VALIDATION OF THE USE OF *SUS SCROFA* (DOMESTIC PIG) DNA AS A RESEARCH TOOL IN A FORENSIC LABORATORY

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ABSTRACT: For a number of years, pigs have been used in a variety of forensic disciplines as a research tool, such as in forensic anthropology. However, pigs have not been used as a research tool in forensic DNA analysis. The use of pig DNA is becoming common in different areas of forensic science such as wildlife management but the transition to forensic DNA research has not happened yet. This paper proposes that pig DNA can be used in a forensic setting for research purposes in a lab that also conducts case work. The methods followed for the development of an STR profile using a commercial multiplex kit for pigs are the same as the standard procedure in a forensic Human DNA laboratory. The validation showed that pig DNA can be analyzed with the equipment currently used for human samples and it does not cross contaminate human samples. These factors would help small labs conduct both research and casework within the same laboratory.

KEYWORDS: Forensic science, Forensic Biology, DNA analysis, Validation study, *Sus Scrofa*, STR, Animal type Pig PCR amplification kit

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE

Glossary

Allele One of two or more forms of a gene that are found in the same region of the chromosome on homologous chromosomes

Locus The specific location on a chromosome where a gene or allele is found

PCR Stands for Polymerase Chain Reaction. It is the process of heating and cooling a double stranded piece of DNA to have it denature and anneal in the presence of *Taq* polymerase and nucleotides to replicate specific regions of the DNA strand.

Primer A short strand of DNA that is capable of binding to a specific region of single stranded DNA known as the complementary sequence.

RFU Stands for Relative fluorescence units. RFUs are the units of measurement for peak heights in a DNA profile to show the relative amount of a certain piece of DNA.

STR Stands for Short Tandem Repeat. A short tandem repeat is an area on a chromosome where a nucleotide sequence 2-13 base pairs long is repeated many times in tandem. These STR regions of the genome are the areas that are targeted for PCR amplification and are the basis of DNA fingerprinting.

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Introduction and Statement of Problem

The use of pigs in forensic research is not a novel idea. The use of pigs can be widely seen in forensic entomology, anthropology, toxicology and other areas [1-3]. In these disciplines, pigs are used instead of human bodies for research on different aspects of decomposition. This type of research is done with pigs because human bodies are often in short supply or difficult to acquire. Despite this fact, the use of pigs in forensic DNA research has been limited. Using pigs in forensic DNA research would be vital in aiding the research of the degradation of DNA on a whole corpse where human bodies are not a practical option. Additionally, pig samples cost far less than human samples.

1.2 General Background

Pigs have been used in forensic DNA analysis for wildlife management and animal protection. For example, Robino *et al* [4] outlined the process of identifying pig samples in a veterinary malpractice case. Another study looked at the same STR kit (Animal-type Pig kit) for wildlife protection and conservation [5]. These articles demonstrate that animal DNA analysis methods have become common practise in certain forensic fields. (e.g., border security meat analysis and the food industry [6].) The possible applications of Pig DNA for forensic DNA research have not been demonstrated. The techniques and procedures to analyze animal DNA are becoming cheaper and quicker, reaching the same level of human DNA analysis is [7]. In

order for the use of pig DNA to become relevant in an accredited DNA laboratory setting, the pig DNA must be able to be analyzed using standard equipment that would be found in a forensic DNA laboratory.

In a standard DNA laboratory there is a typical flow of the samples through the lab. It starts with the extraction of the DNA from the. This step is not species-specific as most nuclear DNA can be extracted using the same method. Once the DNA is extracted the DNA has to be quantified, allowing the analyst to know how much DNA is present. The quantitation step uses a real time PCR instrument and has species-specific primers that look for one or more specific sequences of nucleotides and bind to those sites. The real time PCR instrument will then monitor the quantity of the selected target sequence in real time. The next step is PCR and amplification of specific regions of the genome that will be analyzed with electrophoresis. This step is also species-specific as different primers will have to be used to target different loci for different species. Lastly, the amplified DNA is run through a capillary electrophoresis device to get the base pair size of each of the sequences of DNA. A computer software program then designates each of the sequences of DNA an allele value based on the size and the fluorescent tag associated with the sequence.

Because the analyst has to use the same equipment that is used on human DNA, it must be shown that there is no cross contamination from pig DNA to human DNA. That is to say that the instruments won't detect pig DNA as human DNA and come up with a false positive. This means that there needs to be specific DNA markers that search for and bind to single stranded DNA that has the complementary sequence. The term complementary strand means the sequence is optimal for binding to it, Adenine binds with Thymine and Cytosine binds with

Guanine. (e.g., ATTGC will have the complementary sequence TAACG.) However the marker will need to be found in all Pig DNA sequences [8] and only pig DNA. Novel STR markers have been identified in pigs that would allow them to be classified individually [8]. An STR marker is a sequence of DNA that flanks a region of DNA that has a short sequence (usually a 4-nucleotide sequence) that is repeated multiple times consecutively and that repeats a different number of times in different individuals. One such commercial multiplex kit is the Biotype Diagnostic Gmbh: Animaltype Pig PCR Amplification kit for STR amplification [14]. This kit is capable of being used with the current equipment that is found in the Laurentian University Forensic DNA Lab.

A second reason that pig DNA may not be commonly used in a forensic laboratory is because the reason for using pig DNA is not recognized. With new major areas of study in forensic DNA analysis focusing on touch DNA [9] there is no shortage of touch DNA that can be easily accessed from humans. Yet, the use of pigs will be more relevant when a decomposed or very complex or compromised sample is found at a crime scene. Pigs will be useful in these types of cases because using pigs will permit ethical clearance for the research to commence. Pigs are killed every day in the food industry and research but the number of humans that die and donate their bodies to science are far more limited. Decomposition research using pigs would allow researchers to identify areas of the genome that degrade the slowest, and a proper procedure may be developed that would be done with human DNA that has been exposed to the same degradation factors.

An additional positive to doing research with pig DNA instead of human is that pig DNA cannot contaminate a human sample because the STR markers will not match and are human

specific [10]. Some of the human STR markers that appear to not have any cross contamination from other species are: CSF1PO, TPOX, THO1, HPRTB, FESFPS, vWF and F13A01 [10]. Primate species (Gorilla and Chimpanzee) appear to have STR amplification peaks that fall outside of the bins (Expected base pair sizes to find alleles) of humans [10]. This means that a lab that is primarily research based, but that also does pay as you go service for police agencies, does not need to worry that traces of their research will contaminate the evidence that they are examining.

Because the use of pig DNA in a forensic laboratory is a novel technique, it will need to be validated. The Scientific Working Group on DNA Analysis Methods (SWGDAM) has extensive guidelines for validating any novel DNA analysis methods [11]. The guidelines outline that the method must be species specific, sensitivity studies must be done, precision and accuracy must be shown and it must work with case type samples [11]. These validations are of the utmost importance to demonstrate that pigs are a reliable model in forensic DNA analysis. They are important because any lab must prove that their methods are reliable and reproducible so that they can be tried and tested in court [12]. These high standards are the reason forensic DNA analysis is considered to be the gold standard for forensic laboratories. This is why it is important that the pig DNA analysis can use the exact same, or very similar, procedures as human DNA. If pig DNA research is held to the same standard as human DNA then the model will be extremely reliable.

The implication of this research is that it has the potential to open up opportunities for research for laboratories that do not have access to sufficient funds or access to human samples for research purposes. Research using pig samples will have fewer ethical issues to deal

with than research with human samples thus; pig samples would be more accessible. Whole pig bodies can be bought from a butcher or a farm and then used in research. Some research could include decomposition timelines for the quality and quantity of DNA that can be extracted from hard or soft tissue. The use of pigs could also allow for research into what tissue samples yield the most DNA and best quality after decomposing for long durations. This could create a guide line for pathologists to know which samples they should collect first to ensure that a profile can be obtained from bodies that are found at crime scenes.

1.3 Goal of the Study

The aim of this study is to develop a procedure that will allow pig samples to be analyzed in a forensic setting identical to the way that human samples are currently. The goal is that only some of the materials would change (STR marker kits) and no other part of the procedure would be affected. To achieve this goal, it must be demonstrated that pig DNA can be extracted and that we can generate an STR profile for individual pigs analogous to STR profiles developed for forensic samples of human origin. From there, samples from different tissue types will have to be tested repeatedly to show precision. Different samples from different pigs will also need to be used to assure that the STR kits have alleles that differ from pig to pig yielding different DNA profiles. Discrimination is essential for forensic DNA samples for mixture analysis and also for identification purposes of individuals. Lastly, the validation will have to include samples that are of tissues found at crime scenes such as blood or saliva and the samples will have to be on different substrates and possibly mixed with other samples to

prove that the techniques work with forensic case type samples.

Due to limited resources a small sample size, less than 40 were used. As a result, test statistics were also limited. A comparison of the mean peak heights, and the amount of background noise of the STR profiles, were undertaken. The stutter ratio was also calculated. Stutter is a peak that is 4 base pairs shorter than the actual peak which is created from slippage during PCR.

CHAPTER 2

MATERIALS AND METHODS

2.1 Sample Collection

The samples that were analyzed in this study were acquired from two different sources. The meat and bone samples were from a pork bone that was acquired from a local butcher (Tarini Brothers, Sudbury Ontario). Blood samples were taken from five different pigs that were purchased from Canadian food inspection agency (Canadian Food Inspection Agency, Ottawa, Ontario). The pigs were bled as part of a routine collection of blood by the Canadian Food Inspection Agency in Ottawa. During this routine collection procedure, the pigs were not euthanized. The blood samples were stored and shipped in lavender topped test tubes. Each tube was treated with Ethylenediaminetetraacetic acid (EDTA), a blood preservative and anticoagulant. The five test tubes of pig blood were stored in a refrigerator at 4^{o}C until needed for DNA extraction.

The pork bone that was used for the collection of the meat and bone samples was uncooked and acquired from the Tarini Brothers in Sudbury. To prepare the meat sample the pork bone was thawed as it was previously frozen at -18^{o}C for storage purposes. Once the bone had been thawed the meat on the bone was teased off of the bone using a new sterile scalpel. The meat was divided into 10 approximately equal portions. Each of the portions of the meat was placed into a sterile 1.5 ml microfuge tube and labelled 1 through 10. These samples were then frozen at -18^{o}C until needed for DNA extraction.

The third type of tissue sample, bone was collected off of the same pork bone that the meat samples were obtained from. A hammer was used to strike the bone and dislodge a small

portion. A spherical piece of bone approximately two centimeters in diameter came off the pork bone. The small piece of bone was ground in a black and decker coffee bean grinder (Black and Decker, Towson, Marland). The grinder was cleaned with a water and bleach solution before and after each use. The resulting flakes of bone were collected and divided up into five sub-samples. Each sub-sample was placed into its own 1.5 ml microfuge tube and marked as bone sample 1 through 5 and frozen at -18^{o}C for storage.

2.2 DNA Extraction

The entire set of DNA extractions were carried out with AutoMate Express™ Forensic DNA Extraction system instrument from Thermo Fisher Scientific (Waltham, Massachusetts, United States). The extraction using the meat samples was done with two different magnetic bead DNA extraction procedures: the PrepFiler *Express™* Forensic DNA Extraction Kit and the PrepFiler *Express* BTA™ Forensic DNA Extraction Kit (Applied Biosystems™). The first was called the 'bodily fluid extraction' and the second was called the 'Bone, Tooth and Adhesive (BTA) extraction'. Both of these procedures were preprogrammed into the AutoMate Express instrument. Both of the extraction methods were used because the meat sample seemed to fall within the recommended sample types for each of the extraction techniques. It was unclear which extraction technique would be most beneficial in extracting the DNA from the meat sample. It was expected that the BTA extraction technique would yield more DNA because the process was more vigorous and gain access to more of the nuclear DNA from the muscle tissue.

The procedure of the bodily fluid extraction technique involved some sample preparation beforehand. In order to prepare the samples for extraction of DNA the samples

were placed into a tube called the Lysep elution tube. This elution tube had a hole in the bottom with a filter and a tube connected to the bottom of the Lysep elution tube. The combination of the Lysep elution tube and bottom tube formed what was termed the Lysep column. Approximately 0.6g of the meat sample was placed into each of the Lysep columns. Each of the columns had the addition of 500µl of the PrepFiler lysis solution that was made with 500µl of the provided PrepFiler lysis buffer and 5µl of 1M Dithiothreitol (DTT). Once each of the Lysep columns had the necessary solutions, the samples were placed into the Thermomixer from Eppendorf (Eppendorf, Hamburg, Germany). The Thermomixer was set to 70^{o}C and 750rpm. The five meat samples that were undergoing the bodily fluid extraction were placed into the thermomixer for 40 minutes. At the end of the 40 minutes the samples were taken out of the Thermomixer and were placed into a centrifuge where they were spun at 10,000x the force of gravity for two minutes. After the two minutes of centrifugation the samples were removed from the centrifuge and the two halves of the Lysep column were separated. The bottom half containing the liquid solution was kept and the top half containing what was left of the meat sample was discarded. The bottom tube was placed into row S of the Holder for the AutoMate Express instrument. An empty microfuge tube was placed into row E of the Holder and a clean tip and tip holder was placed into row T2. A second tray held the PrepFiler Express cartridges placed into it, matching each cartridge to a sample tube. A negative sample control was also loaded into the extraction run. Both of the trays were then loaded into the AutoMate Express instrument. The program card was then placed into the instrument as well and the AutoMate Express bodily fluid protocol was selected and carried out. This program took about 30 minutes to complete. The extraction system of the AutoMate Express is a magnetic bead

separation technique. After the extraction run had been completed the extracted DNA of each of the samples was now in the 1.5ml microfuge tubes that were loaded into the tray before the run began. Each of the tubes was then labelled according to the sample that it contained before. The tubes were labelled PS 1, 2, 3, 4 and 5. PS was the identifier for the meat samples that went through the bodily fluid extraction procedure. All of the samples were then placed into the freezer to be stored at -18^{o}C until the samples were needed for quantitation.

The blood samples were also extracted using the bodily fluid extraction methods as outlined above. 40µl of blood was placed into each Lysep column. Two microfuge tubes were created for each pig blood sample. This created 10 samples that were to be extracted. The tubes were labelled PBL (pig blood) 1.1, 1.2, 2.1…. 5.2. two mixtures were also made with the pig's blood. Using pig blood sample 1 and 3 two mixtures were created with equal quantities of each of the blood samples (20µl of each sample). These two samples were labelled PBLM (Pig Blood Mixture) 1.1 and 1.2.

The dilution series for the Blood was created by having a set of blood mixtures that were 1in1, 1in10, 1in25, 1in50, 1in100 and 1in200. The dilution series was done with the blood samples labelled 943:01, (pig 1) 945:02, (pig 2) and 946:03 (pig 3). The 1in1 mixture was made by extracting 100µl of blood into a microfuge tube. The 1in10 dilution was made by adding 10µl of pig blood into 90µl of water. The 1in25µl dilution was created by adding 5µl of pig blood in 120µl of water. The 1in50 dilution was created by adding 5µl of pig blood in 245µl of water. The 1in100µl dilution was created using 5µl of pig blood and 495µl of water. The 1in200 dilution was created using 5µl of pig blood and 995µl of water. This created 18 samples, six samples for each of the three different pig blood samples. After the dilutions were created the microfuge

tubes containing the diluted blood samples were vortexed in the mini vortexer (Fisher Scientific, Waltham, Massachusetts, United States) and spun down in the mini centrifuge (E&K Scientific, California United States). After the centrifugation 75ml of each of the samples was aliquoted by dotting them onto Whatman FTA paper (Whatman, Maidstone, United Kingdom**).** The dotting created circles approximately 2.5cm in diameter. The samples were left to dry for about an hour in the DNA extraction room at room temperature. After the hour was up, two punches were taken of each of the samples for extraction. The punch was circular and was 0.5cm in diameter. The puncher was cleaned with a water and bleach solution between each punch. The two punches were placed in a Lysep column and the extraction procedure that was outlined above for the bodily fluid DNA extraction technique was carried out.

The remaining five meat samples that were teased off of the pork bone went through the BTA extraction procedure. This procedure was very similar to the bodily fluid extraction procedure, however, instead of the sample being placed into the Lysep column the thawed meat samples were placed into the provided bone and tooth lysate tubes. Next, 230µl of PrepFiler BTA lysis solution was added to the tubes with the sample. The BTA lysis solution consisted of 220µl of PrepFiler BTA lysis buffer, 3µl of DTT and 7µl of Proteinase K. The thermomixer was then heated to 56^{o}C and the samples were placed into the Eppendorf thermomixer for 18 hours at 750rpm. After 18 hours of uncubation, the lysate tubes were removed from the Thermomixer, they were centrifuged in the Eppendorf Centrifuge 5424 (Eppendorf, Hamburg Germany) for 2 minutes at 10,000x the force of gravity. These samples were labelled BTA 1 through 5.

The Bone samples were prepared with the BTA extraction as well. The small fragments

of bone dust weighing approximately 0.6g were placed into the bone and tooth lysate tubes. The procedure as outlined above was carried out for the extraction of the DNA from the Bone samples. The extracted DNA from the bone samples was labelled BS (Bones Sample) 1, 2, 3, 4, and 5.

A complete list of all of the samples that had their DNA extracted can be found in Table 2.1.

2.3 Quantification of DNA

After the extraction of DNA was completed, a quantitation of the amount of DNA in each of the extracted samples was performed. The first puantitation was done using the ABI 7500 Real-Time PCR instrument (Applied Biosystems, Foster City, California) and the Quantifiler® Human DNA Quantification Kit from Thermo Fisher Scientific [15], which uses a human-specific assay that targets the 18S region of the chromosome. The quantitation was done to demonstrate that the pig DNA would not be recognized by the human specific primers in the Assay mix. The components of the reaction mix that were added to the sample in real time PCR instrument are outline in Table 2.2.

The quantification for the amount of pig DNA in each of the extracted samples was done with the NanoDrop 8000 spectrophotometer (Thermo Scientific Waltham, Massachusetts, United States)[16]. A 2µl volume of each of the samples was pipetted onto one of the eight optical surfaces of the device. The device then measures the absorbance of each of the samples in the 260-280nm wavelength. The instrument automatically generates a curve and calculates the concentration of the DNA within each of the samples in ng per µl.

Using the results from the NanoDrop 8000 the samples were diluted to a concentration of approximately 2.5ng/ml. The list of the dilutions are summarized in Table 2.3.

Table 2.1. All samples subjected to DNA extraction.

Muscle samples	**Bone samples**	**Blood samples**
PS 1	BS 1	PBL 1.1 ,1.2
PS 2	BS 2	PBL 2.1, 2.2
PS 3	BS 3	PBL 3.1, 3.2
PS 4	BS 4	PBL 4.1, 4.2
PS 5	BS 5	PBL 5.1, 5.2
PS Blank	BS Blank	Pig 1 dilution series
BTA 1		Pig 2 Dilution series
BTA 2		Pig 3 Dilution series
BTA 3		PBLM 1.1, 1.2
BTA 4		PBL Blank
BTA 5		
BTA Blank		

Table 2.2. Reagents and volumes for one sample amplified on the Applied Biosystems real time PCR instrument.

Reagent	**Volume (µl)**
Quantifiler® Human Primer Mix	10.5
Quantifiler® PCR Reaction Mix	12.5
Sample	2.0

Table 2.3. Dilutions of the extracted samples to make the approximate concentration of DNA 2.5ng per µl.

Sample	Sample volume (µl)	TE buffer volume (µl)
PS 1	2.2	122.8
PS 2	2.3	122.7
PS 5	2.1	47.9
BTA 1	4.3	120.7
BTA 3	2.1	47.9
BTA 5	2.6	47.4
BS 1	2.0	248.0
BS 3	2.1	47.9
BS 5	5.0	1245.0
PBL 1.1	2.5	47.5
PBL 2.1	4.9	20.1
PBL 3.1	3.0	22.0
PBL 4.1	5.5	19.5
PBL 5.1	2.8	22.2
PBLM 1.1	2.1	22.9
PBLM 1.2	2.6	22.4
Pig 1: 1:1	6.1	18.9
Pig 1: 1:10, 1:25, 1:50, 1:100, 1:200	2.5	0
Pig 2: 1:1	3.5	21.5
Pig 2: 1:10, 1:25, 1:50, 1:100, 1:200	2.5	0

2.4 PCR Amplification and Electrophoresis

The next step in the process was to amplify the diluted sample using PCR (the polymerase chain reaction). The Kit that was purchased from Germany was called the Animal-type pig kit. The instructions in the user manual [13] called for the reaction volume to be 25µl. Due to limited resources it was decided to make the reaction volume 12.5µl in order to be able to amplify twice as many samples. The volumes of each of the reagents that were used for one PCR reaction were: 6.05µl of nuclease free water, 2.5µl primer mix D, 1.25µl of primer mix, 0.2µl of Taq 2 DNA polymerase and 2.5µl of sample to be amplified.

Once prepared, the samples were placed into the Applied Biosystems 9700 Thermal cycler (Applied Biosystems, Foster City, California, United States). The instrument had been programmed to follow the heating and cooling cycles per the Animal-type kit [14]. One complete heating and cooling cycle is as follows: start at 94^{o}C and hold for 4 minutes (hot start), then 94^{o}C for 20 seconds, then 60^{o}C for 40 seconds followed by 72^{o}C for 30 seconds, this was repeated for 30 cycles. Then the third portion of the cycle was 70^{o}C for 60minutes and then a decrease to 10^{o}C to be held until the samples were removed from the thermal cycler.

In order for the samples to be run on the 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, United States) a spectral calibration had to be performed. The spectral calibration was done to make sure that the dyes were not overlapping in the system. The four dyes that are used in the Biotype, Animal-type Pig kit are 6-FAM, HEX, NED, and ROX [17]. The spectral calibration standard [SD-30 Matrix Standard Kit (Dye set D) Applied Biosystems^{TM}] was

made with 47.5µl of Hi-Di formamide (Manufacturer) and 2.5µl of matrix standard. 10µl of the master mix was aliquoted into 4 adjacent wells in a 96 well plate.

After the spectral calibration had been completed the samples were prepared to go into the genetic analyzer. The samples were added to a 96 well plate. Before the addition of the samples the wells that were to house a sample, had 12µl of master mix added to them. The master mix was created with 12.3µl of Hi-Di formamide and 0.2µl of size standard 550 (ROX, provided in the Biotype Animal-type Pig Kit) per sample that would be injected. Added to the samples were four allelic ladders, two Negative amplification blanks and one positive amplification control. In total 32 samples were run.

Once the raw data came from the 3130 Genetic Analyzer it was transferred to another computer via USB. The other computer had GeneMapper® ID-X v1.4 software from ThermoFisher Scientific (Thermo Scientific Waltham, Massachusetts, United States). The panels and bins that were required to analyze the raw data from the genetic analyzer were downloaded from the Animal-type website [14] and modified for compatibility with the genotyping software version. The panels and bins were then imported into the GeneMapper ID-X software.

The STR profiles were then analyzed and cleaned-up by means of the analyst clicking off peaks that were noise, stutter, pull-up or other kinds of artifacts that were found within the profiles.

Using the cleaned-up data the two most dissimilar profiles were chosen to create the mixture profiles. The two profiles that were the most dissimilar were the sample from the

muscle tissue/bone tissue and pig 946:03 (pig blood 3). Using the extracted DNA from the two previously named diluted samples a mixture profile was created by amplifying mixtures of DNA from the two sources in varying proportions. The mixture profile entailed creating a mixture of different ratios. The ratios that were chosen were: 5% pig blood 3 to 95% bone sample, 10% pig blood, 25% pig blood, 50% pig blood, 75%pig blood, 90% pig blood and 95% pig blood. In total there were seven mixture profiles developed.

The extracted diluted samples then went through the same processes listed above for PCR amplification and capillary electrophoresis. The raw data was then brought over to the gene mapper IDX software to have the profiles examined and cleaned up for examination.

2.5 Statistical Tests Used

The statists were calculated partially by hand and also using an excel workbook to calculate the complex and larger numbers.

To determine whether or not there was a statistical difference between the BTA extraction and the bodily fluid extraction a Wilcoxon Test for nonparametric data was performed.

The mean height of the heterozygous and homozygous alleles was calculated for each of the profiles of the dilution series. A Kruskal Wallis test was used to see if there was a significant difference between the mean peak heights of the alleles for the different dilution

concentrations in the series. To determine where any statistical difference may lay a Nemenyi test was performed.

The mean stutter peak height ratio was calculated for many different profiles and many different alleles. Once the mean was calculated the standard deviation was calculated. The average stutter peak height plus 3 standard deviations was set as the stutter peak height cut off.

CHAPTER 3

RESULTS

3.1 Results

The first set of results that were obtained was from the quantification of the pig DNA with the human primers using the QuantiFiler Human assay in the ABI 7500 real time PCR instrument. All of the quantifications came back with a value of zero.

The second quantitation that took place was done with the NanoDrop 8000. In this quantitation the muscle and bone samples were run to determine the quantity of DNA in the extracted samples. These quantitation data are listed in Table 3.1. Some samples were run twice. The Wilcoxin test found that there was a significant difference at the 0.025 confidence level between the concentrations of the BTA extraction when compared to the bodily fluid extraction.

The third quantitation was with the blood samples; also using the NanoDrop 8000. In this quantitation there were two mixtures, the normal blood samples and the dilution series. These results can be found in Table 3.2.

A positive control that came with the kit was run (Figure 3.1) As well as a negative amplification blank (Figure 3.2). No alleles were found in the Negative amplification blank.

One of the replicate STR profiles generated from the meat sample and from the Bone sample are shown in (Figures 3.3 and 3.4 are respectively).

The Profiles of the dilution series (Figures 3.5-3.10) are present to demonstrate the effects the dilution had on the RFU (Relative Fluorescence unit) heights. As the sample became more diluted the RFUs decreased for each of the peaks. Additionally, as the sample became more diluted allele drop out started to occur.

The average peak heights of the heterozygous and the homozygous alleles are shown for each of the dilutions in the dilution series (Table 3.3) to compare the affect dilution had on the DNA profile. The Kruskal Wallis test found here to be a significant difference at the 0.025 confidence level. The Nemenyi test found that there was a significant difference between the two most dilute samples (1:100 and 1:200) and the least diluted samples (1:1 and 1:10).

Table 3.1. Concentration of DNA in each of the muscle and bone samples.

Sample	**Concentration of DNA (ng/µl)**	
	1st round	**2nd round**
PS 1	8.445	57.59
PS 2	17.68	55.46
PS 3	36.27	80.70
PS 4	2.981	32.82
PS 5	13.26	23.90
PS Blank	-2.743	-2.660
BTA 1	30.46	29.13
BTA 2	23.51	11.46
BTA 3	22.91	24.38
BTA 4	6.559	10.72
BTA 5	57.16	19.04
BTA B	-1.116	-2.340
BS 1	139.2	128.2
BS 2	256.2	247.5
BS 3	27.62	24.08
BS 4	429.6	n/a
BS 5	415.8	n/a
BS B	-3.777	n/a

Table 3.2. Concentration of the extracted DNA from the pig blood samples

Sample	Concentration of DNA (ng/µl)
PBL 1.1	19.61
PBL 2.1	5.065
PBL 3.1	8.272
PBL 4.1	4.550
PBL 5.1	9.087
PBLM 1.1	11.76
PBLM 1.2	9.652
Pig 1 1:1	2.223
Pig 1 1:10	7.643
Pig 1 1:25	24.76
Pig 1 1:50	32.38
Pig 1 1:100	5.285
Pig 1 1:200	27.43
Pig 2 1:1	0.5572
Pig 2 1:10	24.88
Pig2 1:25	10.00
Pig 2 1:50	11.21
Pig 2 1:100	38.95
Pig 2 1:200	7.661

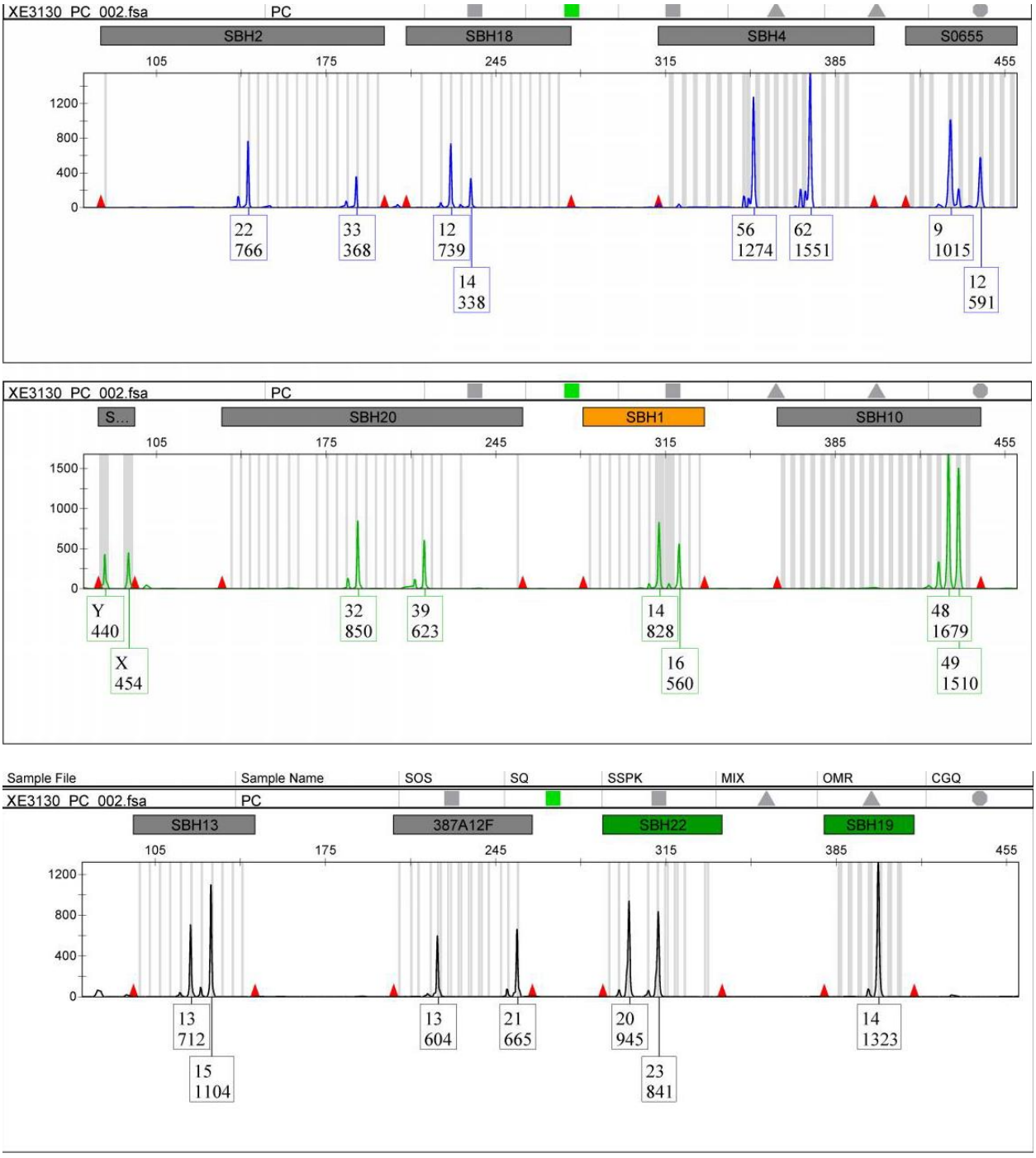

Figure 3.1. STR profile of the positive control that came in the Animaltype pig kit. This sample was electro-kinetically injected for 5 seconds.

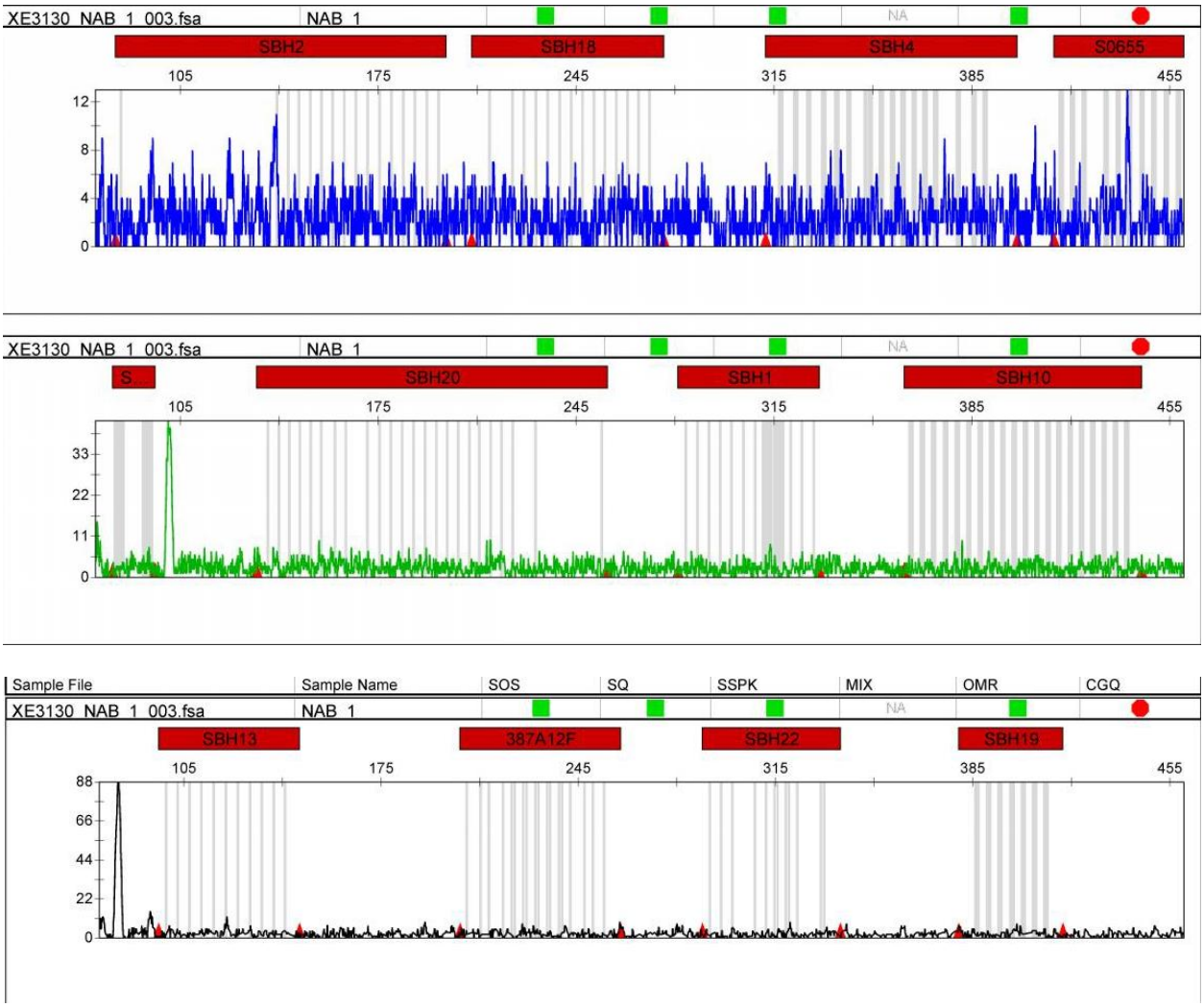

Figure 3.2. The Negative amplification blank STR profile. This sample was electro-kinetically injected for 5 seconds.

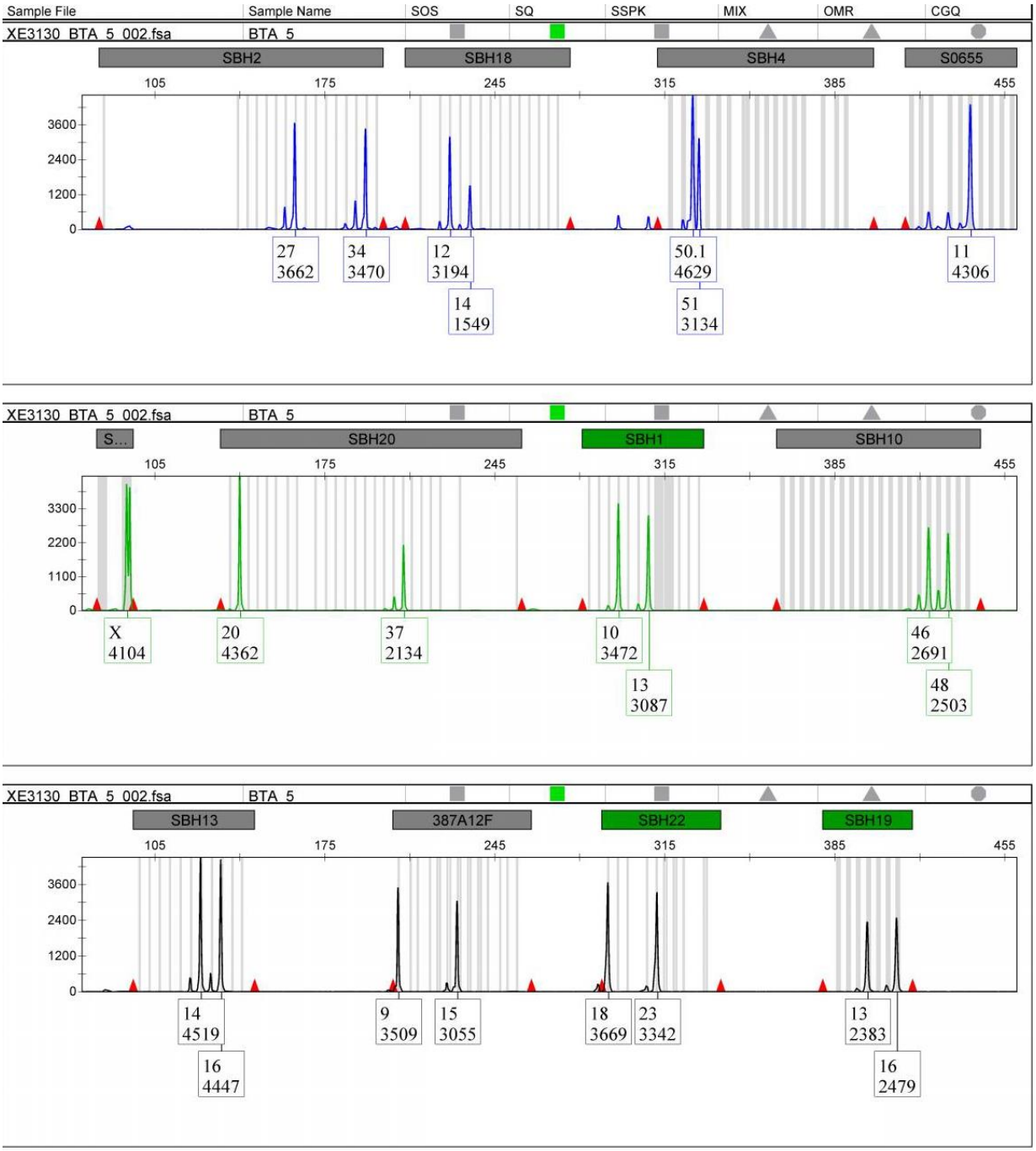

Figure 3.3. STR profile for the meat sample that was obtained from the pork bone using the BTA extraction. This sample was electro-kinetically injected for 5 seconds.

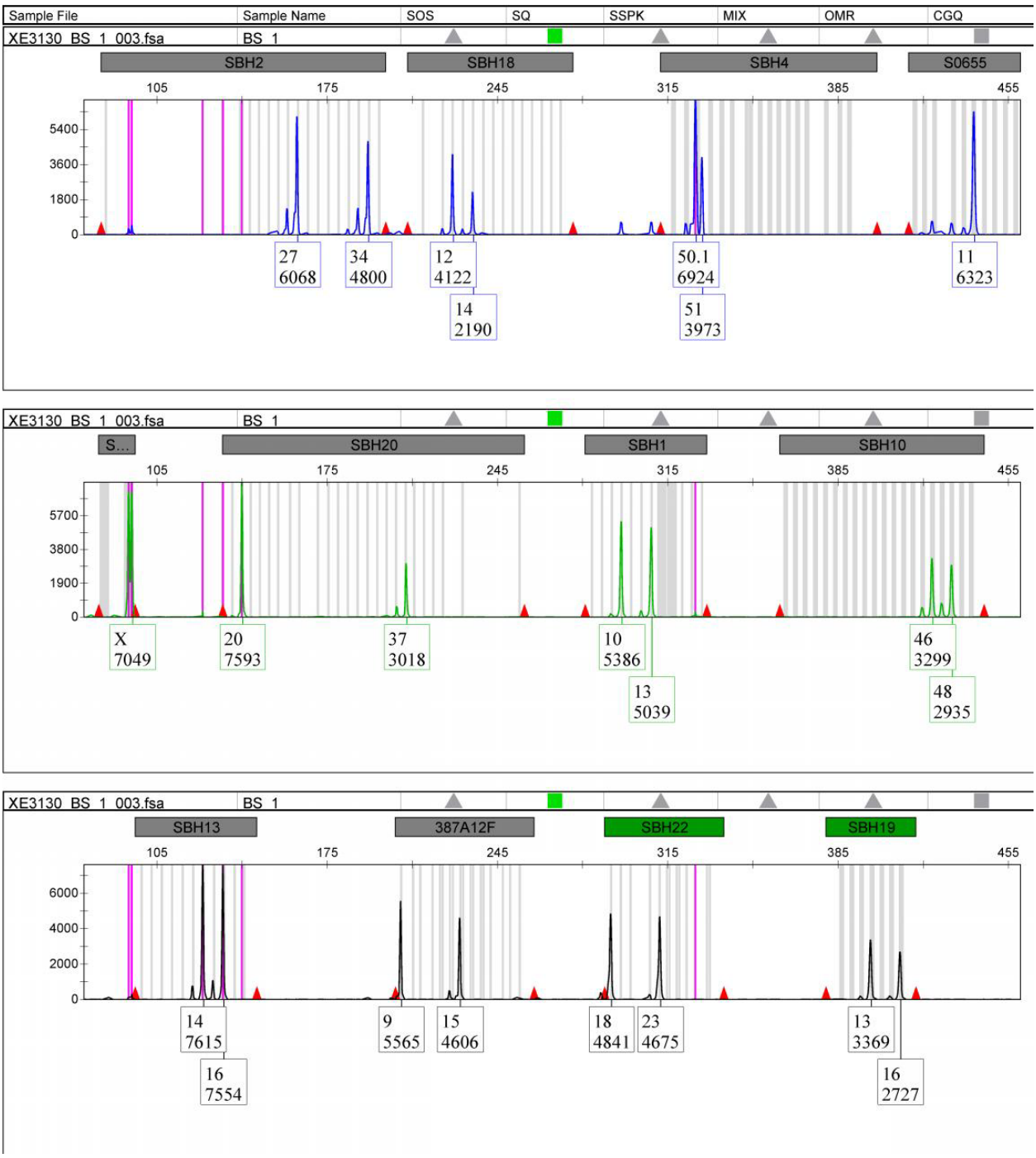

Figure 3.4. STR profile of a bone sample that was taken from the pork bone. This sample was electro-kinetically injected for 5 seconds.

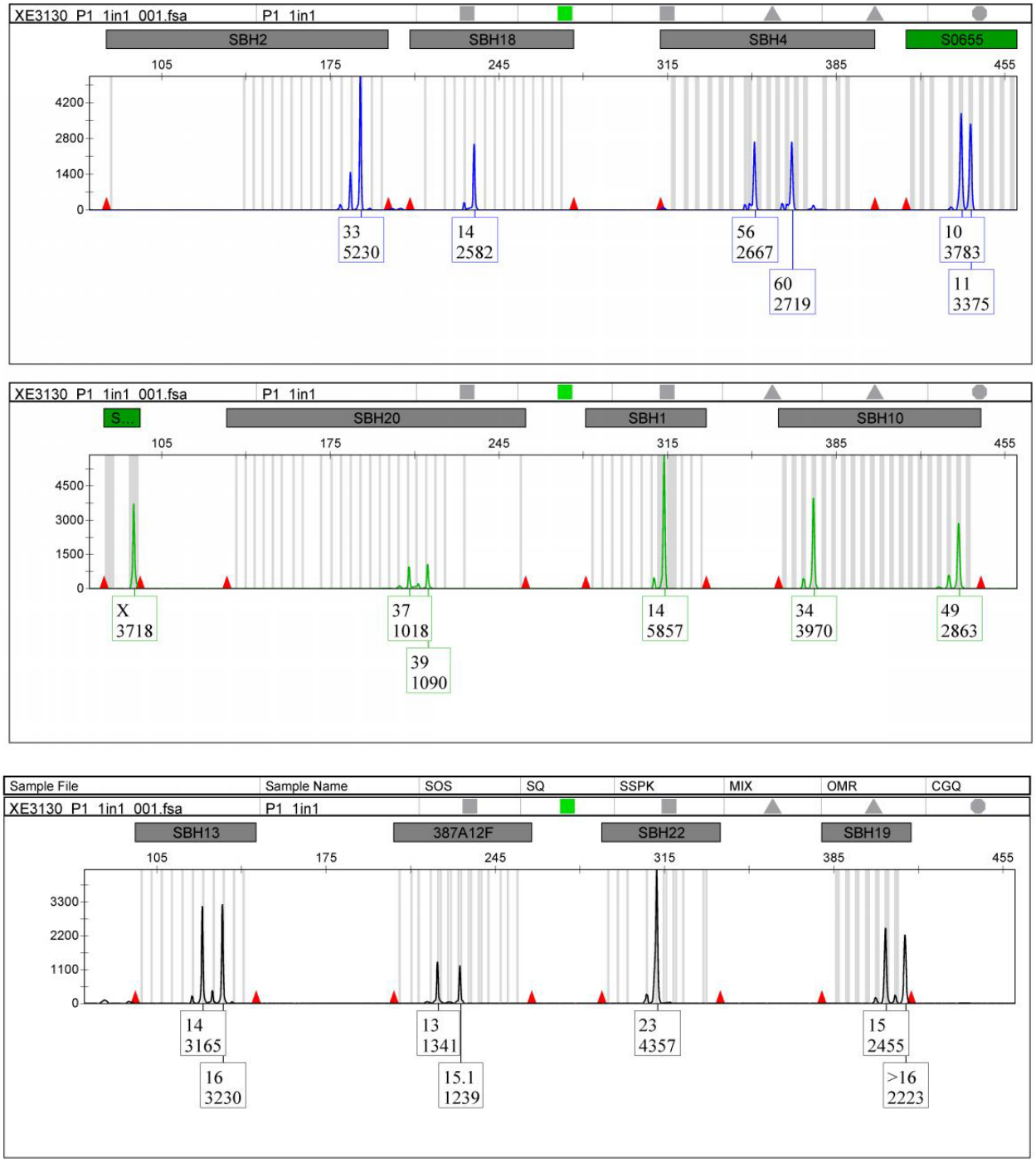

Figure 3.5. STR profile of pig blood sample 1 with no dilution. This Sample was electro-kinetically injected for 5 seconds.

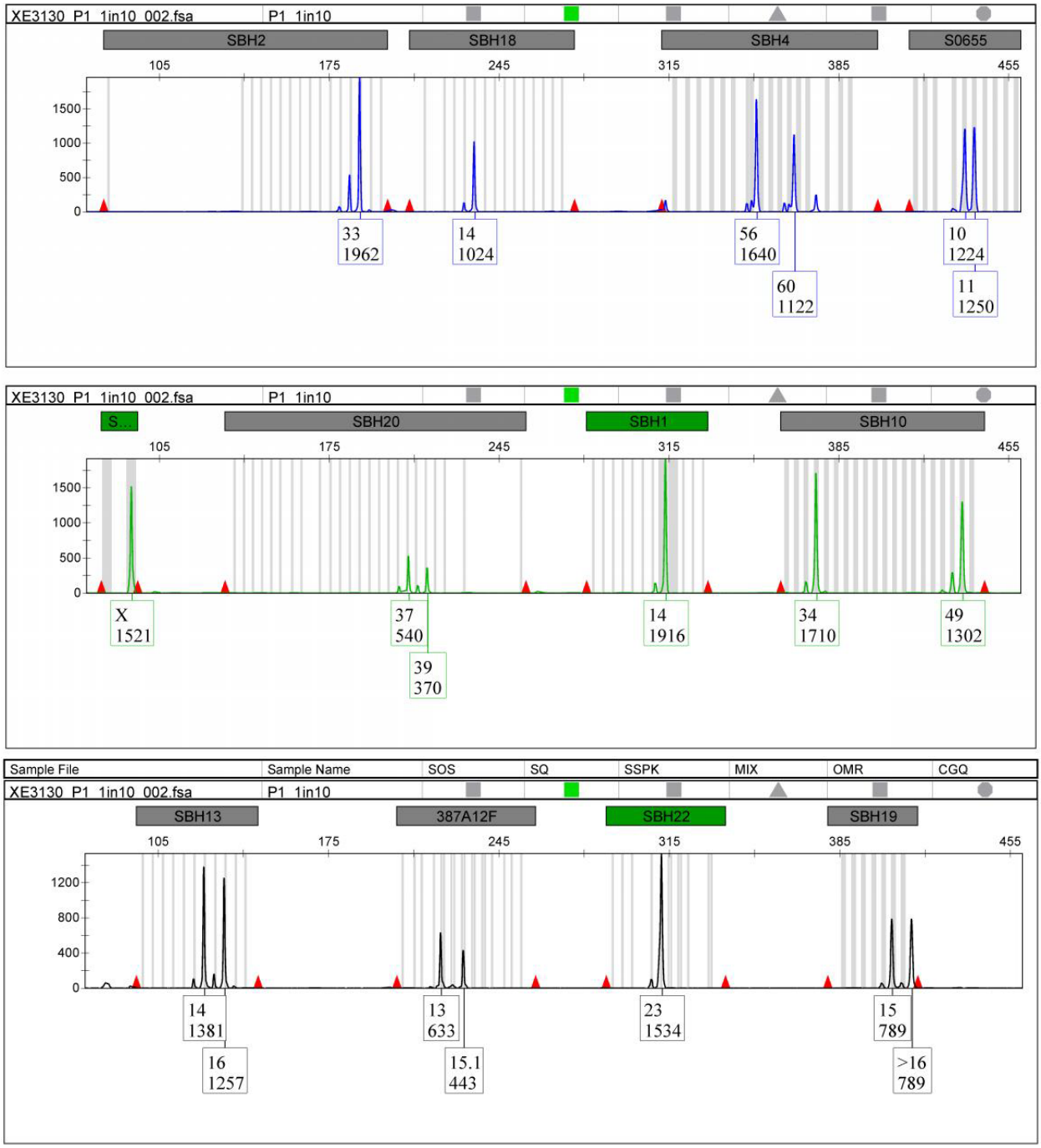

Figure 3.6. STR profile of pig blood sample 1 with a 1:10 dilution. This sample was electro-kinetically injected for 5 seconds.

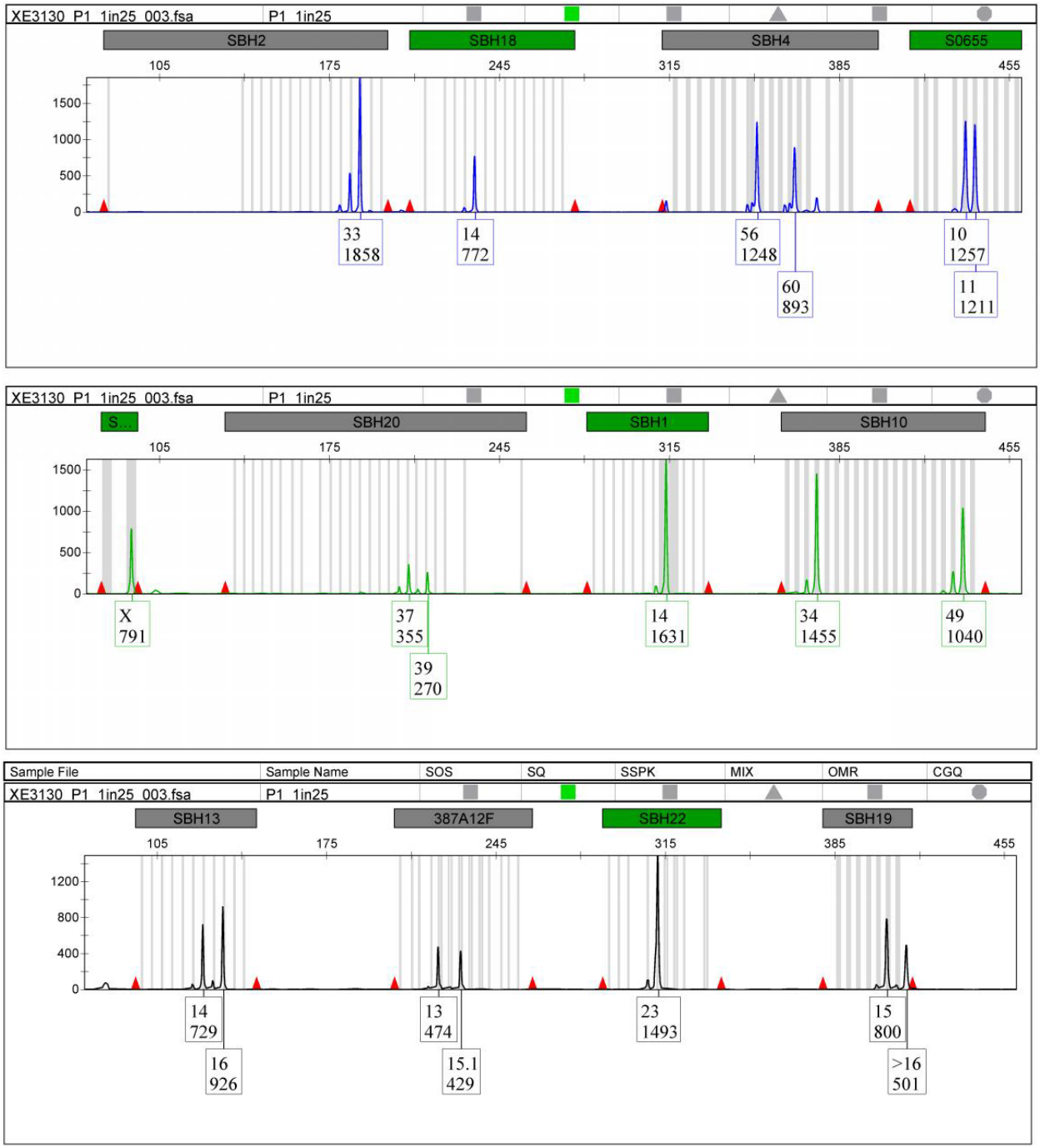

Figure 3.7. STR profile of pig blood sample 1 with a 1:25 dilution. This sample was electro-kinetically injected for 5 seconds.

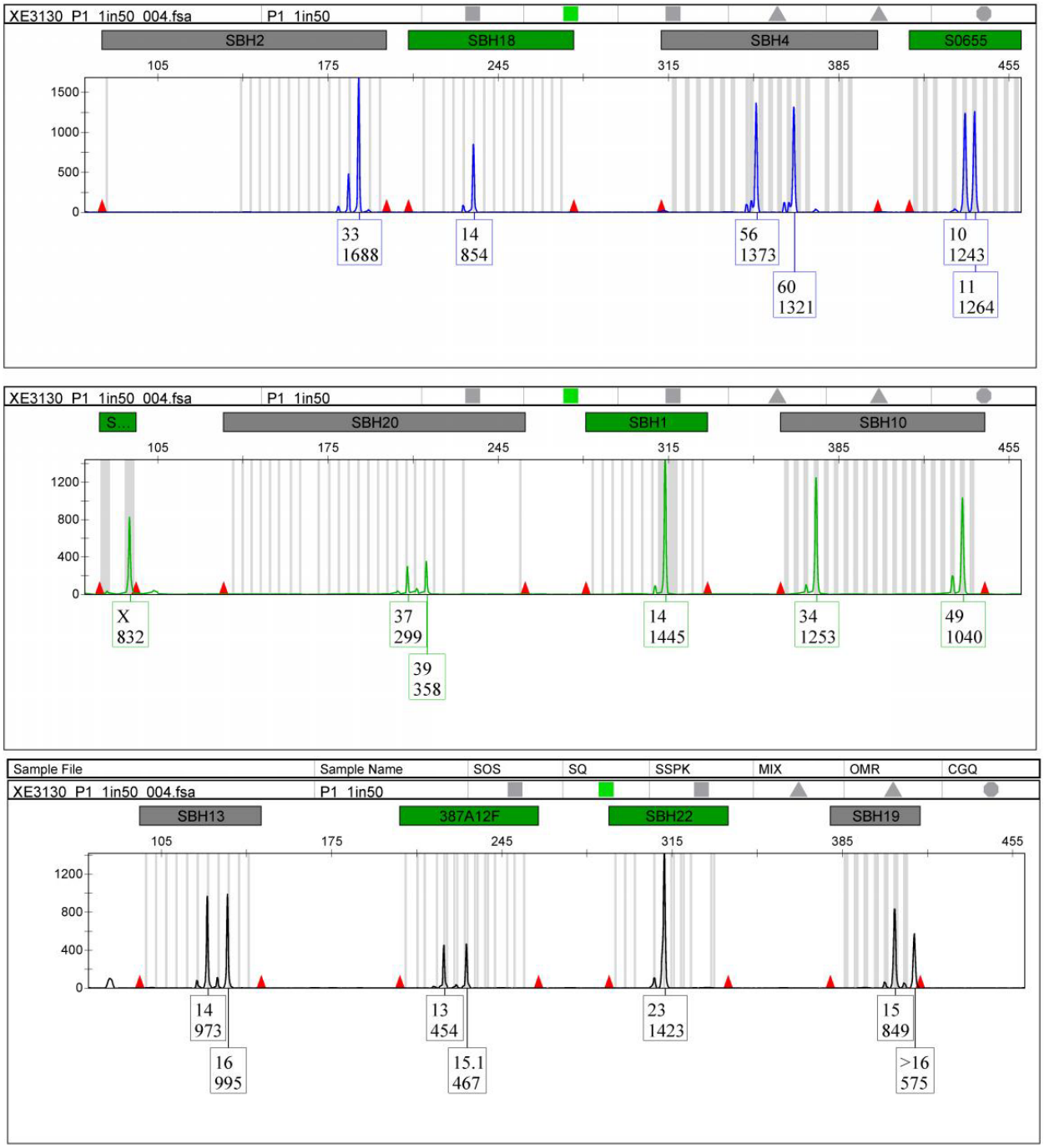

Figure 3.8. STR profile of pig blood sample 1 with a 1 in 50 dilution. This sample was electro-kinetically injected for 5 seconds.

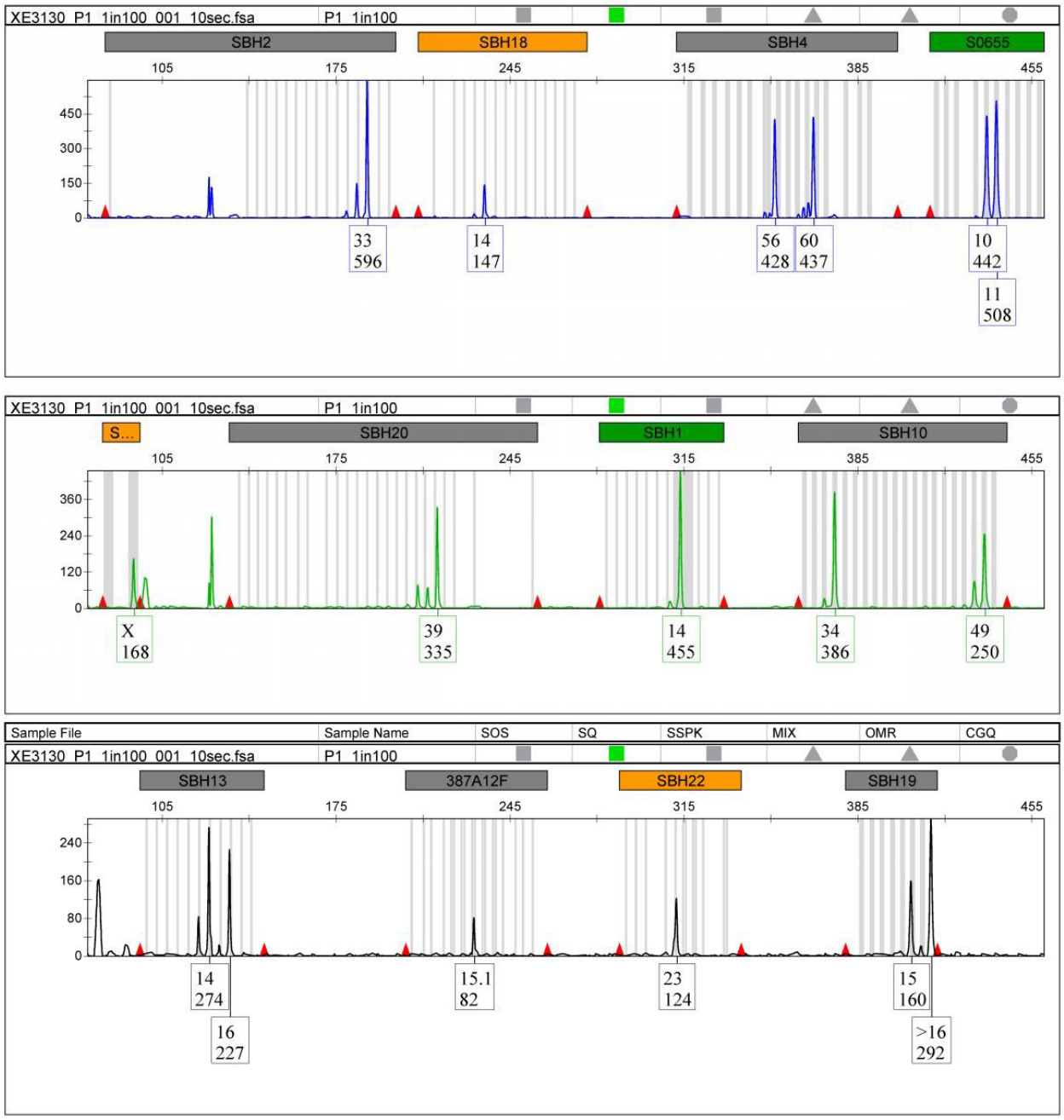

Figure 3.9. STR profile of pig Blood sample 1 with a 1 in 100 dilution. The sample in this profile was electro-kinetically injected for 10 seconds.

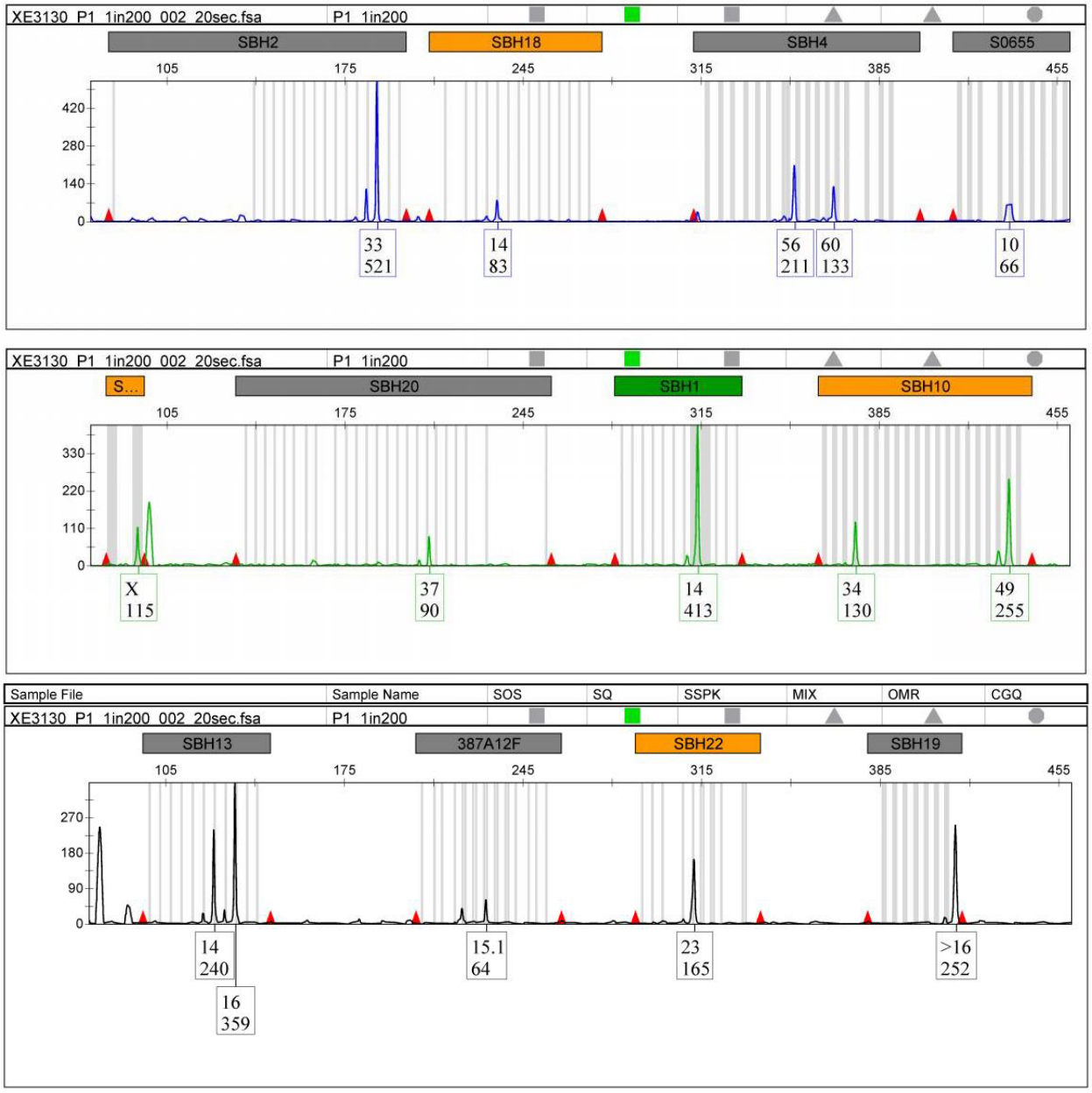

Figure 3.10. STR profile of pig blood sample 1 with a 1:200 dilution. This sample was electro-kinetically injected for 10 seconds.

Table 3.3. Average heterozygous and homozygous Peak heights for each of the dilutions.

Dilution	Mean Homozygotic peak height (RFU)	Mean heterozygotic peak height (RFU)
1:1	4349	2510
1:10	1600	1032
1:25	1309	828
1:50	1248	890
1:100	298	318.4
1:200	259.4	180

The mixture series ranging 5% pig blood to 50% pig blood are shown (Figures 3.11-3.14) to allow a visual representation of when a mixture can still be detected in a DNA profile. The mixture is between two different pig's DNA allowing up to four alleles to be seen at some of the loci.

To determine the cut off for stutter height especially for a mixture profile, the mean stutter height ratio was determined for each locus (Table 3.4).

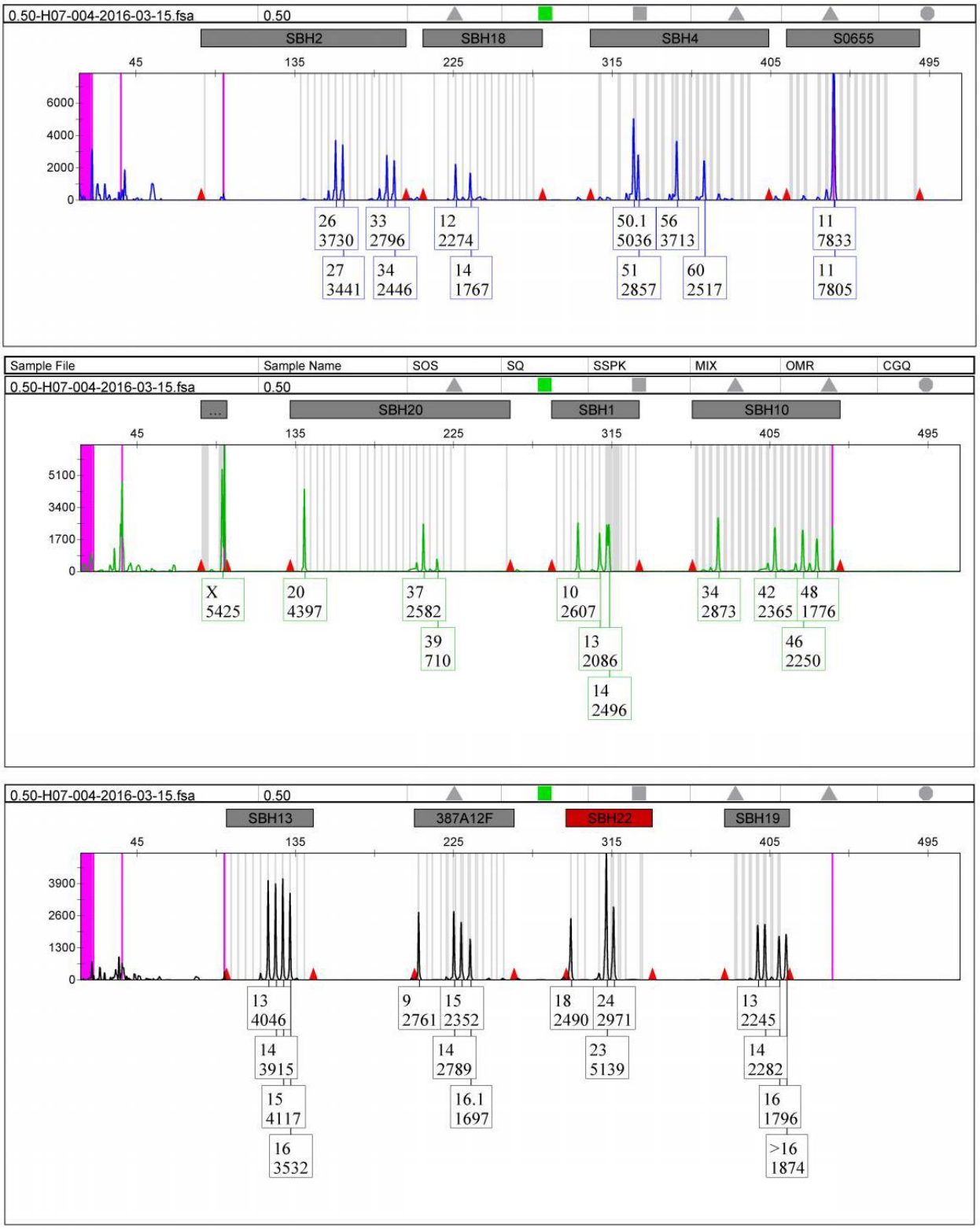

Figure 3.11. 50%/50% mixture of DNA between pig blood sample 3 and bone sample 1

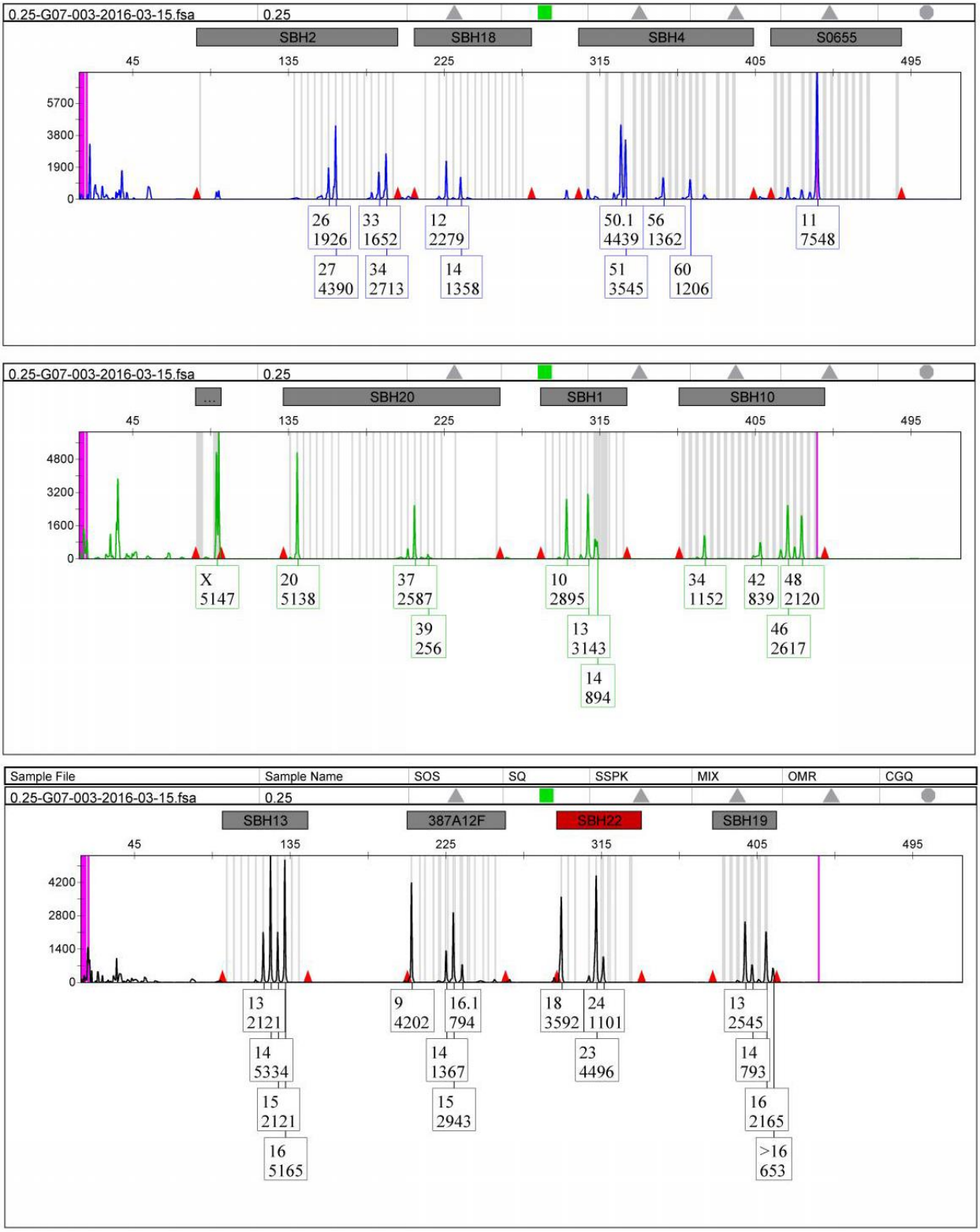

Figure 3.12. 25%/75% mixture of DNA between pig blood sample 3 and bone sample 1

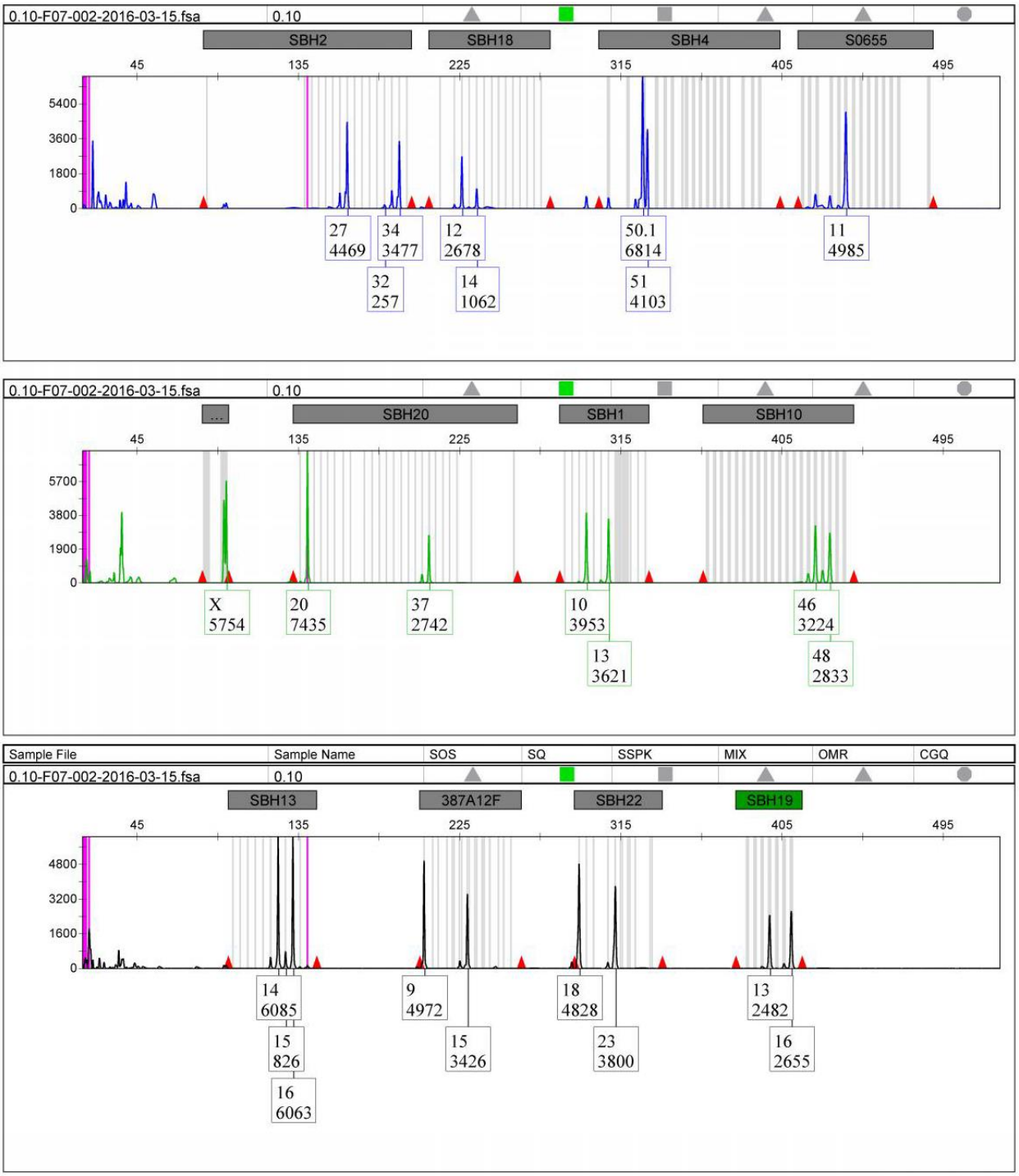

Figure 3.13. 10%/90% mixture of DNA between Pig blood sample 3 and bone sample 1

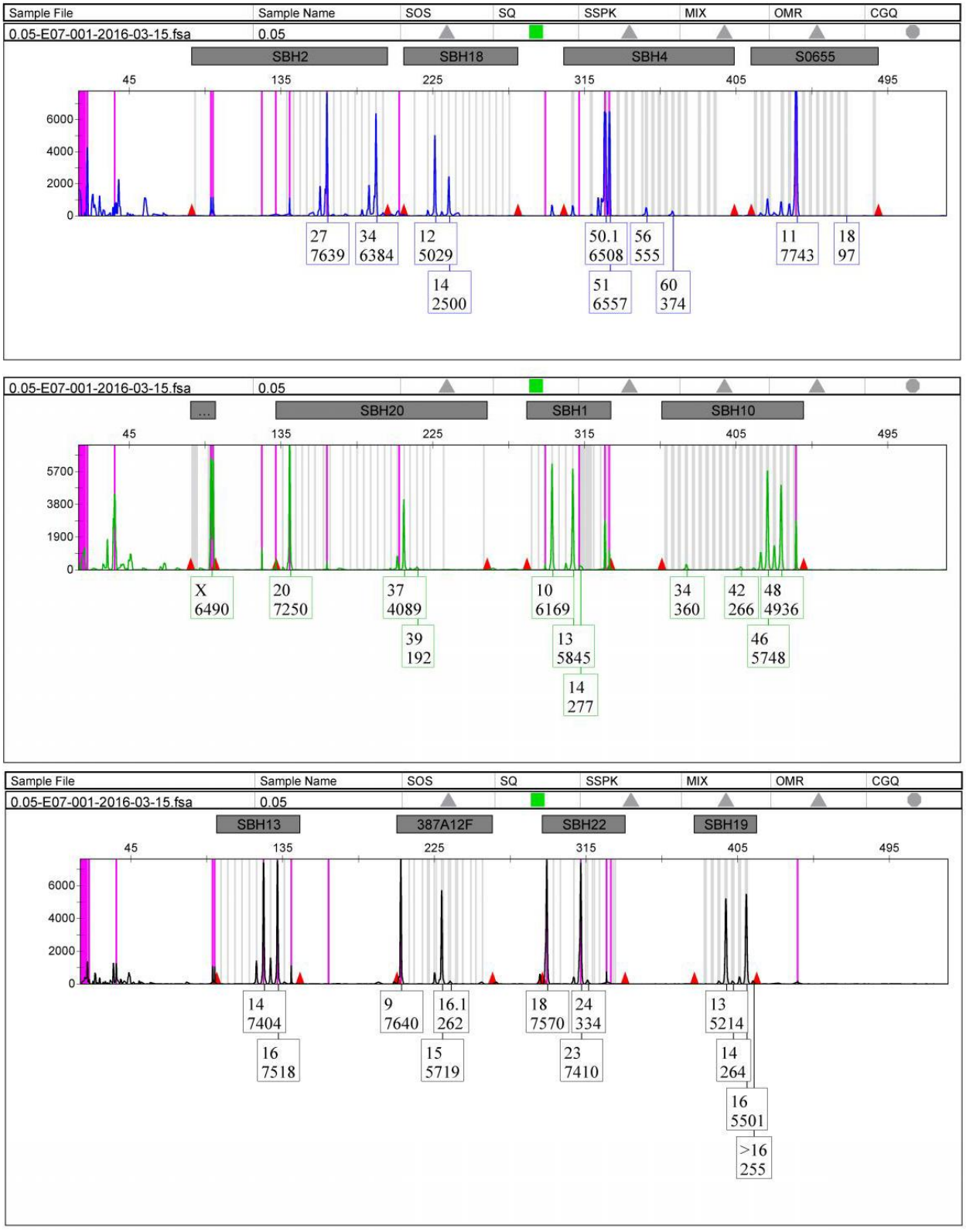

Figure 3.14. 5%/95% mixture of DNA between pig blood sample 3 and bone sample 1

Table 3.4. Stutter to Peak height ratio of the different loci in the STR profiles.

Locus	Mean Stutter Ratio %	Standard Deviation of stutter ratio
SBH2	25.61	4.74
SBH18	11.89	2.39
SBH4	11.73	3.07
S0665	5.69	1.04
SBH20	20.48	9.95
SBH1	7.7	2.13
SBH10	20.97	5.41
SBH13	11.49	2.96
387A12F	6.97	3.22
SBH22	8.4	1.03
SBH 19	9.29	2.38

CHAPTER 4

DISCUSSION

4.1 Points of discussion

The main goal of this study was to determine whether or not an STR profile could be created from pig DNA using the equipment that was commonly found in a forensic DNA laboratory; more specifically using equipment found in the Laurentian University Forensic DNA Laboratory. This goal was accomplished as 6 individual STR profiles were developed from the Kit plus the positive control that came with the kit. The positive control that came with the kit also came with a reference of the STR profile of the Positive control sample. The profile that was generated (Figure 3.1) was identical to the profile that the company gave [19]. Every allele at all of the loci were the same. This indicated that the equipment was functioning properly as the profile that was developed was the same as profile that was supposed to be generated. Additionally, all of the samples that were absent of DNA did not yield a profile (Figure 3.2).

Upon extracting DNA from the pork bone the samples, quantitative PCR was run in the on the samples using the primers that were designed for humans in the QuantiFiler Human kit. Upon the completion of the quantitative PCR there was found to be no detected human DNA in any of the samples. Yet, when the samples were run on the NanoDrop 8000 all of the samples contained DNA. Furthermore, an STR profile was developed for all the samples. The reason that the real time PCR instrument did not recognize that there was DNA in the sample was because the primers did not bind to the pig DNA. Because the primers did not bind to the pig DNA it showed that they were in fact human-specific. This was good news as part of the reason this

research is being conducted is because a laboratory may wish to conduct research and profile case samples. Normally the analyst would have to worry about cross-contamination between the research samples and the case samples. However, if the researcher was using pig DNA the primers would not recognize the pig DNA. In addition, if there happened to be any cross-contamination, the pig DNA would not affect the result as the human specific primers would not be able to bind to the pig DNA.

During this research, one of the questions that was raised was whether or not a muscle tissue sample would be best extracted using the PrepFiler bodily fluid extraction procedure or the PrepFiler BTA extraction procedure. In order to investigate this question, five meat samples from the same piece of meat were extracted with each extraction technique. Once the DNA had been extracted the samples went through quantification with the NanoDrop 8000. The mean concentration of DNA in the 5 samples that went through the bodily fluid extraction procedure was 50.1ng/µl, whereas; the mean concentration of the five samples that went through the BTA extraction procedure was 28.1ng/µl. The Wilcoxon test showed that there was a significant difference to the 0.025 confidence level.

Upon statistical evaluation it was found that the two extraction procedures were significantly different from one another in the quantity of DNA that was produced from the extraction. It was expected that the BTA extraction technique would yield more DNA because it was designed for more complex tissue samples and had the addition of Proteinase K that aids in the breaking down of proteins. Even though the addition of the Proteinase K and the longer time in the thermomixer the BTA extraction was less efficient.

When observing the samples after they were removed from the thermomixer for each of the extraction procedures, the BTA extraction had degraded the sample of meat more than the bodily fluid extraction had. For this reason it was hypothesized that the BTA would yield more DNA than the bodily fluid extraction procedure. This was not the case. The bodily fluid extraction appeared to be more efficient in releasing the DNA from solution.

One possible reason for the BTA extracting less DNA than the bodily fluid extraction is that the BTA actually destroyed the DNA that was in the sample. It is possible that the muscle tissue was not rigid enough to protect the DNA that the procedure actually extracted and denatured/destroyed the DNA when lysing the cells. Then when the sample solution went through the extraction in the AutoMate Express only a portion of the DNA that was lysed from the cells was able to bind to the magnetic particle suspension and the rest was washed out with the buffer and contaminants.

The dilution series was conducted to examine the ability of the method to analyze DNA in low quantities and also to determine at what concentration alleles started to drop out of the profile. Alleles first started to drop out of the profile at a concentration of approximately 1:100. Even when the electro-kinetic injection was 20 seconds long, the longest recommended from the kit, there were still alleles missing. One allele that dropped out was allele 37 at locus SBH20 (Figure 3.9). The statistical analysis, Kruskal Wallis and Nemenyi tests, were done to see which dilutions were significantly different. The 1:1 and 1:10 ratio were significantly different from the 1:100 and the 1:200 profiles, respectively. The 1:1 and 1:10 ratios had much larger peak heights than the 1:100 and 1:200 profiles. This makes sense as the alleles were beginning to

drop out at the concentration of 1:100 and the injection times had to be increased from 5 seconds to 10 and 20 seconds to develop any kind of profile.

The mixture analysis was done to see at what relative proportion the minor component of the DNA profiles could be detected. At a 10% pig blood to 90% bone sample and 5% blood sample to 95% bone sample a couple of alleles at some loci are visible but not a whole second profile (Figures 3.13 and 3.14). From these results it was determined that the presence of a mixture could be determined all the way down to a 1 in 20 mixture component but a reliable profile could not be generated. A complete second profile was visible at a 25% pig blood to 75% bone sample concentration though.

The mean stutter height to peak height ratio was calculated. The mean stutter peak height ratio was found to be 12.75% with a standard deviation of 3.48. The stutter peak height ratio was very close to the manufactures recommendation of 13% for the kit. Three standard deviations were added to the mean stutter peak height ratio. The result of the 12.75% plus 3 standard deviations is a value of 23.2%. With this it was concluded that any stutter peak that was greater than 23.2 % of the actual peak could indicate that there was the possibility of another source of DNA adding to the stutter peak. This information provides a guide line for mixture analysis. It would allow an analyst to have a specific cut-off of what should be considered another peak from a second possible source.

The guidelines that were being followed for this research were the guidelines of a validation study that were set out by the Scientific Working Group on DNA Analysis Methods (SWGDAM) [11]. The necessary components of a validation are: peer reviewed articles,

sensitivity studies, species specificity, precision and accuracy and case type samples. A current article outlining the ability of the AnimalType kit is Caratti *et al.* [5], it shows the ability of the kit to differentiate pigs and the power of exclusion and discrimination of the kit. The species specificity was not shown in the current study. In order to accomplish this, human samples would have to be run using the kit and determine if a profile could be developed from the human samples using the pig primers for amplification. The sensitivity studies were shown in the dilution series as well as the mixture analysis. Extremely dilute samples were run and a profile could still be generated at a dilution of 1 in 200. Additionally, the presence of a mixture could be detected even at 1 in 20 concentration of one type of sample in another. The accuracy and precision of the kit and the procedure was demonstrated through the positive control and the duplicates of the muscle, bone and blood samples that were run. The positive control was identical to the manufacturer's reference profile. This demonstrated accuracy. Precision was demonstrated when the duplicates of the bone and meat samples all generated the same profile and because the bone and meat samples had the same profile of each other because they both came from the same source of DNA. The case type samples requirement of the SWGDAM guidelines was half fulfilled through the mixture and dilution series that were run. The dilution and mixture series showed that the kit and procedure can handle extremely dilute samples as well as mixtures which are common in forensic case work. Mixtures are common in sexual assault type cases and dilutions are common when an individual tries to clean up after they have committed a crime. The case type sample requirement is only half fulfilled because degraded samples and samples on different substrates are also part of case type samples and these types of samples were not tested in this study.

4.2 Limitations of the Study

This study was limited in the number of samples that were run as well as the ability of the study to do an accurate quantification. In order for the study to be strengthened many more samples will need to be run. Some of the samples that will need to be tested are degraded DNA samples, more blanks to determine the analytical and stochastic thresholds as well as samples on different substrates. These additional samples will advance the validation to show that case type samples can be analyzed as well as determine the background noise levels and to determine at which RFU level a peak in the STR profile should be considered an allele. Additionally, The stochastic threshold should be decided on. The kit recommends a value of 75 RFU but on one of the dilutions there was a peak greater than 75 RFU and the other alle that was supposed to be present had dropped out. This means that a stochastic threshold greater than 75 RFU will likely be needed. Human samples will also need to be run to show that the primers in the kit do not bind with human DNA and create a profile. Pig samples should also be amplified with the current human primers. This would show that the pig DNA would not generate a profile with the human specific primers used in the lab.

The quantification in this research was very limited because the NanoDrop 8000 was not very precise. The values varied from run to run even from the same sample by more than 10ng/µl. An accurate quantification will need to be used so that the dilutions for the amplification for capillary electrophoresis are accurate. In this study due to the fact the concentration of DNA was only an estimation the dilutions were not very accurate and many of

the samples appeared to be overloaded, having an excessive amount of DNA and leading to numerous artifacts in the profiles.

CHAPTER 5

CONCLUSIONS

5.1 Summation

This study completed the primary task that it set out to achieve. The task that was achieved was the development of an STR profile using pig DNA with the same methods and procedures that are currently in practise for forensic case work. The study also succeeded in completing most of the necessary requirements for the procedure to be validated and put into practise in a standard DNA laboratory. The parts of the validation that the research completed was that a stutter peak larger than 23.2% of the actual peak should be considered a possible source of another DNA contributor, a partial profile can be determined even if the DNA is extremely diluted and it is possible to determine if a mixture is present even if the minor contributor of the DNA mixture is only 5% of the overall mixture. This research project demonstrated the viability of using pig DNA as a research tool in a forensic laboratory. The pig DNA would not cross contaminate any active case work that may be taking place. Additionally, many other forensic disciplines are currently using pigs as research tools and the use of pig DNA would allow colleagues to collaborate and work together in research.

5.2 Recommendations

The next step for research in this area would be to develop a primer that binds to double stranded pig DNA. This primer would need to have a sequence of DNA that is present in

all pigs and that is specific to pigs. The primer would need to be present in all pigs and be specific to pigs because it would be used for the real time PCR instrument. The development of a fluorescent primer that can bind to a certain region of the nuclear genome the pig would allow for accurate quantification of the pig DNA. Accurate quantification is an essential part of the typical flow of samples in a forensic laboratory and thus, would be needed for the procedure to be validated.

Another step that should be accomplished would be the optimization of the PCR amplification before being run in electrophoresis. The PCR amplification needs to be optimized because there appeared to be a lot of slippage that occurred during the PCR. This was shown when the mean stutter height ratio was 25% (Table 3.5) at the locus SBH2. The stutter peaks are too high and can almost be misinterpreted as being a mixture with peaks from a minor contributor.

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