human	Mis	N/A	Pos
8	human	Auth	N/A	N/A	22	human	Auth	Neg	N/A	36	human	Mis	Neg	Pos
9	human	Mis	N/A	N/A	23	human	Auth	Neg	N/A	37	human	Auth	Neg	Neg
10	human	Mis	N/A	N/A	24	human	Auth	Neg	N/A	38	human	Auth	Neg	Pos
11	human	Mis	N/A	N/A	25	mouse	N/A	Pos	N/A	39	human	Auth	Neg	Neg
12	human	Auth	N/A	N/A	26	bovine	N/A	N/A	N/A	40	human	Auth	Neg	Pos
13	human	Auth	N/A	N/A	27	bovine	N/A	N/A	N/A	41	human	Auth	Neg	Pos
14	human	Auth	N/A	N/A	28	mouse	N/A	N/A	N/A	42	human	Auth	Neg	Neg

Table 7. Summary of cell line authentication and contamination assessment results
N/A-not available, CLA- cell line authentication results, Mus- mouse marker results, Myco- mycoplasma marker results, Mis- misidentified, Auth- authenticated, Neg- negative result, Pos- positive result

Fourteen cell lines were tested for mycoplasma contamination of which nine tested positive. The majority of the tested cell lines (64%) were contaminated with mycoplasma. Based on the sizes of the mycoplasma amplicons detected from the contaminated cell lines, at least two different species of mycoplasma were responsible for the contamination. A zoomed image of the Fluorescein dye channel of the electropherograms focused on 227-275 bp for each mycoplasma contaminated cell line is shown in (Figure 25). Vertical lines are used to help distinguish between peaks. The numbers below the peaks represent their amplicon sizes in base pair.

One mouse, one rat and one hamster cell line were tested for human markers; none of which was contaminated.

From the seven human cell lines tested for human, mouse and mycoplasma markers, six were authentic; however, three of the authenticated cell lines had mycoplasma contamination. One was

misidentified and had mycoplasma contamination as well. Only three cell lines out of the seven were authentic and free of mouse cell line and mycoplasma contamination.

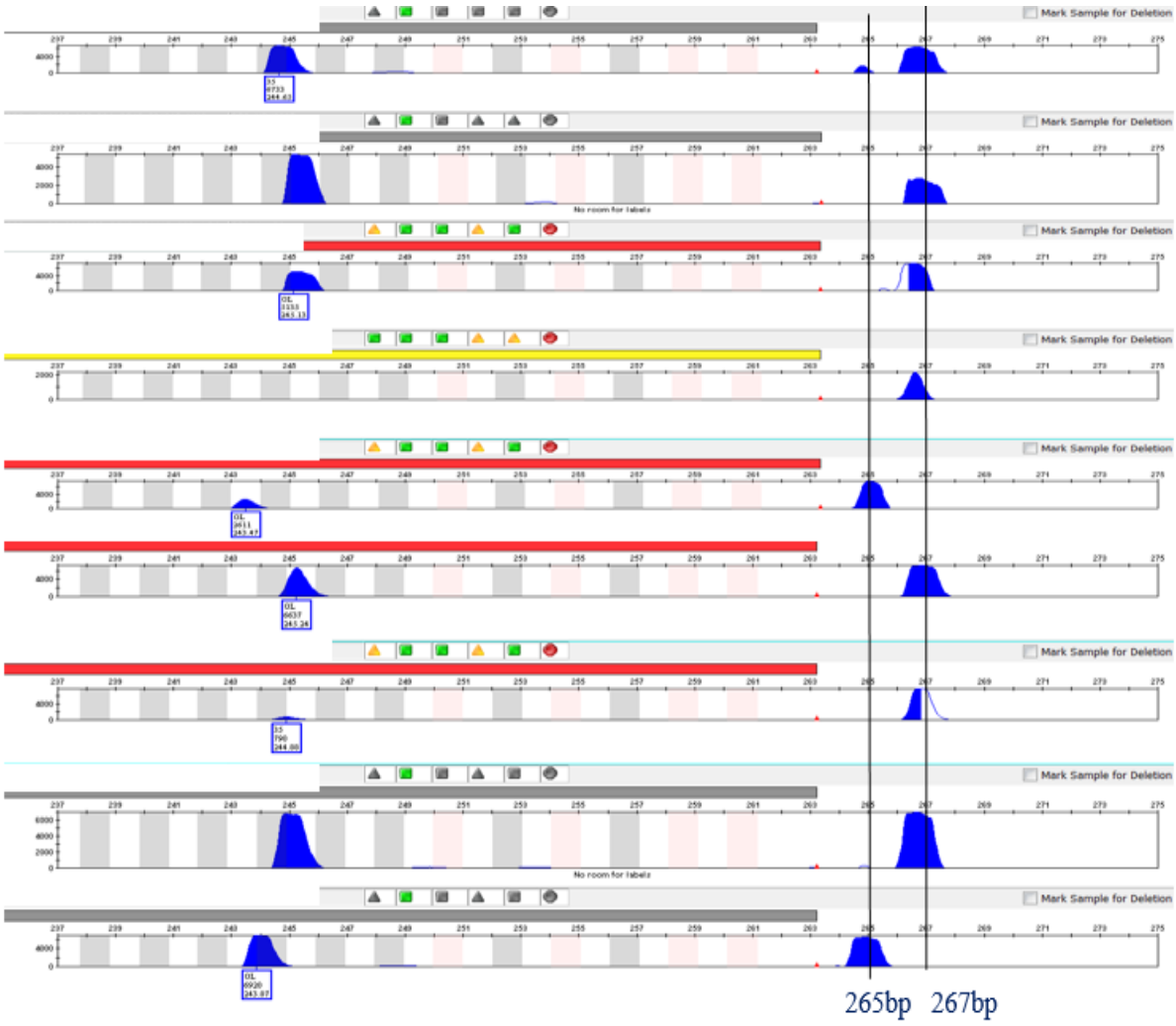


Figure 25. Electropherogram of nine mycoplasma contaminated cell lines

Sixteen of the twenty-one authenticated cell line showed some level of genetic instability. Those cell lines had allele loss or gain. Many instances the additional allele, which was observed in one or two loci had low signal with peak height ratio under 0.3 to the other true alleles in the locus. Genetic instability is well known in cell cultures and a small degree of profile variation, ≤20% is acceptable. Cell lines with ≥80% match to the reference profile are considered authenticated based

on the human cell line authentication standards (ANSI/ATCC ASN-0002-2011). These cell lines accumulate mutations but they are still good representatives of the original tissue type.

Survey

A survey was distributed via email to 100 UNTHSC researchers, with the participation rate of 12%. Twelve responses were evaluated and analyzed. Three quarters of the researchers, nine, who responded use cell lines in their research. All utilize human cell lines, 78% use mouse cell lines and 33% use rat or bovine cell lines as well. The laboratories have a great variation in how many cell lines they have, some less than ten, some over thirty. Based on the responses, the main source of cell lines is obtaining directly from other laboratories followed by purchasing from reputable cell banks and quality controlled repositories. Most (78%) of the researchers expressed concern regarding the authenticity of their cell lines, however only few (22%) use authentication service and only very few (11%) authenticate their cell lines themselves. The cost of authentication they currently use is over $50 per human cell line and does not include contamination assessment. Many researcher (44%) are currently looking for an authentication service while 22% still do not plan to authenticate their cell lines. The majority (78%) of the responders would be interested in mycoplasma screening and many (67%) would be interested in mouse cell line contamination assessment. A responder voiced concern about the proficiency of the tests performed while another survey responder would welcome an in-house cell line authentication service. The Qualtrics analysis report can be found in (Appendix M).

CHAPTER IV

CONCLUSIONS

Cell line authentication and contamination assessment is an essential quality control step for research laboratories using cell cultures. This procedure ensures the reliability and reproducibility of experimental results. It is also a highly recommended procedure for publications using cell lines or requesting funds for research utilizing cell cultures.

In this study a multiplex PCR assay was developed that detects mouse cell line and mycoplasma contamination while performing STR based human cell line authentication in the same time. This assay provides a relatively fast, easy and inexpensive but more comprehensive cell line assessment.

There were several experiments performed to optimize the cell line authentication procedures. Based on the results of the experiments it was found that the optimal amount of cells from the cell culture to blot on the storage card is between 100,000 to 150,000 total cells in 20µl. This cell quantity provides optimal sampling conditions for the direct PCR amplification protocol. A 1.2 mm punch delivers an electropherogram with the least amount of PCR and electrophoretic artifacts while maintaining sufficient DNA to obtain all markers without a possibility of drop out which would reduce the discriminating power or render the results inconclusive. The peak heights of the DNA fragments in the electropherograms with the collection of 100,000 to 150,000 cells in 20µl are the optimal range for the 3130xl DNA analyzer to be detected.

The cell lines can be collected from growing cultures or from frozen stocks for the assay. The experiments showed no considerable difference in the quality of electropherograms. The cells can be resuspended in either PBS or cell line specific medium and still provide quality results.

Artifacts are well known and well characterized in electropherograms from PCR and electrophoretic events. To reduce the occurrence of artifacts due to incomplete denaturation of amplified DNA fragments, a heat denaturation step was tested. Heat denaturation entails the incubation of samples at 95C° followed by snap cooling on ice prior to capillary electrophoresis. Evidence showed that heat denaturation along with the denaturation with HiDi formamide is an essential step to maximize the elimination of artifacts. The number of artifacts decreased approximately 50% especially in TH01 and TPOX loci, improving the quality of the electropherograms. It is necessary to included heat denaturation in electrophoresis set up protocol to ensure optimal results.

The developed cell line authentication and contamination assessment provides three tests in one PCR assay. The GenePrint® 10 System is a kit for the amplification of 10 human markers (CSF1PO, D5S818, D7S820, D13S317, D16S539, TH01, TPOX and vWA) used for cell line authentication. A mouse specific primer set was used for the detection of mouse cell line contamination and a mycoplasma specific primer set to detect various species of mycoplasma contaminants. The primers of the GenePrint® 10 System, the mouse and mycoplasma specific primers perform correctly and reproducibly in one multiplex reaction. The PCR reaction is suitable in half reaction volume (12.5µl), reducing the cost of the cell line authentication and contamination assessment considerably.

There are slight variations in the technique laboratories used to perform cell counting and pipetting, introducing variability in the sample collection. The 20µl cell suspension may not

distribute the cells evenly on the surface of the storage card, which introduces additional variables in sampling conditions. The assay appears robust to these variations and provide reproducible results despite those inconsistencies.

The mouse specific amplicon is detected in the blue dye channel at approximately 84bp. The mycoplasma specific primers were tested on four different mycoplasma species where the sizes of the amplicons were slightly different, detected in the fluorescein (6-FAM) dye channel. There were two amplicons visible when a high concentration of template mycoplasma DNA was amplified. At lower concentrations, only one, the longer (265bp) fragment was detected. In all of the tested samples, the shorter peak (243bp) was detected 22-23 base pair shorter than the longer peak, which also suggests that the shorter peak is a possible artifact. This is important because the shorter fragment is overlapping with the D21S11 locus. The short fragment was not detected without the presence of the long fragment or it did not have a stronger signal intensity in any of the tested samples. The presence of the shorter fragment needs further evaluation. The size of the longer fragments were detected approximately at 264bp for *Mycoplasma hominis* strain 1620, 265bp for *Mycoplasma arginini*, 270bp for *Acholeplasma laidlawii strain PG8* and 273bp *Mycoplasma penetrans*. The differential amplicon sizes suggest that this assay not only detect mycoplasma contamination but also species level can be distinguished. This can be very useful in investigating the origin of contamination, exclude or include possible contamination sources. To accommodate for the mycoplasma and mouse fragments in the new multiplex assay, new bins and panels could be added to the analysis method of the genotyping software to streamline the analysis. In addition, non-nucleotide linker could be added to the mycoplasma primers for better separation of the 6-FAM labelled fragments to provide a better interlocus spacing of D21S11 and mycoplasma locus.

The detection limit for mouse DNA was approximately 1ng in this assay, which corresponds to approximately 200 genomes. The detection limit for mycoplasma was approximately 0.001ng DNA, which corresponds to approximately 2000 genomes.

Cell line authentication was performed on thirty-six human cell lines from which one third was misidentified and four did not have human origin. Fourteen randomly selected cell lines were tested for mycoplasma contamination from which over half was contaminated. Thirteen randomly selected cell lines were tested for mouse cell line contamination but contamination was not found. These results emphasize the importance of this quality control procedure. Cell line authentication is essential as well as the assessment of various contaminants, which can affect cell lines even if the cell line is authentic. This scenario was observed in one quarter of the tested cell lines. The laboratories, which had misidentified cell lines, in this study confirmed that those cell lines were obtained contaminated from other laboratories. They authenticated the earliest passage of the cell lines and found that those were already contaminated. This confirms the importance of cell line authentication at arrival if obtained from other laboratories and emphasize the advantage of obtaining cell lines from reputable cell banks and repositories.

Cell line authentication not only detect cell line cross-contamination but also provide information on the genetic state of the authentic human cell line. The majority (76%) of the tested and authenticated cell lines displayed a low level of genetic instability.

Experiments were planned to verify that mycoplasma contamination could be successfully detected from collected cells without cell culture medium. The cell culture medium is used to perform mycoplasma detection in other common assays. Although, it was not possible to obtain control mycoplasma contaminated human cell lines to evaluate this, several cell line sample were tested positive with high signal, which suggests that it is indeed possible to detect mycoplasma

from infected cells alone. The cell lines, which tested positive for mycoplasma contamination, were collected over three months prior to testing. These findings suggest that mycoplasma along with cultured cells are stable on room temperature blotted on bloodstain card for an extended period of time.

There are a growing number of cell line authentication services available for researchers today. Many of UNTHSC researchers are looking for a cell line authentication service based on the responses from a survey given to one hundred researchers at UNTHSC. Many (66%) would be interested in an in-house service. The majority was interested in the additional contamination screening of mouse cell line and mycoplasma. Based on a cost assessment shown in (Appendix N), the cell line authentication and contamination assessment would be approximately 65$ per human cell line, which is a similar or less expensive what they spend for the cell line authentication service alone without any contamination assessment. The survey could be extended to all research personnel at UNTHSC, the Dallas-Fort Worth metroplex including other universities and research institutes such as University of North Texas in Denton, University of Texas at Arlington, Texas Christian University in Fort Worth, University of Texas Southwestern Medical Center in Dallas as well as Baylor University in Waco. This would assess the regional interest in establishing a cell line authentication and contamination assessment program. The service would allow the local scientists to incorporate cell line authentication and contamination assessment into their research practice while earning more prominence for UNTHSC. If this service is made available at UNTHSC, internal validation of the cell line authentication and contamination assessment assay is necessary to guarantee the accuracy and reliability of the service. Internal validation is an important process in ensuring proper data interpretation and reporting correct results for the requesting scientists.

Many laboratories use cell lines originating from other species such as mouse, rat, bovine, monkey, dog and hamster. Cell line authentication is not readily available for those, non-human cell lines. A species-specific test such as sequencing the CO1 barcode region or expanding the developed multiplex with additional species-specific amplicons would be useful to provide species level identification to those cell lines. Furthermore, additional markers such as gene edited or transformed cell markers could be incorporated into the assay to assess for a more comprehensive cell line characterization. These tests could be a supplementary evaluation to the existing multiplex reaction in the cell line authentication and contamination assessment service.

All these results support the hypothesis that cell line authentication and contamination assessment, which amplifies human STR markers, one mouse housekeeping gene and one mycoplasma housekeeping gene to characterize cell lines and detect most common contamination, is exceptionally useful for genetic and biomedical research laboratories.

APPENDIX

APPENDIX A

Protocol 1. Collection of cell cultures for STR based cell line authentication

Purpose: To collect cell line samples properly and uniformly to ensure the success of downstream cell line authentication. To provide easy to follow instructions for laboratory personnel to prepare cell culture samples for STR-based (short tandem repeat) cell line authentication.

Equipment and Supplies
Laminar flow hood, Pipettes, Pipette tips, Laboratory centrifuge, Basic cell culture laboratory equipment, Hemocytometer and Microscope or Cellometer, Blood storage card (BFC180)

Reagents
Cell culture media, Trypsin, 1X Phosphate buffered saline (1X PBS), Trypan Blue

Procedure

1. Trypsinize cell culture or take a vial of frozen stock.

2. Count cell suspension with hemocytometer, or any other method in your laboratory.

3. Calculate the volume of suspension needed for 200,000 cells.

4. Transfer the volume into 1.5mL Eppendorf tube and centrifuge to collect cells.

5. Resuspend cells in 20µL fresh medium or 1X PBS. (This should be approximately 10,000 cells/µL (1×10^7 cells/mL) concentration.)

6. Label a Whatman® BFC180 Human ID Bloodstain Card (provided) on the designated space on the bottom of the card, located under the circles with the name of the cell line, passage number, date of sampling, and scientist's initials.

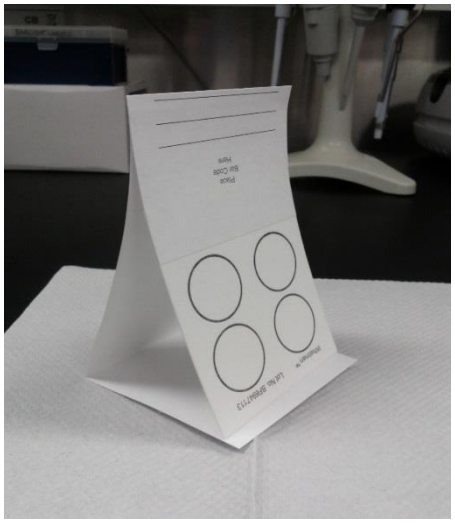
   NOTE: Additional information can be noted on the Excel sheet: "Cell line authentication sample sheet" (provided).

7. Pipette the 20µL of 10,000 cells/µL (1×10^7 cells/mL) suspension to the center of a storage card.

   NOTE: Do not allow wetted spot to touch any surfaces.

8. Dry card on tabletop overnight or until dry at room temperature as the picture shows below. The location should be protected against contamination.

   NOTE: Do not expose spotted card to UV light.



9. Label an envelope with the name of PI, collecting scientist, and laboratory. Place dry card(s) in labeled envelope.

10. Notify Andrea Ormos, Graduate Student, at ao0168@live.unthsc.edu to arrange for drop-off/pick-up of spotted cards.

    NOTE: Spotted cards can be stored indefinitely at room temperature. Multiple cards can be collected at different times and stored in separate envelopes before pick up.

APPENDIX B

Protocol 2. Mycoplasma preparation for DNA extraction

1. Grow mycoplasma in liquid SP4 medium containing fetal bovine serum
2. *M. arginini* and *M. hominis*, the medium is supplemented with 0.25% (w/v) arginine
3. 12mL static cultures grow at 37°C until turbidity or color change in phenol red indicator is observed (toward acidic for *M. penetrans*/*A. laidlawii*; basic for M. *arginini*/*M. hominis*)
4. Centrifuge 10 min at 5000 x g at 15°C
5. Aspirate supernatants, re-suspend pellet in ~1.2mL PBS
6. Place suspension in microcentrifuge tube, re-centrifuge for 5 min at 8000 x g
7. Aspirate supernatants, place ~20 uL PBS over pellet to keep hydrated
8. Freeze overnight
9. Thaw and place at 56°C for 45 min to render non-viable
10. Re-freeze for storage

APPENDIX C

Protocol 3. QIAamp® DNA Mini Kit DNA Purification from Blood or Body Fluids (spin protocol) (Adapted from Manufacturer's Protocol)

Before Starting:
All centrifugation steps are carried out at room temperature (15–25°C).
Equilibrate samples to room temperature (15–25°C).
Heat a water bath or heating block to 56°C.
Ensure that Buffer AW1, Buffer AW2, and QIAGEN Protease have been prepared.

Procedure

1. Pipet 20µL proteinase K into the bottom of a 1.5mL microcentrifuge tube.
2. Add 200µL whole blood sample to the microcentrifuge tube.
3. Add 200µL Buffer AL to the sample. Mix by pulse-vortexing for 15 s.
4. Incubate at 56°C for 10 min.
5. Briefly centrifuge the 1.5mL microcentrifuge tube to remove drops from the inside of the lid.
6. Add 200µL ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s.
7. Centrifuge the 1.5mL microcentrifuge tube to remove drops from the inside of the lid.
8. Carefully apply the mixture to the QIAamp Mini spin column (in a 2mL collection tube) without wetting the rim. Close the cap.
9. Centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2mL collection tube (provided), and discard the tube containing the filtrate.
10. Open the QIAamp Mini spin column and add 500µL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min.
11. Place the QIAamp Mini spin column in a clean 2mL collection tube (provided), and discard the collection tube containing the filtrate.
12. Open the QIAamp Mini spin column and add 500µL Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*;14,000 rpm) for 3 min.
13. Place the QIAamp Mini spin column in a new 2mL collection tube and discard the old collection tube. Centrifuge at full speed for 1 min.
14. Place the QIAamp Mini spin column in a clean 1.5mL microcentrifuge tube. Discard the collection tube containing the filtrate.

15. Add 100 to 200µL Buffer AE to the QIAamp Mini spin column.

16. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.

APPENDIX D

Protocol 4. QIAamp® DNA Mini Kit DNA Purification from Tissues (Adapted from Manufacturer's Protocol)

Before Starting:
All centrifugation steps are carried out at room temperature (15–25°C).
Equilibrate Buffer AE to room temperature (15–25°C).
Heat two water baths or heating blocks one to 56°C, and another to 70°C.
Ensure that Buffer AW1, Buffer AW2 have been prepared.
If a precipitate has formed in Buffer ATL or Buffer AL, dissolve by incubating at 56°C.

Procedure

1. Resuspend pellet in 180µL Buffer ATL.
2. Add 20µL proteinase K and mix by vortexing.
3. Incubate at 56°C for 1 to 3 hours or until tissue is completely lysed. Vortex occasionally.
4. Briefly centrifuge the tube to remove drops from the inside of the lid.
5. Add 200µL Buffer AL to the sample. Mix by pulse-vortexing for 15 s.
6. Incubate at 70°C for 10 min.
7. Briefly centrifuge the tube to remove drops from the inside of the lid.
8. Add 200µL ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s.
9. Centrifuge the tube to remove drops from the inside of the lid.
10. Carefully apply the mixture to the QIAamp Mini spin column (in a 2mL collection tube) without wetting the rim. Close the cap.
11. Centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2mL collection tube (provided), and discard the tube containing the filtrate.
12. Open the QIAamp Mini spin column and add 500µL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min.
13. Place the QIAamp Mini spin column in a clean 2mL collection tube, and discard the collection tube containing the filtrate.
14. Open the QIAamp Mini spin column and add 500µL Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*;14,000 rpm) for 3 min.

15. Place the QIAamp Mini spin column in a new 2mL collection tube and discard the old collection tube. Centrifuge at full speed for 1 min.

16. Place the QIAamp Mini spin column in a clean 1.5mL microcentrifuge tube. Discard the collection tube containing the filtrate.

17. Add 100 to 200µL Buffer AE to the QIAamp Mini spin column.

18. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.

APPENDIX E

Protocol 5. Qubit® dsDNA BR Assay Kit for the quantification of DNA (Adapted from Manufacturer's Protocol)

Before Starting:
Use 0.5mL Qubit® assay tubes.
Bring Qubit® dsDNA BR reagents and buffer to room temperature (22–28˚C).

Procedure

1. Set up the required number of 0.5mL tubes for standards and samples. Label tubes for samples and for the required 2 standards.

2. Make the Qubit® working solution by diluting the Qubit® dsDNA BR reagent 1:200 in Qubit® dsDNA BR buffer for each sample and two standards. (1µL of Qubit® reagent plus 199µL of Qubit® buffer).

3. Pipette 190µL of Qubit® working solution into each of the tubes used for standards.

4. Add 10µL of each Qubit® standard to the appropriate tube, then mix by vortexing 2–3 seconds without creating bubbles.

5. Pipette 198µL Qubit® working solution into individual assay tubes.

6. Add 2µL of DNA sample to assay tube containing the Qubit® working solution for each sample and mix by vortexing for 2–3 seconds. The final volume in each tube should be 200µL.

   Note: Your sample can be 1 to 20µL, therefore, add Qubit® working solution from 180 to 199µL accordingly.

7. Incubate tubes at room temperature for 2 minutes.

8. On the Home Screen of the Qubit® 2.0 Fluorometer, press DNA, then select dsDNA Broad Range as the assay type. The Standards Screen is displayed.

9. On the Standards Screen, select to run a new calibration or to use the last calibration. Press "Yes" to run a new calibration.

10. Insert the tube containing Standard #1 in the Qubit® 2.0 Fluorometer, close the lid, then press "Read". The reading takes approximately 3 seconds. Remove Standard #1.

11. Insert the tube containing Standard #2 in the Qubit® 2.0 Fluorometer, close the lid, then press "Read". Remove Standard #2.

12. Insert a sample tube into the Qubit® 2.0 Fluorometer, close the lid, then press “Read”. After the measurement is completed, the result is displayed on the screen for the diluted sample.

13. To calculate the concentration of the original sample press “Calculate Stock Conc”. The Dilution Calculator Screen containing the volume roller wheel is displayed. Select the volume of your original sample that you added to the assay tube. The Qubit® 2.0 Fluorometer calculates the original sample concentration based on the measured assay concentration. To change the units in which the original sample concentration is displayed, press “µg/mL”. A pop-up window opens, showing the current unit selection.

14. To read the next sample, remove the sample from the Qubit® 2.0 Fluorometer, insert the next sample, then press “Read Next Sample”. Repeat sample readings until all samples have been read.

APPENDIX F

Protocol 6. Cell Line Authentication from Storage Cards Using GenePrint® 10 System: Direct STR Amplification and Capillary Electrophoresis (Adapted from Manufacturer's Protocol)

Purpose
To provide a uniform procedure for direct PCR amplification from Whatman® BFC180 Human ID Bloodstain Card and capillary electrophoresis using Geneprint®10 System for cell line authentication.
The GenePrint®10 system amplifies eight STR loci: CSF1PO, D5S818, D7S820, D13S317, D16S539, TH01, TPOX, vWA required by the standards "Authentication of human cell lines: Standardization of STR profiling" and the gender determining locus Amelogenin.

Equipment and Supplies
Pipettes, Pre-sterilized barrier pipette tips, 0.2mL Strip tubes, 0.65mL and 1.5mL Eppendorf tubes, 96 well semi-skirted PCR plate, Plate rack, Foil seals or strip caps, Crime-Lite™ and safety glasses, UV Stratalinker® 2400, Heat block, Vortex, Eppendorf and Plate centrifuge, Whatman® BFC180 Human ID Bloodstain Card, 1.2mm Harris Micro-Punch, Harris cutting mat, GeneAmp® PCR System 9700, 3130xl DNA analyzer, GeneMapper® ID-X

Reagents
GenePrint® 10 system, 70% Ethanol, 10% Bleach, Ice, UNTHSC Incubation Buffer, HiDi Formamide

Procedure

A. Sampling from Cell Line stained BFC180 Cards

1. Clean bench and cutting mat with 10% bleach, di-water and 70% ethanol. Clean 1.2mm Harris Punch with minimum of three blank punches.

2. Label a UV irradiated 96-well PCR plate or strip tube.

   Note: Use 'GenePrint®10 System Direct Amplification PCR Setup and Layout WS' worksheet for labeling each well on the plate or strip tube.

3. Add 10µL UNTHSC Incubation Buffer to each well.

   Note: Preheat heat block to 70°C.

4. Place one 1.2mm punch per sample into a well.

   Note: Use Crime-Lite™, alternative light source, to visualize the cell line stain when using Harris punch. Yellow protective glasses are mandatory to wear using this alternative light.

5. Incubate plate/tubes uncovered at 70°C for 30 minutes or until dry.

Note: Incubate the full 30 minutes. Shorter incubation times may result in poor performance.

B. PCR reaction set up

1. Thaw reagents, centrifuge tubes briefly and vortex for 15 seconds.

   Note: Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Calculate volumes of reagents for PCR amplification master mix.

   Note: Calculations can be performed on the worksheet. "GenePrint®10 System Direct Amplification PCR Setup and Layout WS". The spreadsheet will automatically calculate the volume needed for each component with a pipetting overage factor. Record lot numbers and expiration dates of reagents on worksheet.

3. Print the completed worksheet for PCR amplification preparation.

4. Prepare PCR amplification master mix for cell line samples and negative control in the no template hood.

Reagent	Volume per Sample (µL)
GenePrint®10 5x Master Mix	5
GenePrint®10 5x Primer Mix	5
Water, Amplification Grade	15
Total reaction volume	25

5. Vortex master mix and aliquot 25µL PCR amplification mix into each well, directly to the processed 1.2mm punch and into the well designated for negative control.

6. Prepare PCR mix for positive control.

Reagent	Volume per Sample (µL)
GenePrint®10 5x Master Mix	5
GenePrint®10 5x Primer Mix	5
Water, Amplification Grade	10
2800M Control DNA,10ng/µL	5
Total reaction volume	25

7. In the DNA hood, add 5µL of 2800M control DNA to the positive control well.

8. Close tubes/seal plate, mix reagents and centrifuge briefly.

C. PCR amplification

1. Place the tubes/plate in the thermal cycler GeneAmp® PCR System 9700.

   Note: Use plate insert when amplifying single tubes or strip tubes.

2. Select the 'cellauth' program. Enter 25µL for the reaction volume and Max mode for ramp speed.

GenePrint®10System thermal cycler program 'cellauth'		
Temperature	Time (minutes)	Number of Cycles
96°C	1:00	
94°C	0:10	26
59°C	1:00	
72°C	0:30	
60°C	10:00	
4°C	Hold	

3. Start the program and sign PCR logbook.

4. After the run is complete, samples can be electrophoresed or store amplified samples at -20°C in a light-protected box.

D. Capillary electrophoresis (CE) set up

1. Prepare instrument, change buffers and water as described in UNT Center for Human Identification Procedure Manual – Research and Development Laboratory "Maintenance and use of 3130xL Genetic analyzer" page 5, buffer replacement. Optional: turn on oven in manual control, protocol page 1.

2. Prepare sample sheet for DNA analyzer described in the manual "Maintenance and use of 3130xL Genetic analyzer" page 12. Click plate manager/new, add plate name, application (GeneMapper Generic), plate type (96-well) , initial for owner and operator then press ok. Fill in sample name in proper wells, result group (GenePrint10) and instrument protocol (GenePrint10_POP6_Long) for all samples.

3. Sign logbook.

4. Thaw reagents, centrifuge ILS600 briefly and vortex for 15 seconds. Centrifuge allelic ladder and mix gently.

   Note: Do not centrifuge ILS600 after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

5. Calculate volumes of reagents for formamide mix.

Reagent	Volume per Sample (µL)
HiDi Formamide	9
GenePrint®10 ILS 600	1
Total volume	10

Note: Calculations can be performed on the worksheet, "GenePrint®10 System Cell Line Authentication Sample Processing Worksheet - CE Plate Set up and Layout" The spreadsheet will automatically calculate the volume needed for each component with a pipetting overage factor. Record lot numbers and expiration dates of reagents on worksheet.

6. Print the completed worksheet for CE preparation.

7. Prepare formamide mix for all samples in the post PCR hood.

8. Vortex formamide mix and aliquot 10µL into each well.

Note: All empty wells in the 16 capillary run need to be filled with 10µL formamide.

9. Add 1µL Allelic ladder to Allelic ladder wells and 1µL PCR product to sample wells.

10. Vortex and centrifuge plate briefly.

11. Heat samples for 3 min at 95°C on heat block or thermal cycler and place plate on ice for 3 minutes.

12. Place the plate in DNA analyzer. In run scheduler, find plate, link plate record to plate and hit run.

13. After run is completed, download data from R&D Data folder from the desktop with designated flash drive. Take the plate off the DNA analyzer and place it in the post PCR fridge.

APPENDIX G

Protocol 7. Amplification of Mouse Amplicon for Cell Line Authentication from Storage Cards Using GenePrint® 10 System: Direct Amplification (Adapted from Manufacturer's Protocol)

This protocol is a supplementary Step B of the Cell Line Authentication from Storage Cards Using GenePrint® 10 System: Direct STR Amplification and Capillary Electrophoresis protocol.

B*. PCR reaction set up for mouse amplicon

1. Thaw reagents, centrifuge tubes briefly and vortex for 15 seconds.

   Note: Do not centrifuge the GenePrint® 5X Mouse Primer Pair Mix and 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Calculate volumes of reagents for PCR amplification master mix.

   Note: Calculations can be performed on the worksheet "GenePrint®10 System Direct Amplification Mouse PCR Setup and Layout WS". The spreadsheet will automatically calculate the volume needed for each component with a pipetting overage factor. Record lot numbers and expiration dates of reagents on worksheet.

3. Print the completed worksheet for PCR amplification preparation.

4. Prepare PCR amplification master mix for cell line samples and negative control in the no template hood.

Reagent	Volume per Sample (µL)
GenePrint®10 5x Master Mix	5
GenePrint® 5x Mouse Primer Pair Mix	5
Water, Amplification Grade	15
Total reaction volume	25

5. Vortex master mix and aliquot 25µL PCR amplification mix into each well, directly to the processed 1.2mm punch and into the well designated for negative control.

6. Prepare PCR mix for positive control.

Reagent	Volume per Sample (µL)
GenePrint®10 5x Master Mix	5
GenePrint® 5x Mouse Primer Pair Mix	5
Water, Amplification Grade	14
Mouse Control DNA,1ng/µL	1
Total reaction volume	25

7. In the DNA hood, add 1µL of Mouse control DNA to the positive control well.

8. Close tubes/seal plate, mix reagents and centrifuge briefly.

APPENDIX H

Protocol 8. Multiplex assay: Amplification of Human STRs, Mouse and Mycoplasma Amplicons for Cell Line Authentication from Storage Cards Using GenePrint® 10 System: Direct Amplification (Adapted from Manufacturer's Protocol)

This protocol is a supplementary Step B of the Cell Line Authentication from Storage Cards Using GenePrint® 10 System: Direct STR Amplification and Capillary Electrophoresis protocol.

B**. PCR reaction set up for multiplex assay

1. Thaw reagents, centrifuge tubes briefly and vortex for 15 seconds.

   Note: Do not centrifuge the GenePrint® 5X Mouse Primer Pair Mix and 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Calculate volumes of reagents for PCR amplification master mix.

   Note: Calculations can be performed on the worksheet "GenePrint®10 System Direct Amplification Mouse PCR Setup and Layout WS". The spreadsheet will automatically calculate the volume needed for each component with a pipetting overage factor. Record lot numbers and expiration dates of reagents on worksheet.

3. Print the completed worksheet for PCR amplification preparation.

4. Prepare PCR amplification master mix for cell line samples and negative control in the no template hood.

Reagent	Volume per Sample (µL)
GenePrint®10 5x Master Mix	5
GenePrint®10 5X Primer Pair Mix	5
GenePrint® 5x Mouse Primer Pair Mix	5
Mycoplasma Primer Pair Mix 10mM each	1
Water, Amplification Grade	9
Total reaction volume	25

5. Vortex master mix and aliquot 25µL PCR amplification mix into each well, directly to the processed 1.2mm punch and into the well designated for negative control.

6. Prepare PCR mix for positive control.

Reagent	Volume per Sample (µL)
GenePrint®10 5x Master Mix	5
GenePrint®10 5X Primer Pair Mix	5
GenePrint® 5x Mouse Primer Pair Mix	5
Mycoplasma Primer Pair Mix 10mM each	1
Water, Amplification Grade	1
2800M Control DNA,10ng/µL	5
Mouse Control DNA,15ng/µL	2
Mycoplasma Control DNA,1ng/µL	1
Total reaction volume	25

7. In the DNA hood, add human, mouse and mycoplasma control DNA to the positive control well.

8. Close tubes/seal plate, mix reagents and centrifuge briefly.

APPENDIX I

Protocol 8. Multiplex assay half reaction: Amplification of Human STRs, Mouse and Mycoplasma Amplicons for Cell Line Authentication from Storage Cards Using GenePrint® 10 System: Direct Amplification (Adapted from Manufacturer's Protocol)

This protocol is a supplementary Step B of the Cell Line Authentication from Storage Cards Using GenePrint® 10 System: Direct STR Amplification and Capillary Electrophoresis protocol.

B***. PCR reaction set up for multiplex assay

1. Thaw reagents, centrifuge tubes briefly and vortex for 15 seconds.

   Note: Do not centrifuge the GenePrint® 5X Mouse Primer Pair Mix and 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Calculate volumes of reagents for PCR amplification master mix.

   Note: Calculations can be performed on the worksheet "GenePrint®10 System Direct Amplification Mouse PCR Setup and Layout WS". The spreadsheet will automatically calculate the volume needed for each component with a pipetting overage factor. Record lot numbers and expiration dates of reagents on worksheet.

3. Print the completed worksheet for PCR amplification preparation.

4. Prepare PCR amplification master mix for cell line samples and negative control in the no template hood.

Reagent	Volume per Sample (µL)
GenePrint®10 5x Master Mix	2.5
GenePrint®10 5X Primer Pair Mix	2.5
GenePrint® 5x Mouse Primer Pair Mix	2.5
Mycoplasma Primer Pair Mix 10mM each	0.5
Water, Amplification Grade	4.5
Total reaction volume	12.5

5. Vortex master mix and aliquot 25µL PCR amplification mix into each well, directly to the processed 1.2mm punch and into the well designated for negative control.

6. Prepare PCR mix for positive control.

Reagent	Volume per Sample (µL)
GenePrint®10 5x Master Mix	2.5
GenePrint®10 5X Primer Pair Mix	2.5
GenePrint® 5x Mouse Primer Pair Mix	2.5
Mycoplasma Primer Pair Mix 10mM each	0.5
Water, Amplification Grade	0.5
2800M Control DNA,10ng/µL	2.5
Mouse Control DNA,15ng/µL	1
Mycoplasma Control DNA,1ng/µL	0.5
Total reaction volume	12.5

7. In the DNA hood, add human, mouse and mycoplasma control DNA to the positive control well.

8. Close tubes/seal plate, mix reagents and centrifuge briefly.

APPENDIX J

Survey questions downloaded from Qualtrics.

Dear Researcher, My name is Andrea Ormos, I am second year forensic genetics master student under the supervision of Dr. Eisenberg. I would like to ask you to participate in my thesis research project by filling out this short survey investigating your interest in cell line authentication. I am evaluating a need for a possible cell line authentication service at UNTHSC. Thank you so much for your time. Sincerely, Andrea Ormos

Q1 INFORMED CONSENT Your responses are anonymously collected and analyzed by Qualtrics. They are used only for the purpose it was collected for. No personal information is linked to your responses. Have you received and read the research statement and do you agree with the conditions and consent to use your anonymous responses for Andrea Ormos's thesis.

- ❍ Yes (1)
- ❍ No (2)

If No Is Selected, Then Skip To End of Survey

Q2 Do you use cell lines in your research?

- ❍ Yes (1)
- ❍ No (2)

If No Is Selected, Then Skip To Any additional comments?

Q3 What type of cell lines do you use in your laboratory? Please mark all that apply.

- ❑ Human (1)
- ❑ Mouse (2)
- ❑ Rat (3)
- ❑ Bovine (4)
- ❑ Hamster (5)
- ❑ Other (6)

Q4 How many cell lines do you have in your laboratory?

- ❍ More than 30 (1)
- ❍ More than 10 less than 30 (2)
- ❍ Less than 10 (3)

Q5 Where do you obtain your cell lines from? Please mark all that apply.

- ❑ Purchase them from cell banks or repositories such as ATCC and Children Oncology Group (1)
- ❑ Obtain them form other laboratories (2)
- ❑ I prepare my own cell lines (3)
- ❑ Other (4)

Q6 Are you concerned about the authenticity of your cell lines?

- ❍ Yes (1)
- ❍ No (2)

Q7 How much do you pay for cell line authentication service per human cell line?

- ❍ $100 or more (1)
- ❍ between $50 and $100 (2)
- ❍ less than $50 (3)
- ❍ I am not using cell line authentication service (4)

Q8 Would you be interested in using a human cell line authentication service at UNTHSC if it was available?

- ❍ Yes, I am authenticating my cell lines elsewhere but it would be more convenient to use an in house service (1)
- ❍ Yes, I am looking for a service to authenticate my cell lines (2)
- ❍ No, I am authenticating my cell lines elsewhere and I am happy with the service (3)
- ❍ No, I am authenticating my cell lines myself (4)
- ❍ No, I don't plan to authenticate my cell lines (5)

Q9 Would you be interested in mycoplasma contamination assessment in addition to human cell line authentication?

- ❍ Yes (1)
- ❍ No (2)

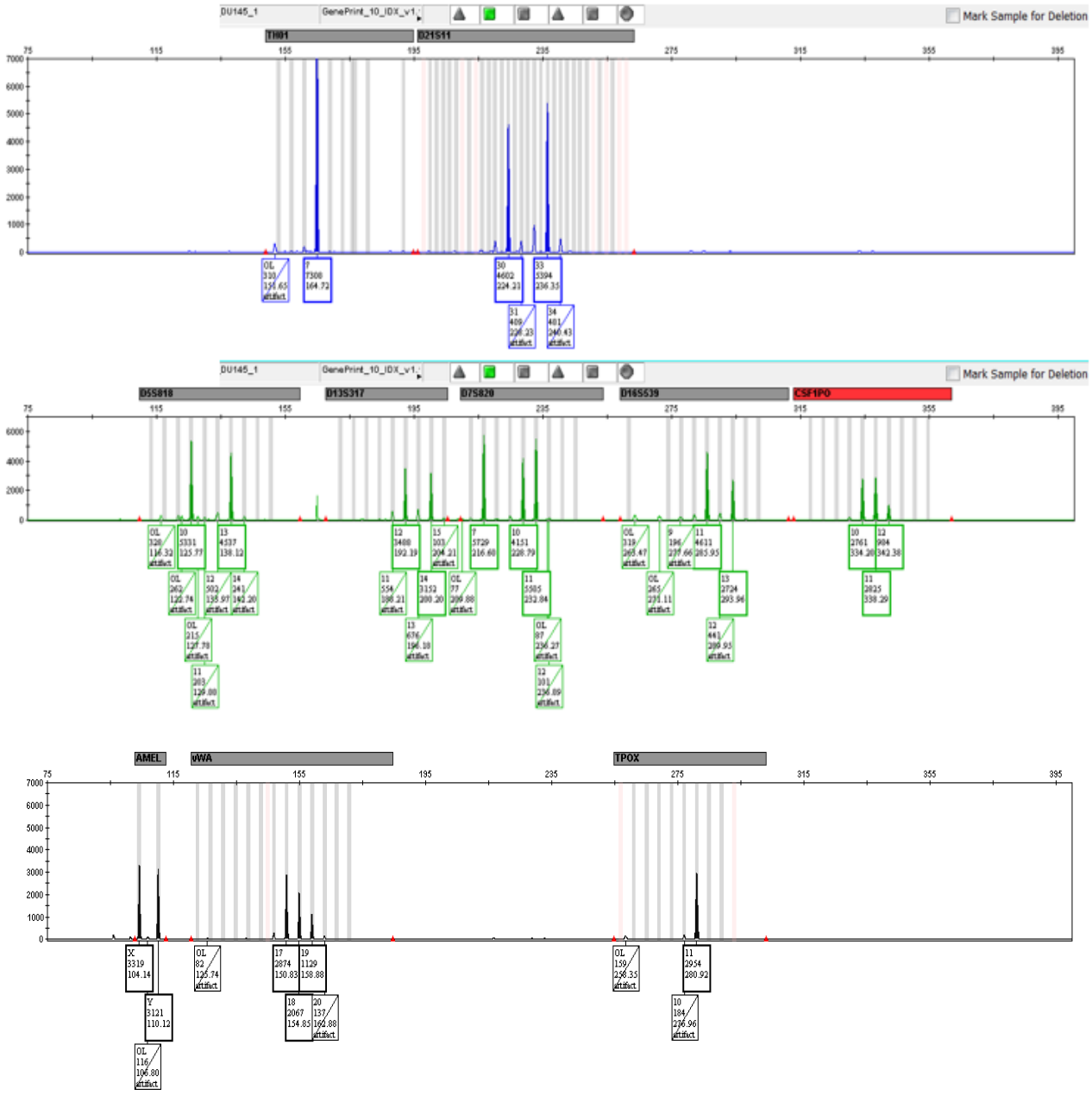
Q10 Would you be interested in mouse cell line contamination assessment of human cell lines in addition to human cell line authentication?

- ❍ Yes (1)
- ❍ No (2)

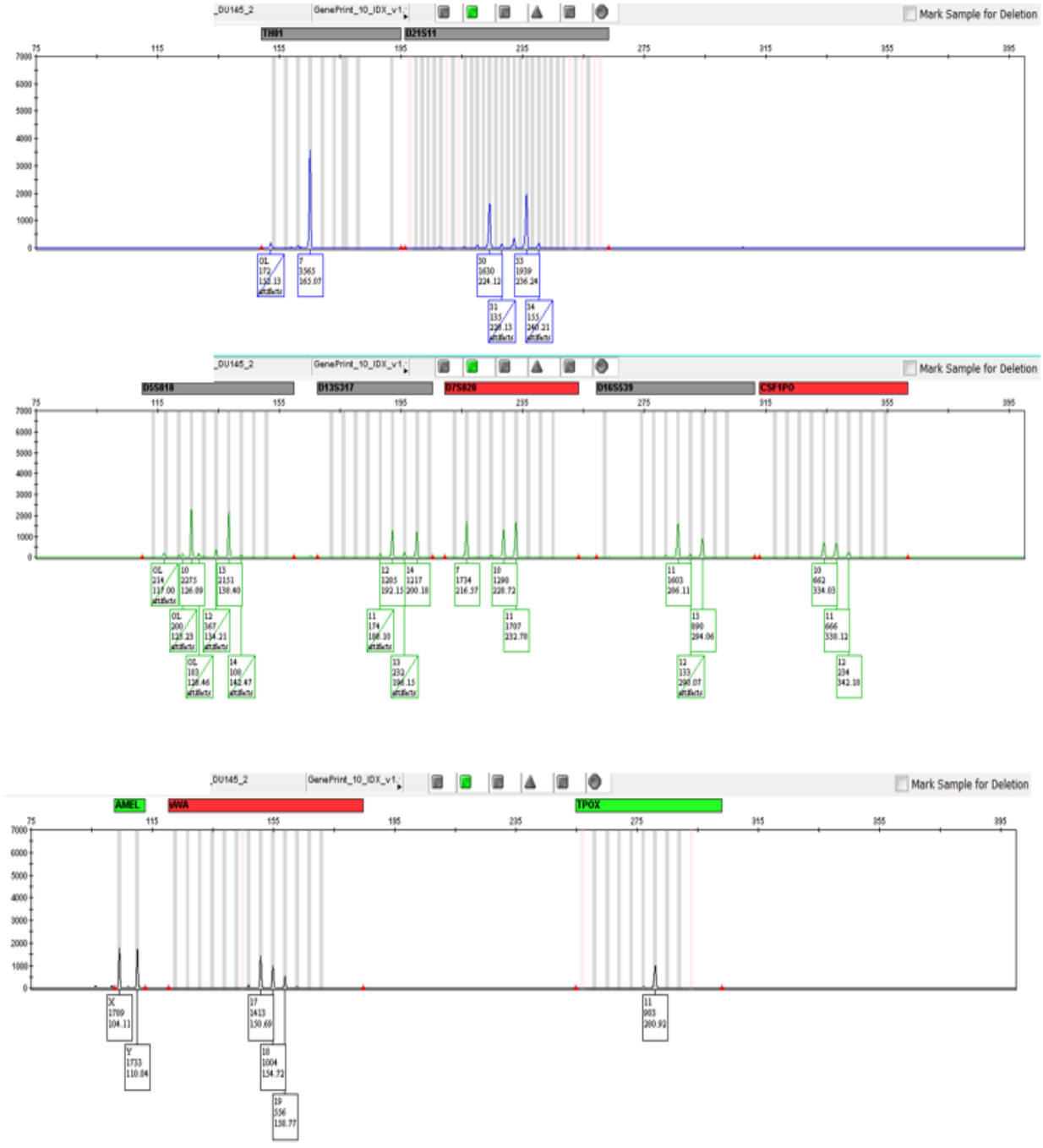
Q11 Do you have any additional comments?

APPENDIX K

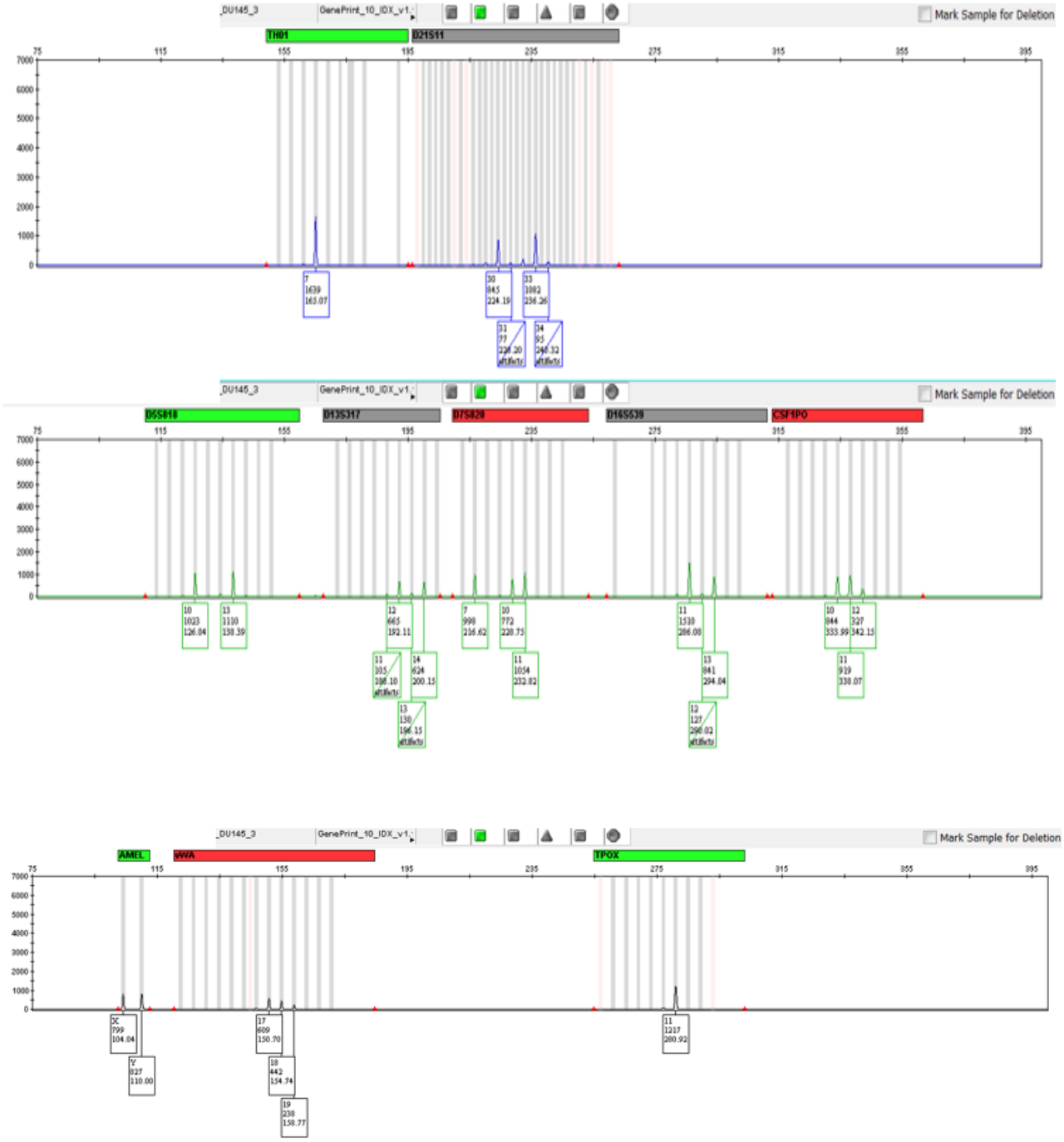
Electropherogram of DU-145 cell line sample from storage cards containing 1,800,000 cells per card. The electropherogram is scaled to 7000 RFU.



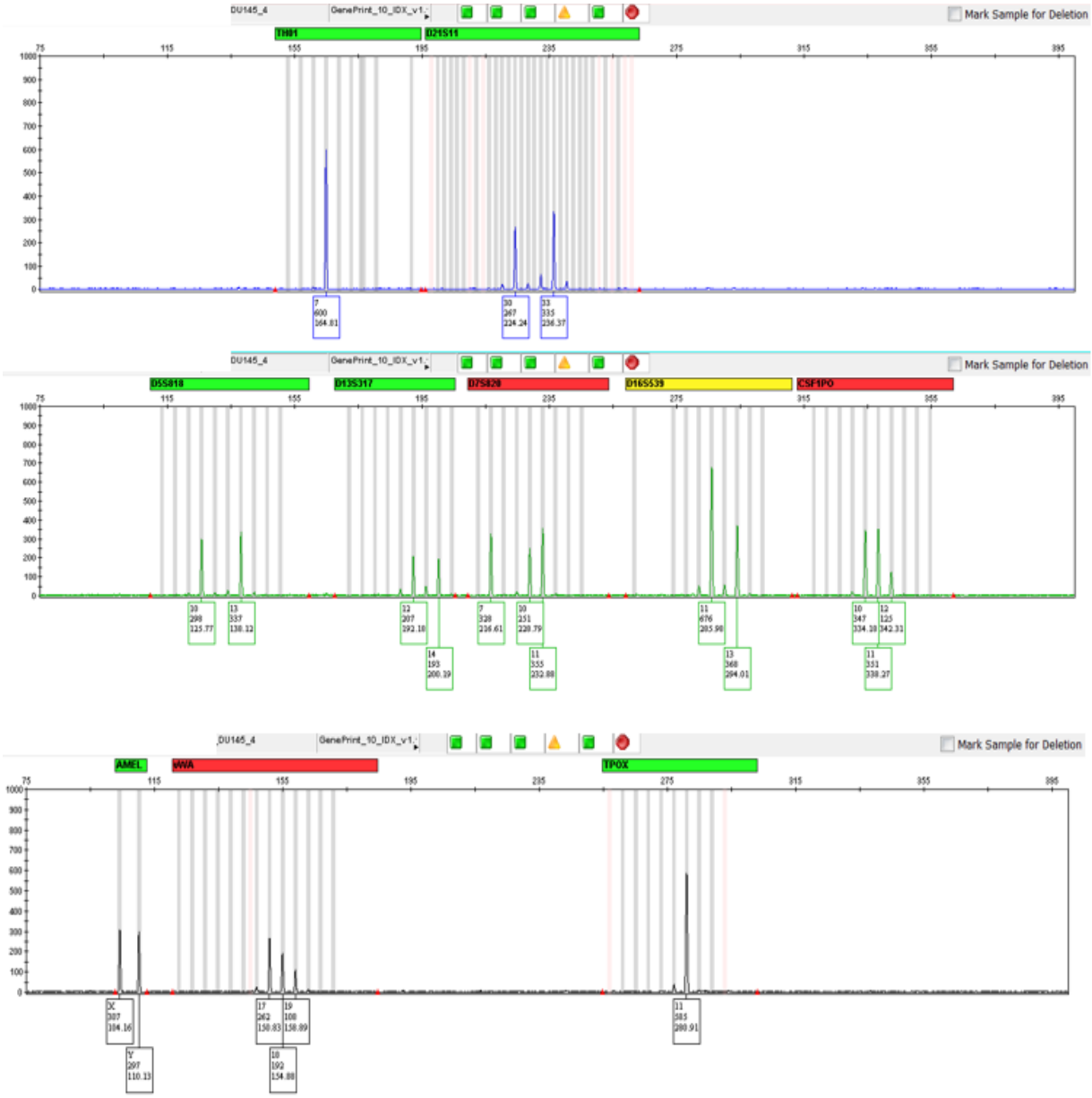
Electropherogram of DU-145 cell line sample from storage cards containing 600,000 cells per card. The electropherogram is scaled to 7000 RFU.



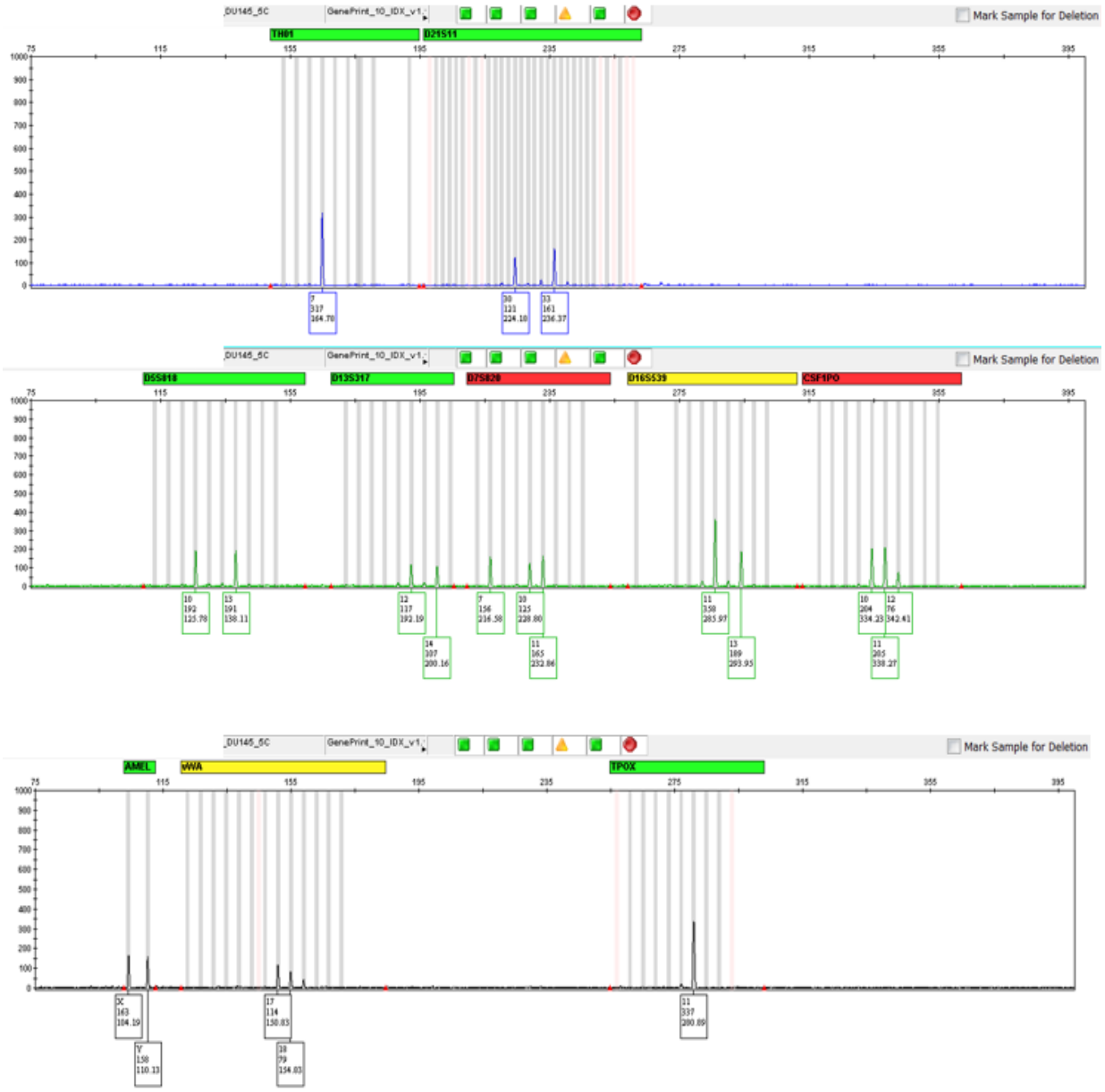
Electropherogram of DU-145 cell line sample from storage cards containing 200,000 cells per card. The electropherogram is scaled to 7000 RFU.



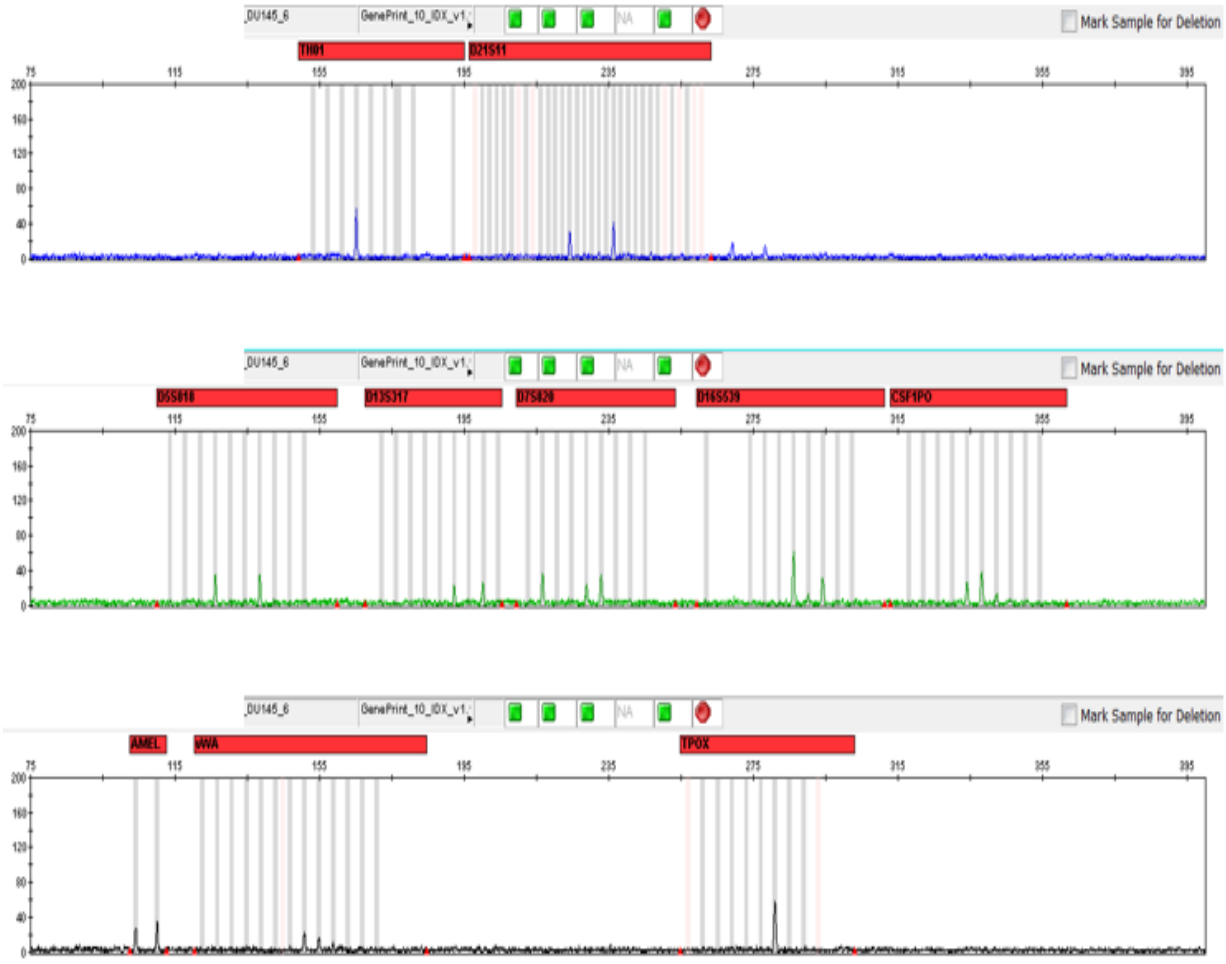
Electropherogram of DU-145 cell line sample from storage cards containing 65,000 cells per card. The electropherogram is scaled to 1000 RFU.



Electropherogram of DU-145 cell line sample from storage cards containing 20,000 cells per card. The electropherogram is scaled to 1000 RFU.

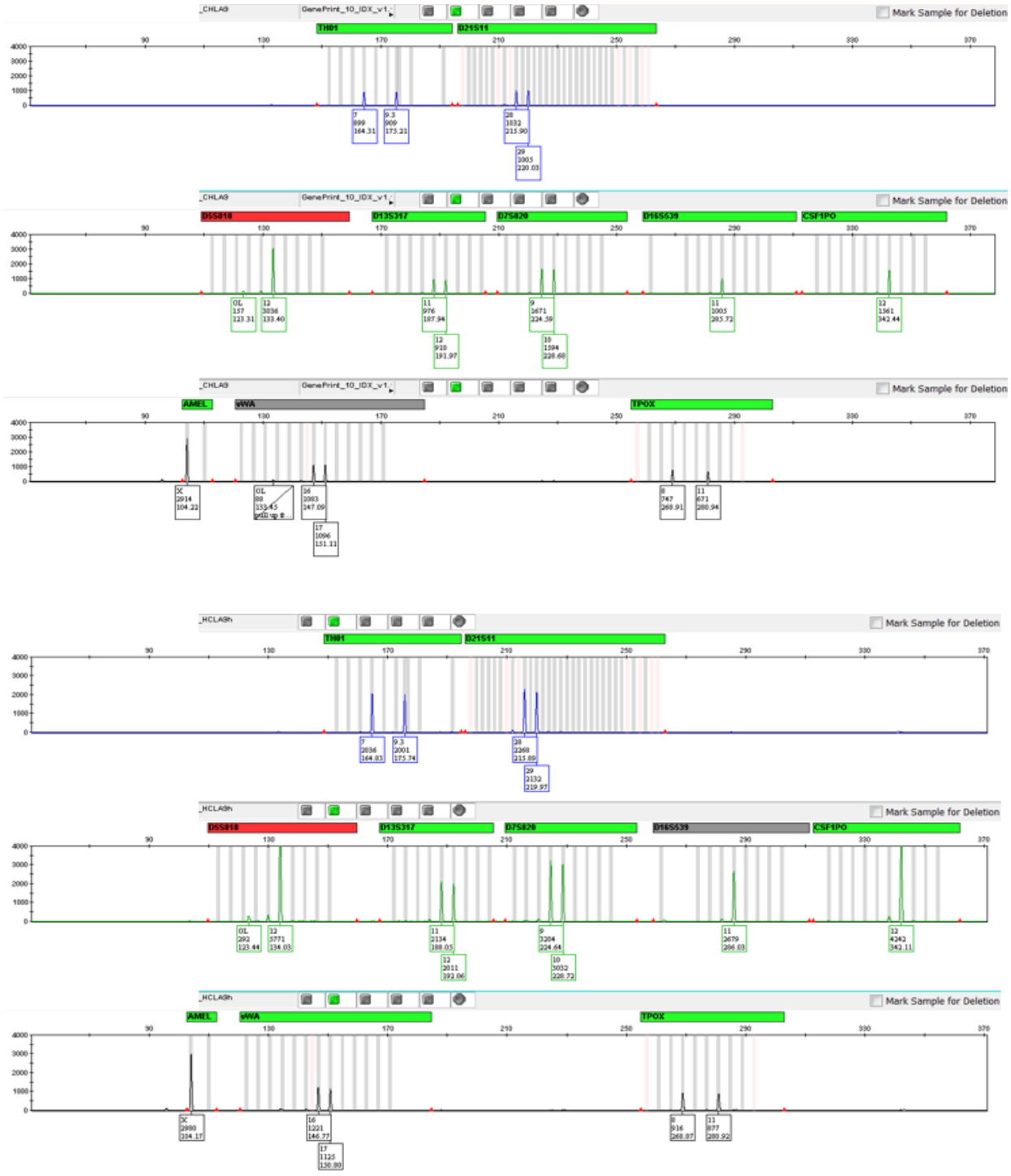


Electropherogram of DU-145 cell line sample from storage cards containing 10,000 cells per card. The electropherogram is scaled to 200 RFU.



APPENDIX L

Electropherogram of CHLA-9 cell line sample from a full PCR reaction (top) and half reaction (bottom). The electropherogram is scaled to 4000 RFU.



APPENDIX M

Analysis of survey question responses downloaded from Qualtrics.

1. INFORMED CONSENT Your responses are anonymously collected and analyzed by Qualtrics. They are used only for the purpose it was collected for. No personal information is linked to your responses. Have you received and read the research statement and do you agree with the conditions and consent to use your anonymous responses for Andrea Ormos's thesis.

#	Answer	Bar	Response	%
1	Yes		12	100%
2	No		0	0%
	Total		12	

2. Do you use cell lines in your research?

#	Answer	Bar	Response	%
1	Yes		9	75%
2	No		3	25%
	Total		12	

3. What type of cell lines do you use in your laboratory? Please mark all that apply.

#	Answer	Bar	Response	%
1	Human		9	100%
2	Mouse		7	78%
3	Rat		3	33%
4	Bovine		3	33%
5	Hamster		0	0%
6	Other		0	0%

4. How many cell lines do you have in your laboratory?

#	Answer	Bar	Response	%
1	More than 30		3	33%
2	More than 10 less than 30		3	33%
3	Less than 10		3	33%
	Total		9	

5. Where do you obtain your cell lines from? Please mark all that apply.

#	Answer	Bar	Response	%
1	Purchase them from cell banks or repositories such as ATCC and Children Oncology Group		7	78%
2	Obtain them form other laboratories		8	89%
3	I prepare my own cell lines		4	44%
4	Other		0	0%

6. Are you concerned about the authenticity of your cell lines?

#	Answer	Bar	Response	%
1	Yes		7	78%
2	No		2	22%
	Total		9	

7. How much do you pay for cell line authentication service per human cell line?

#	Answer	Bar	Response	%
1	$100 or more		1	11%
2	between $50 and $100		1	11%
3	less than $50		0	0%
4	I am not using cell line authentication service		7	78%
	Total		9	

8. Would you be interested in using a human cell line authentication service at UNTHSC if it was available?

#	Answer	Bar	Response	%
1	Yes, I am authenticating my cell lines elsewhere but it would be more convenient to use an in house service		2	22%
2	Yes, I am looking for a service to authenticate my cell lines		4	44%
3	No, I am authenticating my cell lines elsewhere and I am happy with the service		0	0%
4	No, I am authenticating my cell lines myself		1	11%
5	No, I don't plan to authenticate my cell lines		2	22%
	Total		9	

9. Would you be interested in mycoplasma contamination assessment in addition to human cell line authentication?

#	Answer	Bar	Response	%
1	Yes		7	78%
2	No		2	22%
	Total		9	

10. Would you be interested in mouse cell line contamination assessment of human cell lines in addition to human cell line authentication?

#	Answer	Bar	Response	%
1	Yes		6	67%
2	No		3	33%
	Total		9	

11. Do you have any additional comments?

Text Response
My concern is how proficient in preforming the tests
none.
It would be really good to have an in-house cell line authentication core facility.
No.

APPENDIX N

Cost assessment of Cell line authentication and contamination assessment assay for one human cell line.

Expense	Cost	Cost/Sample
GenePrint® 10 System kit	$600	$8
Whatman® BFC180 Human ID Bloodstain Card	$100	$1.50
Mouse primers	$275	$7.00
Mycoplasma primers	$100	$0.10
POP6 Polymer	$560	$1
Consumables	supply	$2
Personnel	salary	$25
Subtotal		$45
UNTHSC surcharge	45%	$20
Total cost		$65

REFERENCES

1. Gey GO, Coffman WD, Kubicek MT. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res 1952;12:364–5.

2. http://www.atcc.org/Products/Cells_and_Microorganisms/Cell_Lines/Animal.aspx

3. Davis JM. Basic cell culture. 2nd ed. New York: Oxford University Press, 2002.

4. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res 1961 Dec;25:585-621.

5. Skloot R. The immortal life of Henrietta Lacks. New York: Random House, 2010.

6. Chatterjee R. Cases of mistaken identity. Science 2007 Feb;315(5814):928-31.

7. Freshney RI. Culture of animal cells: A manual of basic technique and specialized applications. 6th ed. New Yersey: Wiley-Blackwell, 2010.

8. Capes-Davis A, Theodosopoulos G, Atkin I, Drexler HG, Kohara A, MacLeod RA, et al. Check your cultures! A list of cross-contaminated or misidentified cell lines. Int J Cancer 2010 Jul;127(1):1-8.

9. Marx V. Cell-line authentication demystified. Nat Methods 2014 Apr;11(2932):483-8.

10. http://www.vanderbilt.edu/viibre/CellCultureBasicsEU.pdf

11. Ryan J. Understanding and managing cell culture contamination. Corning, NY: Corning Inc., 2008.

12. Henderson GP, Jensen GJ. Three-dimensional structure of *Mycoplasma pneumoniae's* attachment organelle and a model for its role in gliding motility. Mol Microbiol 2006 Apr;60(2):376-85.

13. Young L, Sung J, Stacey G, Masters JR. Detection of mycoplasma in cell cultures. Nat Protoc. 2010 May;5(5):929-34.

14. Nelson-Rees WA, Daniels DW, Flandermeyer RR. Cross-contamination of cells in culture. Science 1981;212(4493):446-52

15. Fogh J, Holmgren N. A review of cell culture contaminations. In Vitro 1971;7(1):26-41

16. ATCC Standards Development Organization. Authentication of human cell lines: Standardization of STR profiling. Manassas, VA: ATCC Standards Development Organization, 2011.

17. http://iclac.org/

18. Capes-Davis A, Freshney I. Database of cross-contaminated or misidentified cell lines.Version 7.2, International Cell Line Authentication Committee (ICLAC); 2014.

19. Hammond HA, Jin L, Zhong Y, Caskey CT, Chakraborty R. Evaluation of 13 short tandem repeat loci for use in personal identification applications. Am J Hum Genet 1994 Jul;55(1):175-89.

20. Stacey G, Bolton B, Doyle A, Griffiths B. DNA fingerprinting--a valuable new technique for the characterization of cell lines. Cytotechnology 1992;9(1-3):211-6.

21. Promega Corporation. GenePrint® 10 system Technical Manual TM392: Instructions for use of product B9510; 2013.

22. http://grants.nih.gov/grants/guide/notice-files/NOT-OD-08-017.html

23. http://www.nih.gov/about/reporting-preclinical-research.htm

24. http://www.npr.org/blogs/health/2014/12/09/368491013/mistaken-identities-plague-lab-work-with-human-cells

25. http://www.npr.org/blogs/health/2014/12/10/368776420/scientists-often-skip-a-simple-test-that-could-verify-their-work

26. http://gbsi.org/summitwebcast

27. http://www.sciencemag.org/content/347/6225/938.summary

28. Reid Y, Storts D, Riss T, Minor L. Authentication of human cell lines by STR DNA profiling analysis. In: Sittampalam GS, Coussens NP, Nelson H, et al., editors. Assay Guidance Manual. 2013;1-22

29. http://www.qiagen.com/us/resources/resourcedetail?id=67893a91-946f-49b5-8033-394fa5d752ea&lang=en

30. http://www.science.smith.edu/cmbs/documents/QubitdsDNABRAssay.pdf

31. http://www.promega.com/resources/pubhub/enotes/stem-cell-line-authentication-and-contamination-detection/

32. http://www.cstl.nist.gov/strbase

33. http://www.atcc.org/~/media/D6191AD299424C3AB5E5F5538C369A0E.ashx