NDLERF

# Assessment of the utility of obtaining human profiles from drug seizures

## Final report

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An Initiative of the National Drug Strategy

# Assessment of the utility of obtaining human profiles from drug seizures

## Final report

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# Contents

# Acknowledgements

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# Acronyms

| | |
|---|---|
| **bp** | base pairs |
| **BSA** | Bovine serum albumen |
| **HVI** | human mitochondrial hypervariable locus 1 |
| **HVII** | human mitochondrial hypervariable locus 2 |
| **DNA** | deoxyribonucleic acid |
| **dTNP** | deoxyribonucleotide |
| **mtDNA** | mitochondrial DNA |
| **PCR** | polymerase chain reaction |
| **SDS** | Sodium dodecyl sulphate |
| **SNP** | single-nucleotide polymorphism |
| **SSC** | Standard saline citrate. |
| **STR** | short tandem repeat (in DNA) |
| **UV** | ultraviolet radiation |
| **WGA** | whole genome amplification |

# 1 Summaries

## 1.1 Executive summary

Our earlier work conducted by us on behalf of the National Drug Law Enforcement Research Fund (NDLERF) indicated that illicit drug powders contain traces of human and non-human DNA. These most likely arise as a result of unintentional contamination during manufacture, cutting or distribution. Although DNA has been detected on drug packages before (indicating who had most recently handled them), the discovery of DNA within the drugs themselves had not previously been reported. This offered the potential for a new way to tactically compare seizures and to identify individuals situated higher up the distribution chain.

This report reflects a more detailed examination of human DNA in drugs and the feasibility of extracting from it useful information. The expected outcomes of the project were to:

- identify how frequently drugs contain human DNA;
- assess the relative merits of various methods for profiling this DNA and make recommendations on which to use; and
- establish the conditions under which DNA survives in drug powders.

The project achieved these outcomes and made some new fundamental findings.

A key finding is that most seizures contain at least a trace of human DNA—in fact it is uncommon for a seizure to be free of human DNA. However, we did not observe any seizures that contained DNA from only a single human. The study also demonstrated that the current standard forensic short tandem repeat (STR) DNA profiling technique, Profiler Plus$^{tm}$, is impracticable as a routine operational screening method for illicit drug powders when a mixture of donors exists. These findings led to trialling alternative methodologies.

Profiling using STR methods will only be useful in the rare circumstance that DNA from only one donor is present. However, analysis of mitochondrial DNA was found to be effective. Unlike the case for STR DNA, there is no national mitochondrial DNA database, so using this technique is restricted to tactical comparisons rather than identifying individuals involved in supply chains.

As most seizures were found to contain DNA, the condition of the powders does not seem to be a major issue. Below a pH of 4.5, extraction does not appear to be profitable.

It was found that handling drug powders, even under laboratory situations, can cause contamination—indeed it is this very ease with which drugs pick-up DNA that means criminals cannot easily stop their DNA ending up in powders. However, it does mean that if DNA extraction from drugs is to be useful, samples for analysis must be taken in an appropriate manner before police or laboratory personnel handle the powder for other purposes.

We have presented two simple, rugged methods for extracting DNA that are ready for immediate operational use and we indicate how properties of the seizures reflect which of the two is the better option in specific circumstances. This report presents a series of recommendations on handling and processing seizures. It identifies sequencing mitochondrial DNA as a direction for further developing methodology to routinely derive information on the distribution pathway of illicit drugs.

The price of DNA sequencing is dropping at an astonishing rate. Given that Australian forensic laboratories are not equipped to conduct mitochondrial sequencing, an arrangement where forensic laboratories extract and interpret data while DNA analysis is outsourced, is expected to be a practical option within a few years.

## 1.2 Technical summary

This two-year project had broad terms of reference. It investigated a wide range of aspects of seizure DNA, albeit relatively shallowly, in order to establish the most favourable approach to extracting forensically useful data from human DNA present in illicit drug seizures. Two DNA extraction procedures were developed specifically for street-grade seizures that gave profiles from a wide range of both street-drug seizures and mock seizures; the imidazole-modified carbonate procedure, as partly developed in the previous report, and one that used a modified silica-filter procedure of a commercial kit. The modification of the carbonate procedure was chosen to deal with the physical difficulties that heroin-containing seizures sometimes presented. The modified silica-filter procedure was selected as an alternative method of general applicability providing cutting agents were not highly alkaline (such as sodium bicarbonate).

A new method of measuring human versus non-human DNA was tested on a batch of seizure DNA. It focused initially on classic STR multiplexes, examining various methods of estimating the 'usefulness' of the DNA for forensic purposes.

Partial STR profiles from two STR multiplexes; Profiler Plus™ and an in-house quintuplex, were quite commonly observed but there had to be one or a very small number of contributors for the STR profiles to be of solid value. It had been surmised that whole genome amplification (WGA) might be routinely applicable to seizure material and so one method was tried but dismissed as unlikely to be of value with seizure material due to the presence of non-human DNA, a problem that would probably apply to all WGA methods.

The pH dependence of the survival of DNA in seizure-like powder mixes was examined and recommendations generated concerning the usefulness of a seizure after a pH check.

Human mitochondrial DNA was readily amplified from street-grade seizure material and there were, as usual, multiple human contributors. Cloning the amplification products allowed haplotyping of individual contributors generating results of potential use to the law-enforcement community. From the small number of samples examined, this type of approach appeared to be the most likely to have practical value to the law-enforcement community. However, as cloning is an unfamiliar technology to the forensic community, an alternative more direct method for haplotyping based on DNA sequencing was explored. A set of sequence-labelled primers for use in 454 sequencing of two mitochondrial loci (HVI and HVII) and protocols for their use were developed as the basis for an alternative technology.

A high yield of informative mitochondrial reads was obtained whenever polymerase chain reaction (PCR) products gave a clear 'band'. An adequate number of reads was often obtained even when a clear band was not seen. The average length of reads obtained was usually longer than half the length of the HV loci; the condition necessary for combining sequences into consensus contigs. The error rate in reads generating false single-nucleotide polymorphisms (SNP) is low and is cancelled out by contiging the reads. However, the systemic error rate generated by long homo-polymer runs is a real issue. For example, 454 sequencing made miscount errors in the adjacent pair of poly-C runs in the HVII locus.

The study built considerable experience with contamination issues and produced insights into how seizures may acquire human material. This may be useful in assessing the evidential/intelligence value of profiles of any sort that are obtained from seizures.

## 1.3 Considerations for casework

DNA can be extracted by either of the two procedures described in this report. One is cheaper, the other involves the least effort for a casework laboratory, and both are of similar usefulness.

From the experience gained in this project, the number of donors of human DNA in seizures of 'street-grade' drugs in Australia is highly variable. It is unlikely that it will be profitable to routinely extract and profile DNA on the assumption of a single human source.

Profiling using STR loci is of limited value and best suited to those isolated cases in which high levels of DNA with few donors are indicated (see results section for a discussion of this point). Mitochondrial loci are probably the most appropriate for routine cases where a low amount of DNA is expected and maps of supplier/handler networks are being assembled.

The outside of seizure tablets are commonly richer in DNA, presumably due to handling, than the inside of a tablet, offering the possibility of discriminating between contributions made prior to tablet-making and those after tablet-making.

Seizures with a nominal pH of 4.5 or less are unlikely to have amplifiable DNA and when seizures have a pH below 7.0, amplification products should be checked for indications of template switching and the generation of spurious sequences during amplification; for example, discontinuities or strange insertions in otherwise well known sequences such as human DNA sequences.

# 2 Introduction and background

The terms of reference for this project set by NDLERF in the schedule to the agreement are 'when undertaking the Project the Participant must:

- artificially set up experiments with seizures that have had human cells deliberately added;
- conduct experiments with seizures containing "inherent" DNA;
- collect and correlate both dependent and independent data; and
- explore data-relationships to determine the level of successes expected for different types of drugs, and determine the usefulness, or otherwise, of a pH check.'

In a previous project examining DNA contained within drug seizures (Burgoyne et al 2008), we found that human DNA was present in some seizures. It was proposed that this source of DNA should be investigated to look at its potential for obtaining human DNA profiles. This would determine if it was worth exploiting and, if so, how.

It was envisaged that this investigation would involve examining a representative sample of Australian street-grade seizures for their content of profile-able DNA using both a selection of DNA extraction technologies and a selection of profiling technologies. Selection of extraction technologies was reasonably straightforward but selection of the most effective profiling technology was less clear because the field of profiling technologies is currently highly dynamic.

It was also envisaged that many well-controlled experiments would be carried out with artificial mixtures of seizure material with defined amounts of DNA added. However, a late change in regulations governing the amount of drug we could hold at any one time forced the experiments to be based on aliquots of 'street-grade' material each comprising about one gram. These proved to be almost individually unique. Experience showed that it usually required at least 250 mg for an extraction to be useful. Consequently, experiments using well-controlled, high-quality multiple replicates with model systems using defined blank levels of DNA and defined added DNA were found to be logistically difficult and, eventually, almost unacceptably wasteful of seizure material. Large amounts of big seizures that would make this much more practical required secure storage beyond our current capacity and, it was judged, would give much less return to the forensic community than would further experiments with the intrinsic DNA of seizures. As a result, the recommendations arising from this project are based on experience with, and observation of, the intrinsic DNA content of seizures—as described in the results section—rather than formally controlled experiments.

This survey has been focused on identifying what is likely to be the most practical approach in a forensic setting where economic and logistic variables are large, jurisdiction dependent and evolving with time. Consequently, the conclusions we come to from our observations are, to a degree, subjective in their assessment of the value of a specific approach in a forensic context.

The core of this project is not the question of human DNA in drug seizures because that has been settled—a significant proportion of them *do* have human DNA within them. **The core-question of this project was and is: 'How can the forensic community get useful information about the human donors represented in seizure DNA first, in a financially efficient way and second, in a parsimonious way. In other words, how do we squeeze out the most information for the least cost and effort?**

The key consideration for this project was determining what form of analysis any given seizure should be subjected to. Forensic standard STR multiplexes such as Profiler Plus™ would, if successful, provide the most useful information because results could be directly compared against the national DNA database. However, the success of STR analysis is dependent on a number of metrics including the:

amount of DNA present in a sample;

- proportion of DNA that is human;
- number of contributors; and
- average size of the DNA fragments.

Without knowledge of these, applying STR analysis may fail and in doing so, may consume the total sample, rendering alternative but still useful analyses impossible. We considered ways of gaining some of these metrics to inform the decision on whether to apply STR analysis.

In respect of the amount of DNA present in a sample, estimation of the human DNA content using the industry standard systems for Human-DNA, Quantifiler™ (Applied Biosystems), a real-time amplification method, is likely to be misleading. Quantifiler™ detects human genomic DNA by amplifying a fragment of 62 base pairs in length and is expected to overestimate the content of useful DNA, as it amplifies fragments well below the size of the smallest alleles in some current multiplexes. As a result the degree of degradation of the DNA would influence the applicability of the result. A two dimensional array approach used early in the project did give information on the amount of DNA present and the ratio of human to non-human DNA but was too resource intensive for routine use.

Variations on the standard STR approach, applied to a small subsample of the DNA extracted from a seizure, can in principle yield information relevant to determining if standard STR analysis would give useful information. For example, early in the project, we trialled high cycle number amplifications using a quintuplex of STR loci run on the Bioanalyser™ as a means of indicating both the amount and diversity of human DNA genotypes present. This approach is dependent on peak height for estimating yield and relative peak height for estimating the number of donors. However, separation based on this low-resolution capillary electrophoresis proved only partially successful since allele resolution was insufficient to reliably estimate donor numbers. Appropriate resolution is achievable using Profiler Plus™ with standard instrumentation and an extended number of amplification cycles. However, we found that patterns derived from artificially constructed complex mixtures showed that amplification of mixed alleles did not preserve allele ratios, even when using the standard number of amplification cycles, defeating the expectation of resolving individual profiles. Although the direct use of STR analyses such as Profiler Plus™ certainly gives profiles, the probability of them being useful is low. We found that when human DNA is present in seizures it is rare that only one donor was present, yielding mixed profiles of limited value.

A search was then initiated for an alternative test that would indicate the utility of a seizure and would also give some information that would also relate to identifying a donor. We found that mitochondrial loci are, as expected from their relative copy number, much easier to amplify out of drugs seizures than nuclear loci. This immediately offered the prospect of limited but hard data on both the number and identity of the humans contributing and so offered the prospect of being the first and best estimate of the ‘usefulness’ of the DNA in a particular drug sample. Here, ‘usefulness is a practical concept that integrates all considerations plus case-importance into a single decision—“proceed further” or “do not proceed further”’.

In summary, the more obvious approaches that could become forensic industry standards for profiling human DNA in drug seizures have been considered for their suitability for that purpose. It is suggested that assessing a seizure for either evidential or intelligence value begins with a mitochondrial amplification. A suitable locus currently used in the industry is hypervariable locus 2 (HVII), which has a size–range similar to that of the short-amplimer multiplexes in use in minifiler. Amplification of HVII by real-time polymerase chain reaction (RT-PCR) could be used to estimate overall amounts of DNA and further analysis of the RT-PCR product is capable of giving a good estimate of the diversity of donors by, for example, examining the SNPs within it. Further, if mitochondrial amplification indicates that the DNA is not suitable for obtaining a multiplex STR nuclear DNA profile, then usually it would have directly provided intelligence-grade information.

# 3 Experiments and conclusions

## 3.1 Finding the non-human to human DNA ratio

The project surveyed the ratio of human DNA to non-human DNA content in typical seizures and displayed this using a scatterplot.

### *Why this was done*

To estimate the ratio of human DNA to non-human DNA in seizures that were to be subject to other investigations.

### *What was done*

DNA was extracted from a number of seizures (detailed in Section 5.2.1) and each was amplified with a selective primer (antiseq05) and spotted separately onto an array (described in Section 5.3) along with a control and spots of known DNA content. As listed in Appendix 1 and described in the previous report (Burgoyne et al. 2008), some of the spots are single sequences and some are mixtures derived from real seizures. The array was hybridised with two materials each carrying different fluoresce labels. These were amplification products from human DNA and amplification products from detritus mixture GR170 known to contain non-human DNA typical of that found in seizures but having much less human DNA. The antiseq05 primer was used to non-specifically amplify a pseudorandom sample of sequences from the template. The use of antiseq05 and the origin of detritus mixture GR170 are described in our previous report *The bioprofiling of illicit drugs* 2008.

### *What was observed*

Fluorescent signals from the arrays were presented as a scatterplot (see Figure 1). The light red area of the scatterplot is where spots will fall that are rich in human sequences. The light green area is where spots will fall that are poorer in human sequences but rich in the non-human sequences commonly found in seizures of illicit substances (see previous report [Burgoyne et al. 2008] for a discussion of the spectrum of non-human sequences).

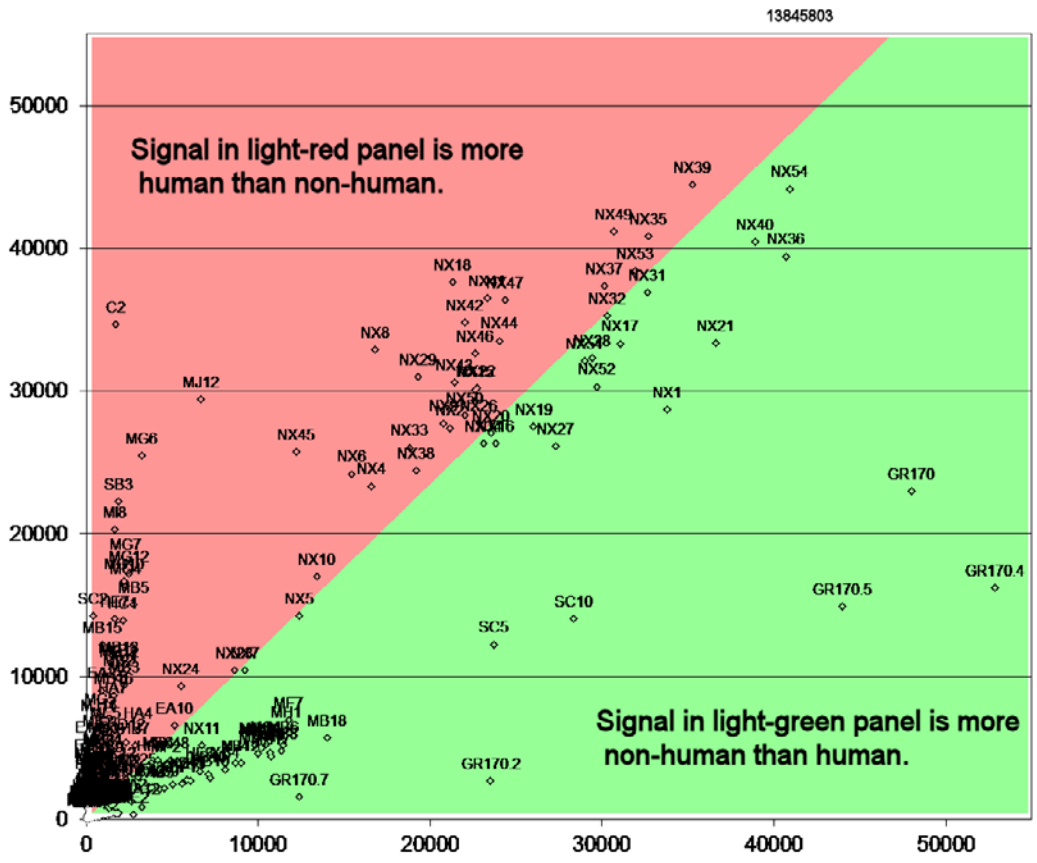


**Figure 1** Scatter plot of fluorescent signals from an array containing DNA amplified from illicit drug seizures and a variety of controls. Seizure material is from ecstasy tablets listed in appendix 3. The experimental points, derived from tablets and blank controls, all have the prefix NX and controls with known content of human DNA and DNA from detritus all have sets of other characters as prefixes. Sample C2 is human only, the first character G denotes derivatives from old seizures that have behaved as if they contain common non-human detritus, the character S denotes a sample derived from a soil and the character E denotes a sample derived from environmental dust. H and M denote samples derived from heroin or methamphetamines respectively. The X-axis is the fluorescence elicited by challenge with GR170, DNA from a detritus mix known to be mainly a complex mixture of sequences from non-human sources. The Y axis is the signal elicited by pure human DNA. The relative content of human to non-human sequences determines the two dimensional position of any spot in the area of the scatterplot

## *General comments*

Human DNA was found in some seizures and not in others, confirming the pilot work conducted in our previous NDLERF project. Combining the results of our pilot work, which involved a small number of seizures and the present work, it is apparent that most seizures contain human DNA. The amount of human DNA was correlated to a considerable degree with the amount of non-human DNA present in a seizure, a finding in accord with results reported for house dust [Toothman et al 2008]. However, no indications of degree of degradation or multiplicity of donors can be deduced from this type of data.

## Discussion and conclusions

- The array is a useful way of determining the relative content of human and non-human DNA in a seizure, placing the human control C2 well into the red sector of the scatterplot and the known sources of non-human DNA mainly in the green sector. Even some apparently inappropriately placed spots, for example spot SB3 (derived from a soil sample) on close study and submission of its sequence to Genbank was found to be from 'Homo sapiens genomic DNA, chromosome 18' as implied by the array and so was actually a stray human sequence, presumably present in the soil or as a result of contamination during preparation of DNA from soil.
- The scatterplot demonstrates that most tablets that contain DNA have a slight to moderate predominance of human DNA. A greater predominance of human content would have placed a spot nearer the Y-axis, closer to the positive human-mix control, C2. From this, it was concluded that the non-human DNA is probably a normal concomitant of the human DNA. This is unsurprising as human surfaces are known to contain microbes and their DNA from a number of sources, including the intrinsic skin flora. This conclusion comes from the observation that the NX spots cluster around the diagonal and are distant from both the positive controls for non-human DNA in the far right of the green sector and human DNA C2 in the far left of the red sector.
- The scatterplot was not designed to measure absolute amounts of human DNA, only relative amounts; however, it indicates that there is a large and almost continuous variation in the DNA content of seizures. The larger the amount of DNA, the more amplification product is loaded onto the array, resulting in a stronger signal placing the spot higher up the diagonal. After examining related data such as loci and alleles present, a routine objective standardisation for total amount was not considered worthwhile. Other features of the quality of the DNA became recognised as more significant issues, most notably the multiplicity of donors, which are discussed later below.
- The data showed some blank control samples, the worst being that NX54 (a supposedly negative control) had been heavily contaminated. The contamination was most plausibly traced to the properties of commercial filters, probably involving electrostatic attraction of detritus, leading to contamination after UV decontamination during DNA extraction. This forced us to change our anti-contamination procedures to control this source of intermittent contamination (see Section 5.6 Artefacts and contamination issues).

## Implications for the next stage of work

The finding that the content of human DNA tended to be correlated with similar amounts of non-human DNA lowered our expectations for finding circumstances suitable for the efficient use of whole-genome-amplifications (WGA). The concomitant non-human DNA will always degrade the WGA performance and, although it did not justify the abandonment of the WGA option, it did indicate that in the absence of a rapid demonstration of utility, limited effort should be invested in WGA.

The scatter of seizure spots along the diagonal of the scatterplot confirms the expectation that there would be continuously variable DNA levels; that is, there was no indication that seizures either had no DNA or 'sufficient DNA'.

The finding that we had a previously unrecognised contamination source triggered the search for a technical resolution of this (see Section 5.6 Artefacts and contamination issues).

Overall, we needed to establish an estimation procedure for human DNA that also took into account the size– or profilability of the DNA fragments and could give, at least in principle, a preliminary indication of the number of donors. This suggested the use of a very simple multiplex such as a quintuplex we had developed for other purposes to quickly measure the general 'profilability' of the DNA content of a seizure.

## 3.2 Using STR loci to study quality issues

### *Why this was done*

To see if a simple STR amplification for the presence of human loci could act as a useful pre-sort to eliminate samples that might contain human DNA but were of a size unsuitable for full STR profiling. This was proposed as an alternative to directly proceeding to, for example, Profiler Plus™ because a custom multiplex could be quickly and cheaply analysed on fast capillary-electrophoresis-on-a-chip technology available on the market as the 'Bioanalyser™'.

### *What was done*

DNA from amplification of six seizure samples (NX31 to 36) with the quintuplex primer set, together with controls of human DNA and a blank were resolved by agarose gel electrophoresis or the Bioanalyser™ (see Figure 2A) enabling a subset of the standard STR human loci to be observed. No attempt was made to examine alleles as neither the agarose gels nor the Bioanalyser™ system is capable of resolving them. The quintuplex amplification, gel electrophoresis systems, primers used and the product size ranges are detailed in the *Methods* section.

### *What was observed*

Gel analysis of the full set of amplimers and controls (see Figure 2A) provided low-resolution separation of the product DNA. Bionalyser™ scans of amplimers from seizure sample NX31 (see Figure 2B) and of human control DNA (see Figure 2C) provided greater detail, including high molecular weight products not visible on the agarose gels.

### *General conclusions*

In accord with expectations from the array, most samples clearly contained amplifiable human loci, albeit in highly variable amounts. The longer loci seemed to be slightly less amplifiable than the shorter loci but this was not consistently so for the samples examined.

When estimating DNA using the quintuplex, both the Bioanalyser™ and gels gave similar estimations of the amount of DNA and, as a bonus, similar estimations of locus drop out. However, as neither the Bioanalyser™ nor the gels could completely resolve alleles, there was no practical gain in using the more expensive technology of the Bioanalyser™ merely because it gave much more attractive looking results. The apparent advantage of the Bioanalyser™, with its partial resolutions of alleles, could not be translated into a practical gain.

Overall, agarose gel analysis of test amplifications on the NX series of seizure samples was deemed more practical than either the Bioanalyser™ or the array.

## Discussion

The quintuplex is a multiplex generating product in the forensically relevant size classes, so is expected to give a better measure of the amount of profile-able human DNA than methods based on real-time PCR of fragments of smaller size such as Quantifiler™. The quintuplex displayed the number of loci available and scans of these tracks provide an objective estimate of the amount of DNA in arbitrary units that could be standardised to an estimate of nanograms of DNA available in a sample derived from a seizure. While agarose gel technology is convenient and cheap, it cannot indicate the number of donors. To generate a normogram of 'utility', we would need at least the quintuplex system plus a scoring protocol for it and also a measure of number of donors. However, estimating the number of donors requires resolution of alleles, which neither the agarose gel nor the Bioanalyser™ is able to provide.

The quintuplex amplification and analysis on a Bioanalyser™ is cheaper and faster than direct use of current industry-standard multiplexes. However, the techniques are not standard and laboratory time is so expensive that the materials cost-saving of a cheap multiplex is probably not justified for a working laboratory.

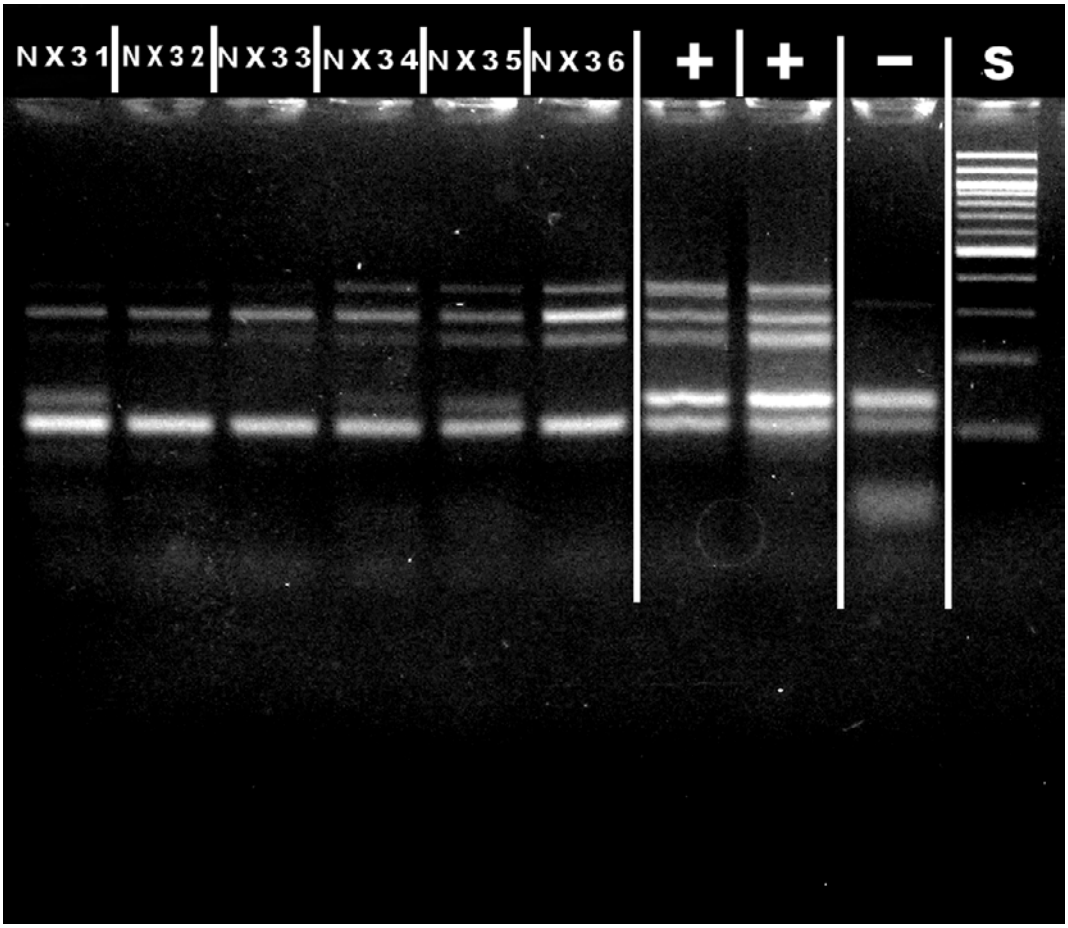


**Figure 2A** Agarose gel assessment of loci yield and quality. DNA from amplification of six seizure samples (NX31 to 36) are compared with amplifications of purified human DNA (+) and a negative control (-). As in this case, negative controls almost always contained some amplified material, not necessarily human. The standard track (S) is a 100 bp ladder. Most observable products are below 400 bp, the expected size range.

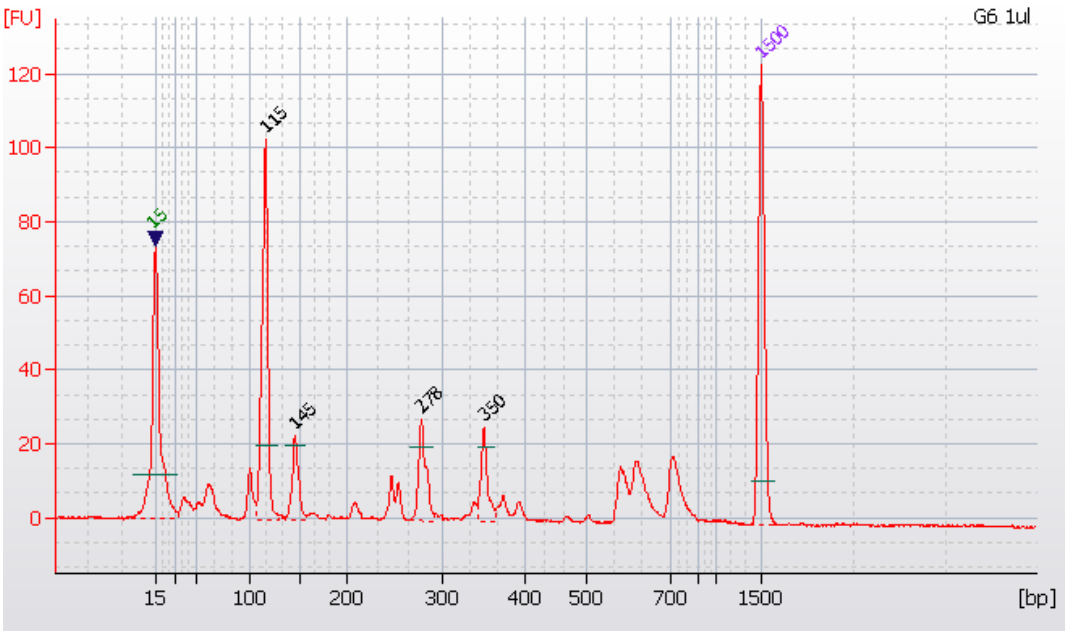


**Figure 2B** The Bionalyser[tm] CE electrophoresis system output for the amplification product of NX36. The numbers associated with a peak are the nominal base-pairs length of that peak. The vertical axis is arbitrary fluorescence units as a measure of DNA. The horizontal axis is nominal base pairs. The peaks at 15 and 1,500 base pairs are the manufacturer's internal standards. The resolution of this system varies with the peak position and can be as poor as ±10 base pairs.

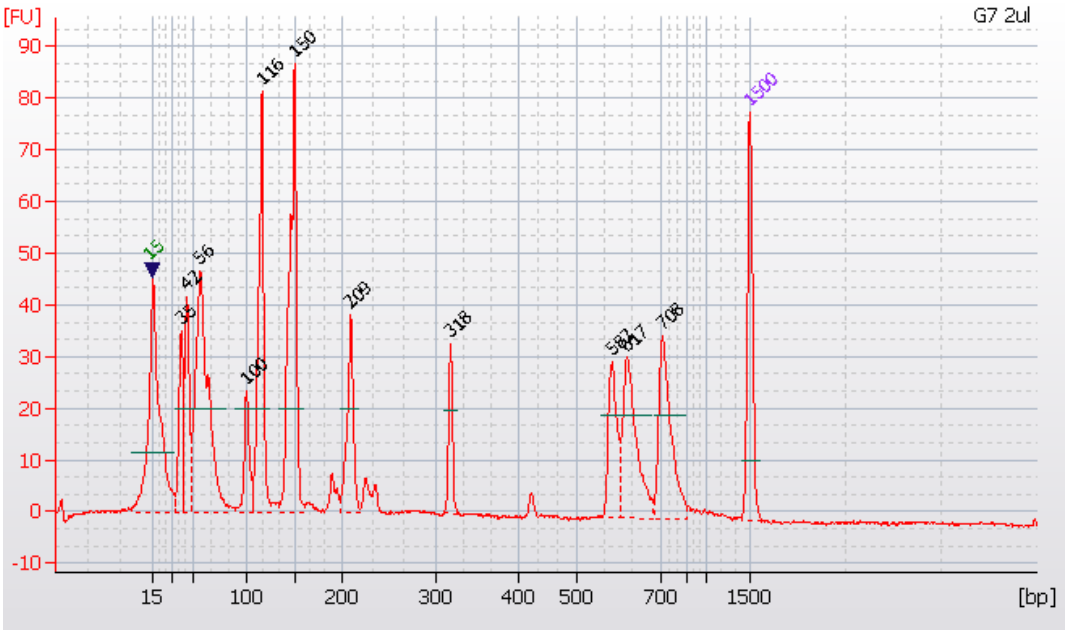


**Figure 2C** The Bionalyser[tm] CE electrophoresis system output for the human DNA control (+) immediately adjacent to sample NX36 on the agarose gel. Other details as for figure 2B.

## 3.3 Brief survey of the utility of pre-amplification

A brief survey of was made of the potential value of pre-amplification, primarily whole genome amplification (WGA), as a first step in profiling DNA extracted from illicit drugs.

### *Why this was done*

To see if a pre-amplification with a commercial WGA kit or an arbitrary primer would appreciably improve the DNA for diagnostic amplification.

### *What was done*

A number of seizures plus blank and positive controls were amplified using the Sigma WGA1 GenomePlex® kit (Section 5.4.4) system or an arbitrary primer. The amplified DNA was then examined for its utility in human profiling. The quintuplex system (Section 5.4.2) or a sub-set of it was used for the evaluation.

### *What was observed*

In some samples improvements were observed with WGA but not after amplification with the arbitrary primer. This improvement was inconsistent and the extra handling the technique required led to seriously increased risk of contamination. It was found that

- controls in which human DNA was amplified worked well and gave patterns typical of human DNA;
- pre-amplification by WGA improved the final yield of product from some seizures but mostly did not; and
- blank controls commonly had abnormal bands in them. In contrast, and as expected, the presence of real template, suppressed the production of these high molecular weight artifacts.

The data were only qualitative examinations of the products of amplification with the quintuplex primer set. These were analysed on both agarose gels and by capillary electrophoresis using the Bionanalyser$^{tm}$ (data not shown).

### *Conclusions*

The arbitrary primer pre-amplification was effectively useless and WGA did not seem to offer advantages, if anything, rather the opposite so it was decided to abandon the use of pre-amplification at this time. Non-human DNA tends to dominate the WGA and we concluded that if there was enough human DNA to make WGA work as expected, then it was more efficient to go straight to a sensitive multiplex like Profiler Plus$^{tm}$. This is in de-facto agreement with the curious dearth of reports of general usage of WGA on dirty material.

## Discussion

Pre-amplification with WGA systems has long been considered as an option in forensic DNA technology. It may well have application under some circumstances but in our experience, the crude, degraded and impure mixtures of template found in drug seizures does not appear to be one of them.

# 3.4 Considering the number of donors

## 3.4.1 Examination of standard STR alleles

This examination used a major, high discriminatory-power multiplex (Profiler Plus ™).

### *Why this was done*

To assess if examining the number of human alleles at STR loci of the type used in industry-standard STR could provide an insight into how utility degenerates as donor-numbers increase. Although this section is primarily concerned with assessing the effects of the presence of a multiplicity of donors, it also has a number of secondary aspects including examination of quality issues.

### *Background*

In donor mixtures, an infinite number of experiments are possible using an undefined number of donors and all possible ratios of donors to each other. To encapsulate that reality as thoroughly as possible within a realistic number of trials, a sample of DNA from a single male donor was mixed in various ratios with a mixture collected from more than 600 male donors in Adelaide in 1984 (the exact number of donors is unknown due to confidentiality requirements).

### *What was done*

The mixtures along with controls of single donor DNA and the ~600 donor DNA were constructed for 28 cycles to examine the effects of the poly-donor DNA on the profile of the major donor obtained with Profiler Plus ™. Amplification for 28 cycles was chosen, as this number of cycles is the least distorting used in forensic practice.

Mix 1—Single donor only (Figure 3A)

Mix 2—Single donor plus ~600 donor DNA at a ratio of 1:1 (Figure 3B)

Mix 3—As for mix 2 except the ratio was 1:10 (Figure 3C)

Mix 4—As for mix 2 except the ratio was 1:100 (Figure 3D)

Mix 5—The ~600 donor DNA alone (Figure 3E)

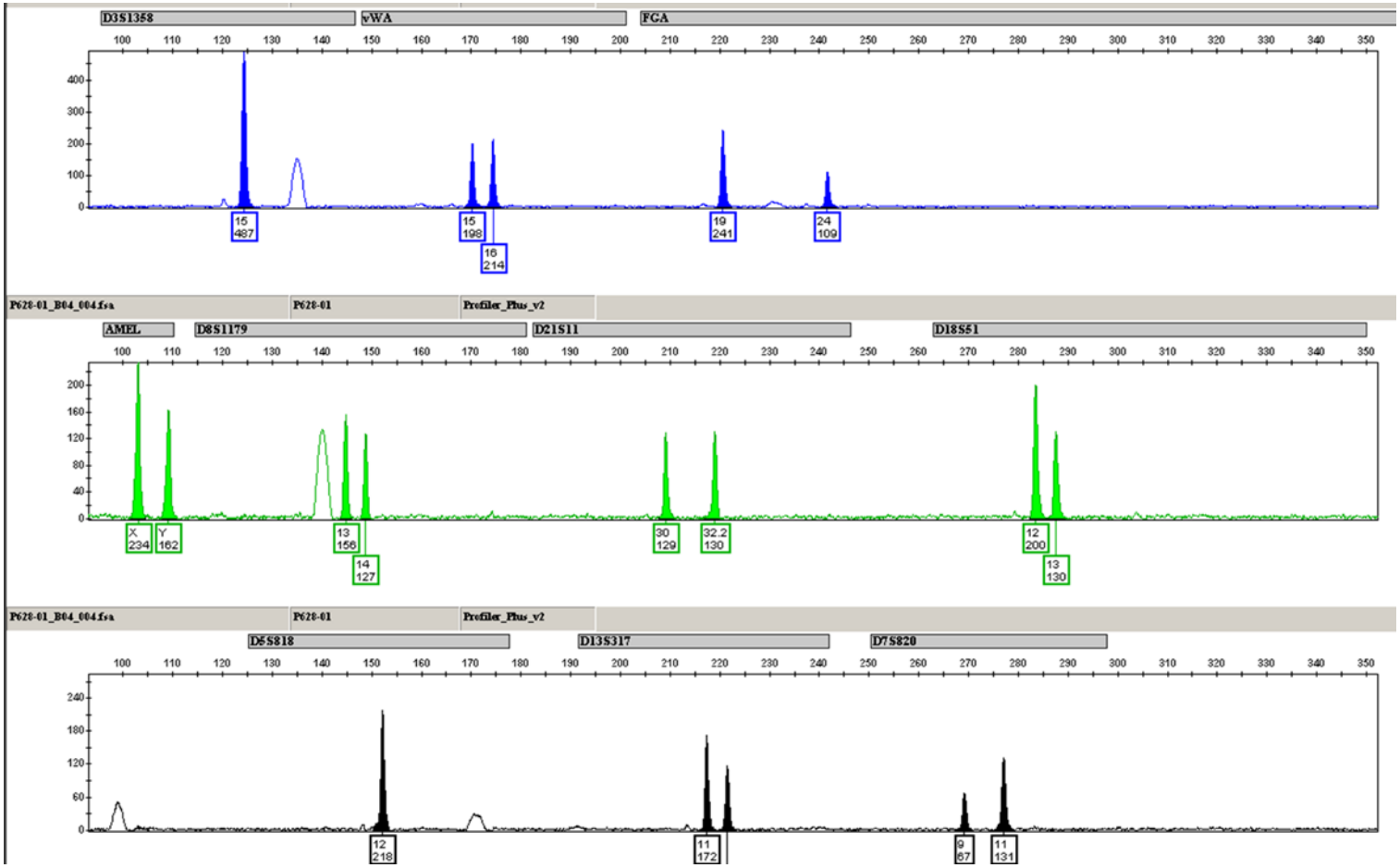


**Figure 3A** The single donor profile showing the genotype to be:

D3S1358 15 (homozygous)

vWA 15, 16

FGA 19, 24

D8S1179 13, 14

D21S11 30, 30.2

D18S51 12, 13

D5S818 12 (homozygous)

D13S317 11, 12

D7S820 11, 9

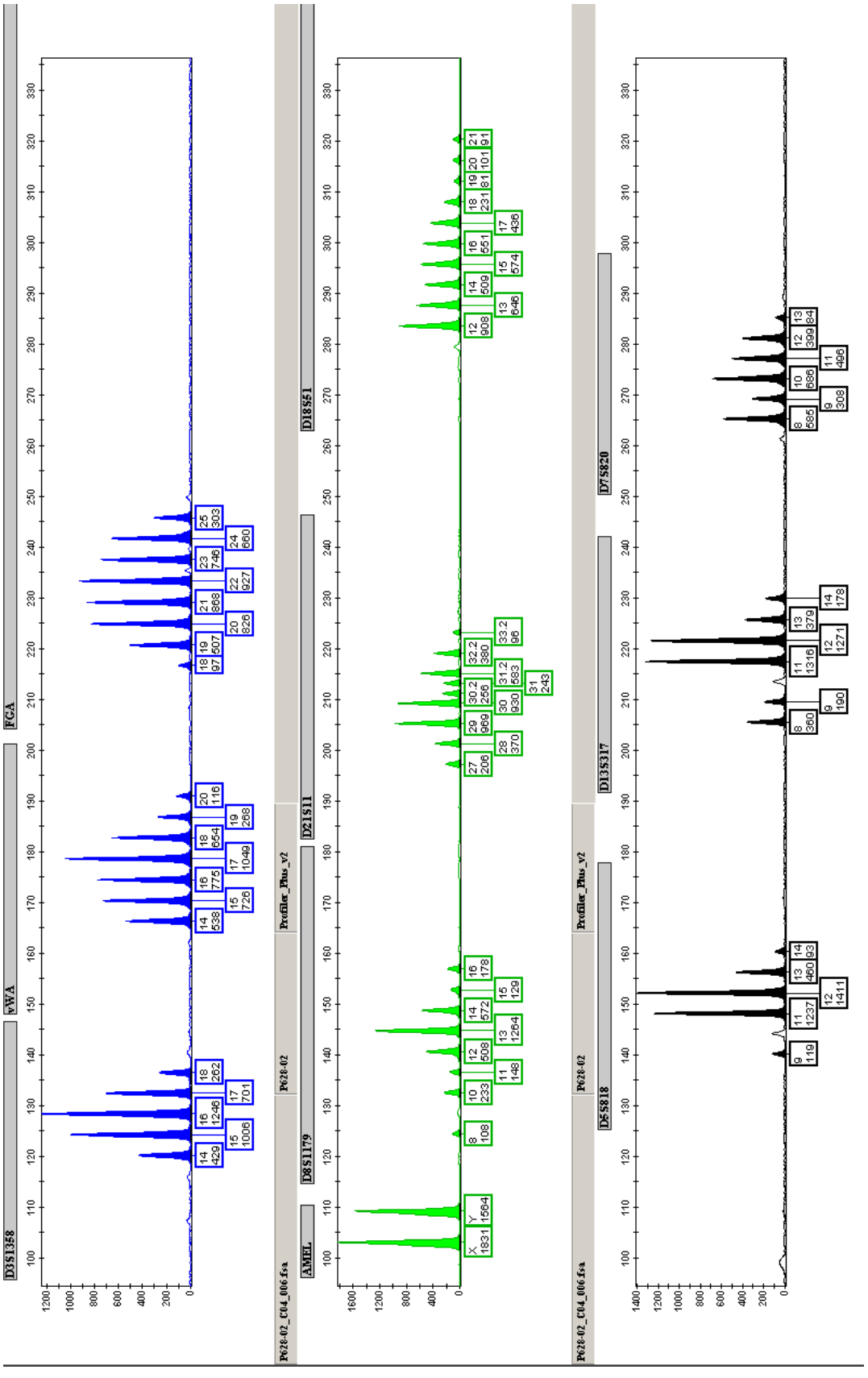


**Figure 3B** Single donor: ~600 donor DNA at ratio of 1:1 profile is not markedly different from that of the profile of the ~600 donor DNA alone (Figure 3E)

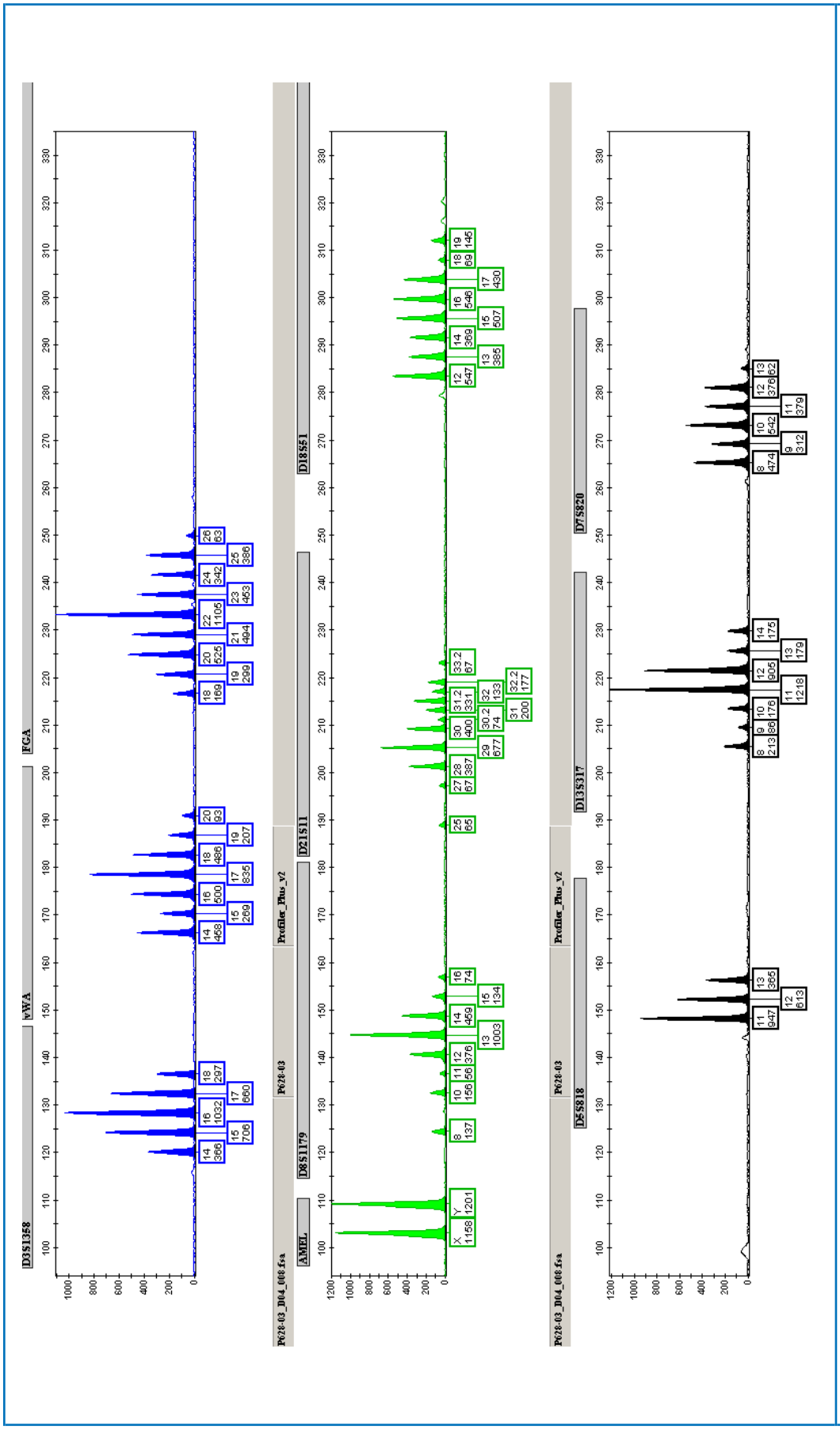


**Figure 3C** Single donor: ~600 donor DNA at ratio of 1:10 profile is also not markedly different from the profile of the ~600 donor DNA alone (Figure 3E).

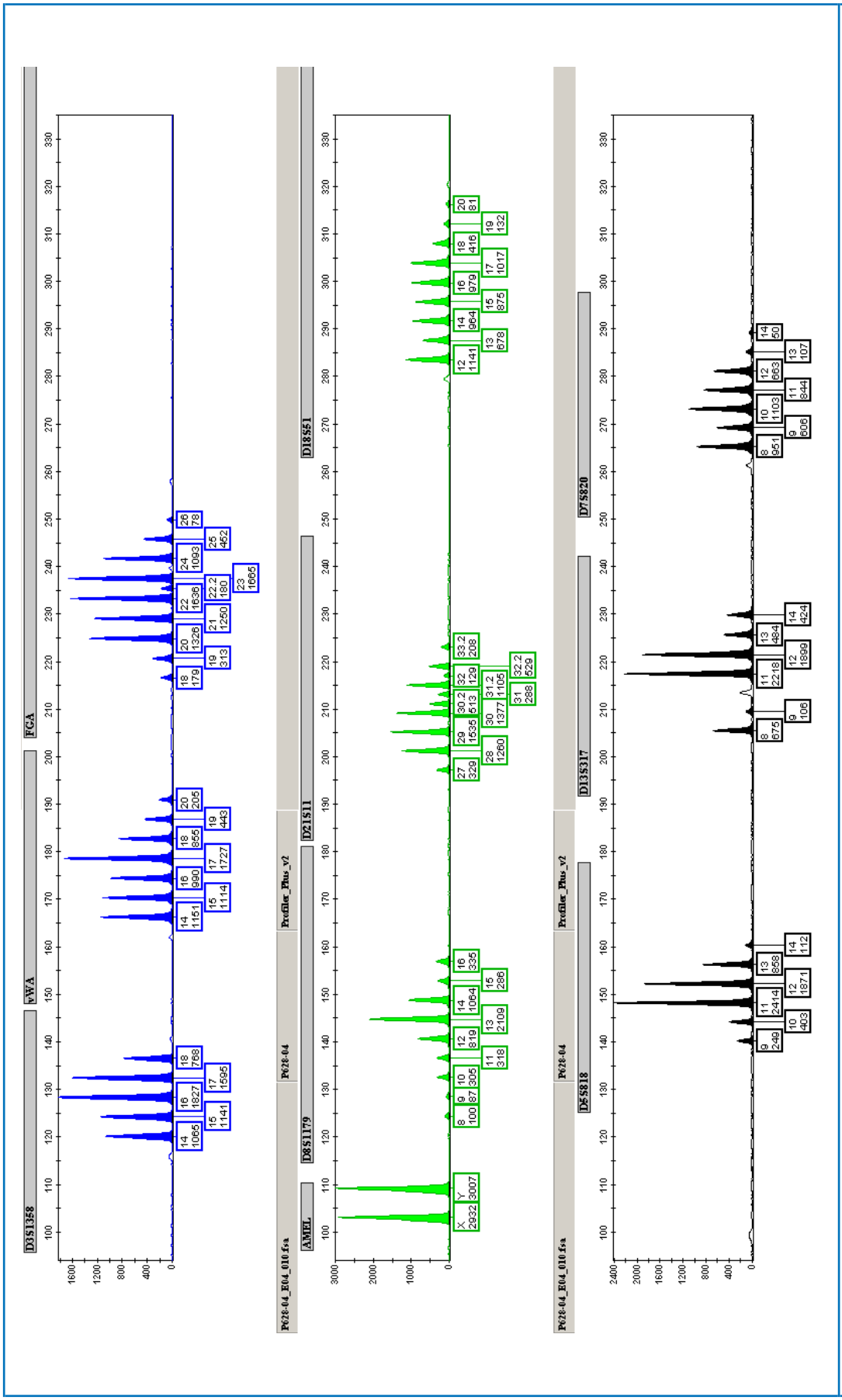


**Figure 3D** Single donor: ~600 donor DNA at ratio of 1:100 profile is essentially a duplicate of the profile obtained with the 1:10 mix, showing the stability of the result.

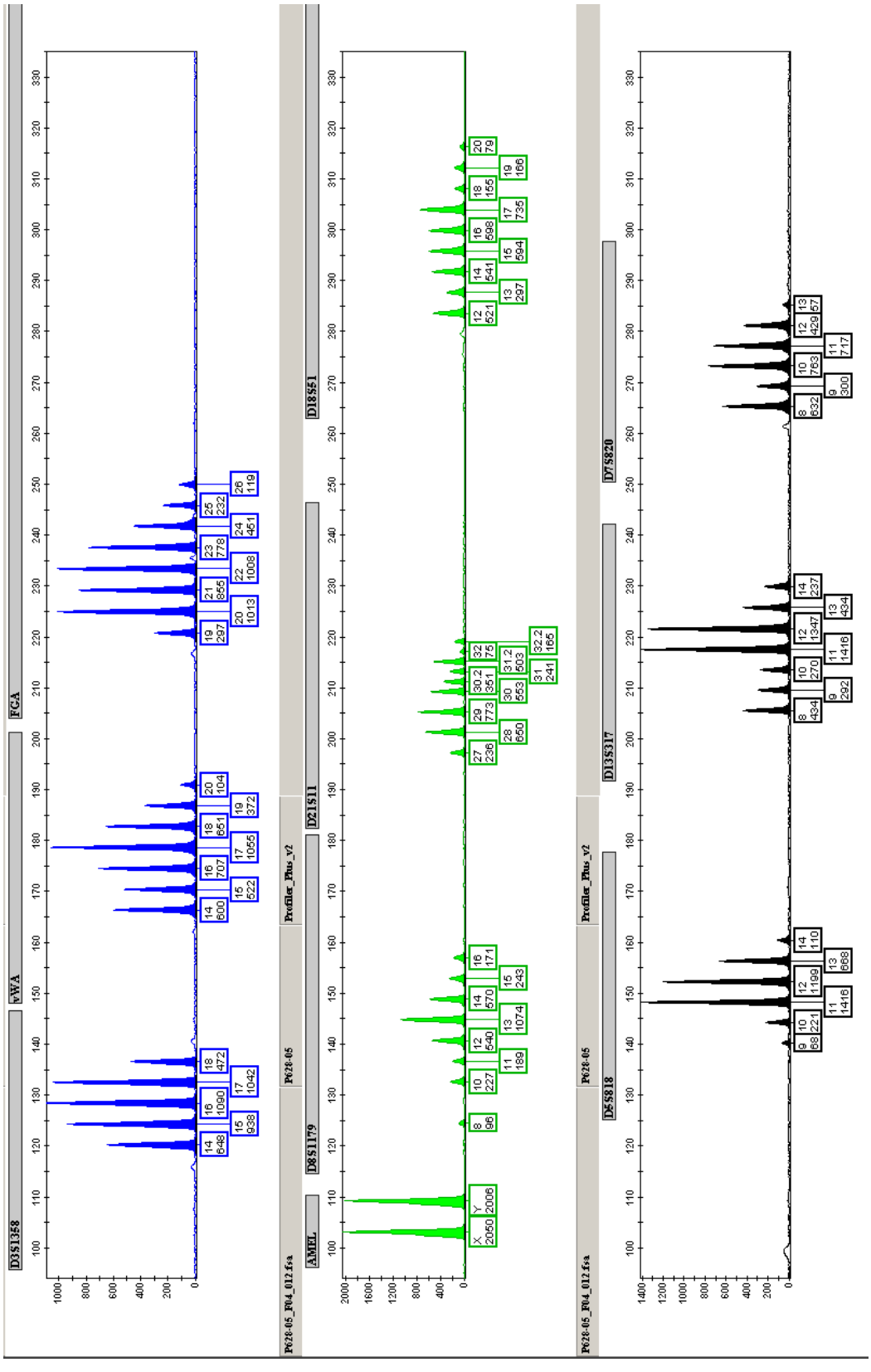


**Figure 3E** Profile obtained with the ~600 donor DNA alone.

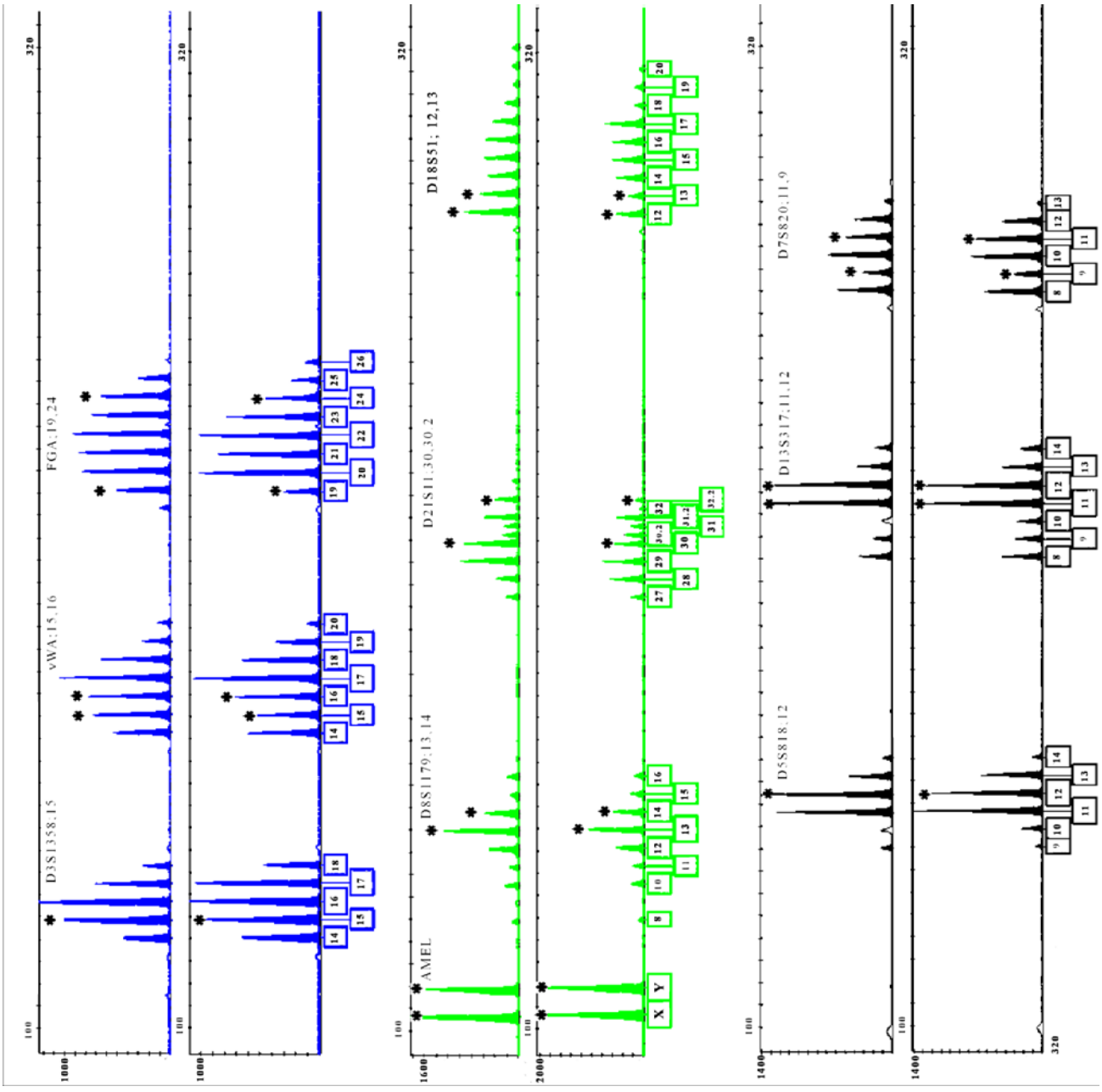


**Figure 3F** Alignment of the electropherograms obtained from single donor plus ~600 donor DNA at a ratio of 1:1 (top panel of each pair, from Figure 3B) with that obtained from the ~600 donor DNA alone (bottom panel of each pair, from Figure 3E). The asterisks and the allele numbers are those of the single donor. The degree to which the top track looks different to the bottom track within each track-pair gives the degree of separation of the single donor from the multiple donors. Even simple inspection shows that such differences are barely observable when present at all. In a 1:1 mixture, they are already, effectively inseparable.

**Table 1** The alleles at the listed loci that most dominate the profile of a massively diverse DNA amplified with Profiler Plus™. The most prominent alleles are in rank-order of prominence.

| Locus | Predominant alleles |
|---|---|
| D3S1358 | 16, 17, 15 |
| VWA | 17, 16, 18 |
| FGA | 22, 20, 21 |
| D8S1179 | 13, 14, 12 |
| D21S11 | 29, 28, 30 |
| D18S51 | 12, 16, 15 |
| D5S818 | 11, 12, 13 |
| D13S317 | 11, 12 |
| D7S820 | 10, 11, 8 |

### *Conclusions*

At equal total DNA concentrations, a sample of diverse human DNA almost completely overwhelms the signal from a single donor. This is true whether the allele from the single donor is a common allele or a relatively uncommon allele. Thus, if a profile were derived from more than one donor, it would be prudent to assume that is a spurious profile derived from any number of donors. Table 1 lists the most frequently expected alleles in spurious profiles that Profiler Plus™ would tend to synthesise out of DNA derived from multiple donors. Comparing figures 5 to 9 also demonstrates the stability of the amplification results.

## Discussion

In real seizures, when DNA was present, multiple alleles indicating multiple donors were the norm. Consequently, the results from these artificially constructed mixes were sobering as they demonstrated that even when DNA from a single donor was present in large excess over that from any of a large number of minor donors, the population of minor donors speedily overwhelmed the signal from the single donor even when the single donor contributed 50 percent of the total DNA. The conditions selected for this trial (28 cycles of amplification) were chosen to be the least distorting protocol used in forensic practice. These results imply that a background of high diversity precludes useful results with Profiler Plus™ and that the ideal now becomes one or two donors at the most. Samples showing evidence of highly diverse sources of donor DNA should simply be discarded. Additionally, the multiplicity of donors is not, in practice (and probably principle) determinable if multiplicity is greater than two or perhaps three. With larger numbers of donors, profiles will tend to show—disproportionately and misleadingly—only the common alleles in the human population.

The longer loci did not seem to be less amplifiable than the shorter loci. If anything, the reverse appeared to be the case, indicating that in the two multiplexes (Profiler Plus™ and the quintuplex) other factors are more important in determining which loci will predominate. These are likely a result of the relative primer amounts used to balance the multiplex and the number of amplification cycles used.

No matter how complex a mixture was there was never any loss of loci. Consequently loss of loci observed in amplifications from seizures reflects the absence of amplifiable amounts of DNA.

An obvious implication of the results presented in this section is that the profiles obtained from mixed DNA do not reflect the proportional contribution of individuals whose DNA is present. In the artificial mixture with 50 percent single donor DNA and the diverse spectrum of alleles present in an equal amount of the ~600 donor DNA, the single donor profile was not retained as a doubling in the height of relevant peaks but was lost among the alleles amplifying from the near full spectrum of alleles present in the population. This leads to the question; if the highly irregular and distinctive peak heights at the various loci *do not reflect population frequencies, then what do they reflect*? The answer to this question is not specifically a remit for this report

but is reasonably obvious. If the distinctive peak heights do not reflect population frequencies then they must reflect an intrinsic sequence property, the unique amplifiability of each allele. This is not a highly original reflection but it is a critical point to bear in mind in interpreting complex mixtures that may be found in seizures. Put simply, as any mixture of human profiles becomes more complex, then the relative peak heights shift towards reflecting the sequence-specific physics of amplification of each sequence much more than their relative contributions to the mixture. The selective processes that occur during amplification are quite important.

## 3.4.2 DNA profiles obtained using Profiler Plus$^{tm}$

### *Why this was done*

To survey a small sample of Australian seizures of amphetamine tablets for the diversity of amplifiable products, given that the main issues concerning mixed amplifications have been clarified.

### *What was done*

Seizure DNA was extracted from multiple samples from multiple seizures (Section 5.1). Initially seizures were evaluated using the quintuplex (Section 5.4.2) at a resolution high enough to separate alleles and then amplified with Profiler Plus$^{tm}$ (Section 5.4.1). However after using the quintuplex for the first few samples it was abandoned in favour of direct use of Profiler Plus$^{tm}$. Samples were processed and profiled as seizures became available, and the results scored for the general properties of the profiles.

### *What was observed*

Profiles obtained were diverse with many having negligible numbers of alleles at sites in the profile where loci were expected. A few seizures yielded too much DNA for efficient amplification and no seizures were observed with a single well-profiled donor and only rarely were seizures observed with absolutely no human DNA. However, even the most complex mixtures were not as ‘diverse’ as the ‘highly diverse’ DNA created and used in in Section 3.4.1

### *Conclusions*

In our limited experience, multiplexes seem unsuitable for the analysis of the donor DNA present in drug seizures. Many fragmentary profiles were observed (data not shown) and none of the profiles were easy to analyse. In the long-term it may be more productive to start with non-STR loci in the mitochondrion genome and use the results to nominate the very few DNA samples that will be suitable for STR analysis.

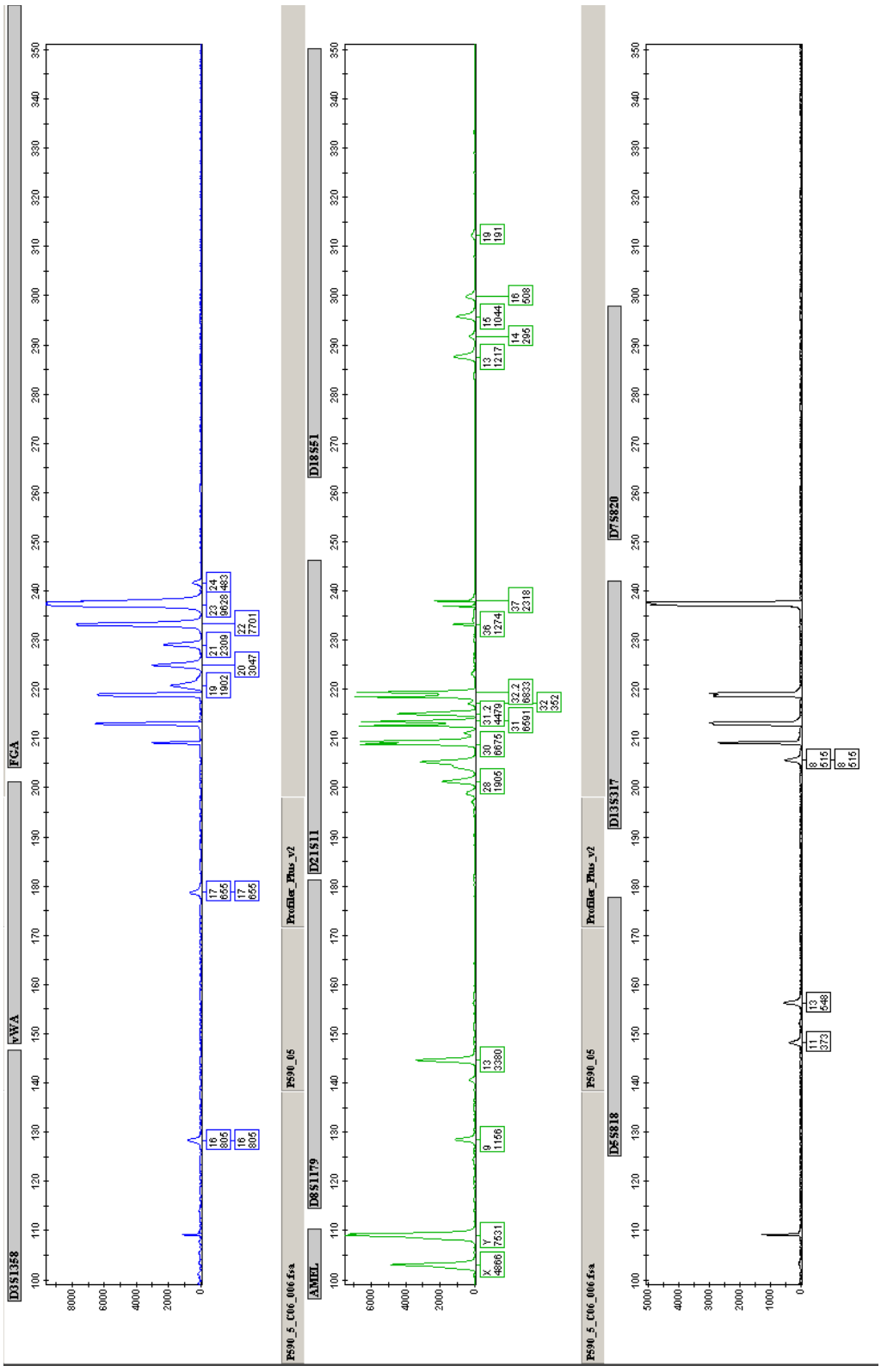


**Figure 3G** An example of a complex and poor profile obtained from a seizure, in this case from seizure NX41. See Appendix 2 series profile samples for other examples.

## *Summary of example Profiler Plus™ profiles from the NX series of seizures*

Images of the profiles are shown in Appendix 2, and their provenances in Appendix 3.

**Name and comment**: NX15 Six loci dropped out

D3S1358

VWA

FGA 19, 20, 21, 22, 23, 24

AMEL Y only

D8S1179

D21S11 Too complex to call.

D18S51 12, 13, 14, 15, 16, 17

D5S818

D13S317

D7S820

**Name and comment:** NX16 Five Loci dropped out

D3S1358

VWA 16

FGA 118, 19, 20, 21, 22, 23, 24

AMEL XY

D8S1179

D21S11 28, 29, 30, 30.2, 31, 31.2, 32, 32.2

D18S51 13, 14, 15, 17, 20

D5S818

D13S317

D7S820

**Name and comment**: NX17 Six loci dropped out

D3S1358

VWA

FGA 19, 20, 21, 22, 23

AMEL XY

D8S1179

D21S11 28, 29, 31, 32.2

D18S51 13, 14, 15

D5S818

D13S317

D7S820

**Name and comment:** NX18 Six loci dropped out

D3S1358

VWA

FGA 19, 20, 21, 22, 23

AMEL XY

D8S1179

D21S11 28, 29, 31, 31.2, 32.2

D18S51 17

D5S818

D13S317

D7S820

**Name and comment:** NX19 Three loci dropped out

D3S1358 16

VWA 19

FGA 19, 20, 21, 22, 23

AMEL XY

D8S1179 9

D21S11 28, 28.2, 29, 30, 31, 32.2

D18S51 13, 14, 15, 17

D5S818

D13S317

D7S820

**Name and comment: NX20** Three loci dropped out

D3S1358 16

VWA 19

FGA 19, 20, 21, 22, 23

AMEL XY

D8S1179 9

D21S11 28, 28.2, 29, 30, 31, 32.2

D18S51 13, 14, 15, 17

D5S818

D13S317

D7S820

**Name and comment: NX37** Two loci dropped out

D3S1358 15, 16

VWA 15, 19

FGA 20, 21, 22, 23

AMEL XY

D8S1179 13, 14

D21S11 28, 31, 32.2

D18S51 13, 14, 15, 16

D5S818 11, 13

D13S317

D7S820

**Name and comment: NX38** Three loci dropped out

D3S1358

VWA

FGA 19, 20, 21, 22, 23

AMEL XY

D8S1179 13, 14

D21S11 28, 30, 31, 32.2

D18S51 12, 13, 15, 16, 21

D5S818 11, 12, 13

D13S317 8

D7S820

**Name and comment: NX39** Two loci dropped out

D3S1358 15, 16

VWA 17, 20

FGA 19, 29, 21, 22, 24

AMEL XY

D8S1179 8, 9, 10, 14

D21S11 28, 29, 31, 32.2

D18S51 13, 14, 15, 16, 17

D5S818

D13S317

D7S820 12

**Name and comment: NX40** Six loci dropped out

D3S1358

VWA

FGA 19, 21, 22, 23, 24

AMEL XY

D8S1179

D21S11 0.2, 31, 32, 32.2, 33, 33.2

D18S51 12, 13, 15

D5S818

D13S317

D7S820

**Name and comment: NX41** One locus dropped out

D3S1358 16

VWA 17

FGA 19, 20, 21, 22, 23, 24

AMEL XY

D8S1179 9, 13

D21S11 28, 30, 31, 31.2, 36, 37

D18S51 13, 14, 15, 16, 19

D5S818 11, 13

D13S317 8

D7S820

**Name and comment: NX42** A very confused profile that may have contained animal DNA

D3S1358 15, 17

VWA 15, 16, 20

FGA 19, 20, 21, 22, 23, 24, 25

AMEL XY

D8S1179 9, 13, 14

D21S11 28, 29, 30, 31.2, 32, 32.2, 33.2

D18S51 12, 13, 14, 15, 16, 17, 20, 23

D5S818 8, 11, 13

D13S317 13, 16

D7S820

**Name and comment: NX43** Meaninglessly complex

D3S1358 15

VWA 15, 19, 20

FGA 18, 19, 20, 21, 22, 23

AMEL XY

D8S1179 9, 14, 16

D21S11 28, 29, 30, 31, 31.2, 32, 32.2, 33.2, 37

D18S51 12, 13, 15, 16, 17, 18

D5S818 11, 13

D13S317 8, 11

D7S820 11, 12

**Name and comment: NX44** Five loci dropped out

D3S1358

VWA 19, 23

FGA

AMEL XY

D8S1179

D21S11 Too complex to call

D18S51 12, 13, 14, 15

D5S818

D13S317 16

D7S820

## 3.5 Human mitochondrial loci from drug seizures containing very low amounts of DNA

### *Why this was done*

It became clear that STR-multiplexes had difficulties in discriminating between donors in a mixture. This was exacerbated by seizures commonly containing DNA from more than one donor; a problem that became impossible to solve where DNA was present but at low DNA levels. It was anticipated that the most desirable technology used to profile human mitochondrial loci—, T-vector-cloning and sequencing of PCR products, one of the industry standards for research molecular biology—could be applied to a forensic problem as a variant of it might be devised which was suitable for forensic DNA technology.

### *What was done*

Primers were selected to amplify both the hypervariable regions in the human mtDNA genome (HVI and HVII, Section 5.4.3 and Appendix 4). Results obtained using these primer sets have been published by others (e.g. Divine et al. 2005) when amplifying mtDNA for sequencing of the hypervariable regions. These were used to

amplify HVI and HVII in separate reactions and the same primers used to sequence the resulting products from both ends. Reference sequences and precautionary sequences from laboratory staff at locus HVII are in Appendix 4 (mitochondrial reference sequences). Amplifications were sequenced by the classic techniques of cloning and sequencing or by the cruder method of direct sequencing of the product mixture to get the major (consensus) profile. A third method used was to sequence-coded amplification products in order to assess the utility of 454 sequencing as an alternative to cloning.

### *What was observed*

Cloning following amplification worked as expected, and gave mitochondrial haplotypes out of a small but significant proportion of seizures that were giving no useful Profiler Plus™ nuclear profiles. In our hands, the HVII locus amplified much better than HVI but both loci gave products that could be haplotyped and these products were not from the usual or likely sources of contamination. The profiles of at least five 'stranger' donors were observed from heroin and methamphetamine seizures. Successful amplifications were frequently obtained but not from most seizures. On the basis of less than 50 samples, the success rate from any single sample is expected to be about 10 percent. The uncertainty of this estimate arises from the diverse sample sizes and analysis procedures used during these pilot trials.

## Conclusions and discussion

There is certainly mitochondrial genome in drug seizures and the sequences are often amplifiable. The HVII primers appeared to give the best results and are thus the logical candidates for a pre-assessment of seizure DNA content and utility, by, for example, real-time PCR of this locus. The success rate was satisfactory and the data look to be much more useful than that obtained by proceeding directly to STR analysis. Based on the limited extent of trials so far, the sequence results from cloning are most informative and clear-cut. Data from trials where the PCR products were sequenced without cloning, although much simpler to obtain, are not reliable. The inferiority of the cheaper alternative, analysis of uncloned HVII amplicons was apparent even in the small dataset generated in this project and more extensive testing of the superiority of data to be obtained by adopting the cloning approach is not warranted since it is expected to be the superior approach based on the fundamental principles of molecular biology.

It seems clear that mitochondrial loci have a major role to play in assessing seizure DNA when DNA content is low, as is the usual case. The cloned sequences should provide very useful data for police intelligence although, of course, not for evidence.

Since the manipulations required for cloning are not routine in a forensic laboratory, a more suitable alternative to cloning is desirable for forensic use. Thus it is not suggested that the forensic community should engage in the unfamiliar and specialised technology of T-vector-cloning and sequencing of PCR products. However, since T-vector cloning showed unequivocally that mitochondrial loci were available for human typing in some of the least promising seizures, development of an alternative methodology appropriate for forensic use is warranted. Next generation DNA sequencing a technology well suited to the forensic laboratory is likely to offer a suitable technical pathway to this sort of data. We have started exploring 454 sequencing for examining HVII amplicons as a prelude to developing a routine protocol for forensic examination of DNA evidence from drug seizures. The 454 or other next generation sequencing technology is much more likely than T-vector cloning to become an acceptable standard forensic technique.

## 3.5.1 DNA profiles by Sanger sequencing of mitochondrial loci—with and without cloning

### From clones

The HVI locus was amplified from priming sites F15971 and R16410 (where F=forward and R=reverse and the numbers are the sites in the mitochondrial genome) and the sequence of nine clones compared (Table 2). GMQ36 is the reference number for an amphetamine sample. The data are consistent with DNA from two individuals being present in the sample. Neither is a match to the two reference individuals nor to the three other individuals seen in samples GMQ04 and GMQ10 (Table 3).

**Table 2** SNP polymorphism in HVII clones from the amphetamine seizure GMQ36. Shading indicates the replicated readings of the two profiles detected. The nucleotide positions and polymorphisms are with respect to the cannonical sequence (Ingman et al. 2006 and see sequences from this source in appendix-4)

| Clone | Nucleotide position and change | | | | | |
|---|---|---|---|---|---|---|
| | 152 | 263 | 310 | 311 | 317 | 432 |
| 1 | T—C | A—G | C—ins | - | C—ins | - |
| 2 | T—C | A—G | C—ins | - | C—ins | - |
| 3 | T—C | A—G | C—ins | - | C—ins | - |
| 4 | T—C | A—G | C—ins | C—ins | C—ins | T del |
| 5 | T—C | A—G | C—ins | - | C—ins | - |
| 6 | T—C | A—G | C—ins | - | C—ins | - |
| 7 | T—C | A—G | C—ins | C—ins | C—ins | T del |
| 8 | T—C | A—G | C—ins | C—ins | C—ins | T del |
| 9 | T—C | A—G | C—ins | - | C—ins | - |

### From uncloned bulk product sequencing

The sequences were derived from heroin and methamphetamine sequences. They were aligned with the HVI and HVII loci of the revised Cambridge Reference Sequence (NC_012920) and the differences, in relation to the reference sequence, noted as polymorphisms (Table 3).

The first three samples (Table 3) were taken from heroin seizures, however sample GH1D22 was from a different seizure to GH1D34 and GH1D35 which were each 200 mg samples taken from the same one gram bag. Samples GH1D34 and GH1D35 were 'spiked' with a small amount of skin detritus donated by individual#1 to act as a positive control to test the hypothesis that human cells originating from a person handling drugs could not only provide a 'profilable'DNA source, but a 'known' profile might be discriminated in the presence of 'unknown' contributors which may be present in the sample. Samples GMQ04 and GMQ10 were taken from different bags within the same methamphetamine seizure and yet they seem to clearly originate from different individuals. Regrettably, no HVI material was obtained from GMQ04 and GMQ10.

**Table 3** Polymorphisms in HVI and HVII amplimers from methamphetamine and heroine seizures. The red-background asterisks mark allele-drop-out and the grey-shaded cells reflect that HVI amplifications were unsuccessful in these samples. Control samples, individual#1 and individual#2, are shown both for comparison and for elimination.Symbol-Key: The numbers in the top row are the reference sites in the reference sequence and terms like A-T are shorthand for an A in the reference sequence being replaced by a T in the sequence being discussed. Terms like C-ins mean that a base, C in this case, has been inserted with respect to the reference sequence. The terms like A-del means that with respect to the reference sequence, an A has been deleted. Other like A-G are in conformity with this system. The letters na mean that data was not available for those sites in those samples.

| Sample | Nucleotide position and change | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HVII | | | | | | | | | | | | | | | HVI | | | | |
| | 152 | 156 | 263 | 265 | 267 | 310 | 311 | 314 | 315 | 317 | 319 | 321 | 325 | 422 | 430 | 16031 | 16311 | 16315 | 16288 | 16402 |
| GH1D22 | - | - | - | - | - | - | - | - | - | - | - | - | C-ins | - | - | A-del | - | - | - | - |
| GH1D34 | T-C | - | A-G | - | - | C-ins | C-ins | - | C-ins | - | - | - | - | A-ins | * | - | T-C | - | - | A-C |
| GH1D35 | T-C | - | A-G | - | - | C-ins | C-ins | - | C-ins | - | - | - | - | - | * | - | T-C | T-C | - | - |
| GMQ04 | - | T-C | - | - | A-G | - | - | C-ins | - | - | - | C-ins | - | - | - | na | na | na | na | na |
| GMQ10 | - | - | | A-G | - | - | C-ins | - | - | - | C-ins | - | - | - | - | na | na | na | na | na |
| Individual#2 | T-C | - | A-G | - | - | C-ins | - | - | - | C-ins | - | - | - | - | - | - | - | - | T-C | - |
| Individual#1 | T-C | - | A-G | - | - | C-ins | - | - | C-ins | - | - | - | - | - | T-A | - | T-C | - | - | - |

The results are consistent with a single contributor for sample GH1D22 and a different contributor/s for samples GMQ04 and GMQ10. Evidence also suggests more than one contributor in the form of double peaks in the sequencing chromatograms (data not shown).

While the 'spiked samples, GH1D34 and GH1D35 look mostly like individual#1, as expected, the asterisk with red background at position 430 in HVII indicates an allele drop-out which reflects failure to observe individual#1's (T–A) at that position in the sequence.

## 3.5.2 Exploring 454 sequencing of human mitochondrial profiles

## Sequence-coding mitochondrial amplimers

### *Why we did this*

To explore an emerging technology that could provide an alternative to T-vector cloning to obtain single molecular species. This approach allows the sequence of quite different molecules present in a single sample to be read individually, an approach that dramatically reduces the cost per sample. Sequence-coding allows multiple samples to be sequenced as a batch, and allows a forensic lab to use a secure label for each sample within a batch. The label is undecodable by other parties because the sequence-codes, contain no information whatsoever about the samples without access to the coding key.

### *What has been done*

sequence-codes were devised for separating samples after massed sequencing. Poor and smeared primary-amplification products were submitted to 454 sequencing. The results were examined to see, first, if Sequence-coding them would corrupt the results. To this end, two quite different classes of sequence-codess were used in the secondary amplification. As security, a sequencecode was placed on both forward and reverse primers. Two quite different strategies were tried. In one, the two barcodes were complementary, and for the other, they were non-complementary. Sequence-coded primers were then used to re-amplify the (HVI/HVII) loci so that multiple samples could be sequenced simultaneously using 454 technology.

The products from non-sequence-coded primer amplifications using the standard, mtHVI and mtHVII, primers were used as a template for barcoding amplification.

The 9mer sequence-codes were embedded into the truncated 5' end of the primer sequences for HVI (F15971, R16410) and HVII (F15, R429) in such a way that they all had similar melting points and could all be amplified with the same settings on a PCR machine. All sequences used for barcoding, the diagnostic gels and the final construction of the mixes sent for 454 sequencing are available in Appendix 6.

On examining product at the end of the first amplification and the subsequent barcoding amplification, the initial (non-sequence-coded) amplifications from real seizures that were known to be poor templates, were so smeared that it was clear that much of the product was not from the desired loci. However, this was potentially an advantage as, by contrast, the positive control (post-barcoding) was quite satisfactory. It showed that the technique itself was satisfactory and thus the poor quality of the primary product from the seizures would now stringently test the concept of using 454 sequencing to extract haplotype data from the amplimers.

Comparing the first amplification to the sequence-coded amplifications showed little obvious effect of sequence-coding. It was concluded that there was nothing to indicate that sequence-coding in the secondary amplifications was having a distorting effect.

As the two sequence coding strategies trialled both gave similar profiles when analysed by agarose gels; smears from the drug samples and bands from the positive controls, amplimers obtained using the non-complementary set of primers were arbitrarily selected for sequencing. The sequence-coded amplimers were bulked into a single mix and sent to the Australian Genome Research Facility in Brisbane for 454 sequencing.

### *What was observed*

The raw data comprising 291,791 reads were assigned to the relevant sample using the sequence-codes (Appendix 7). This shows the reads obtained for each sequence-coded batch, the average read length for each batch and the maximal read length for that batch. It also shows images of random samples of the raw reads lists to illustrate the nature of the data.

There were no notable technical difficulties. The HVI and HVII sequences in each sample were constructed by alignment (Appendixes 8 and 9) allowing detection of multiple haplotypes in a single seizure; for example, four in seizure P53061764 (Appendix 9).

As expected, HVI and HVII sequences formed the overwhelming predominance of reads. The small number of extraneous bacterial and human nuclear reads observed, reflecting occasional priming from irrelevant sites were mainly detected in seizures containing very little DNA. However, the predominance of the target sequences meant there was no possibility of confusing these rarities with mitochondrial HV haplotypes. Further confirmation of the origins of all sequences was obtained by comparing with the National Center for Biotechnology Information (NCBI) database. Appendix 8 shows a sample of such comparisons.

The primer set for HVII gave a result with lower quality template than the primer set for HVI.

## Conclusions and discussion

The barcoding strategy worked and long read 454 sequencing was shown to be a viable alternative to cloning and sequencing multiple mitochondrial haplotypes present in a seizure. This approache could be adopted for future forensic applications.

Perhaps the only fault of 454 sequencing for this application is that it is intrinsically error-prone in determining the length of homopolymer runs above about four bases; for example GGGGGGG. However, this is a well-known systemic error of pyrophosphate sequencing technology and was expected. Ultimately there may be ways of handing this type of SNP but at present, they should not be used. This is not a major shortcoming as other SNPs do not have this issue. The problem of ordinary single-base misreads is not a serious matter as they are easily cancelled out by overlapping multiple reads using suitable software (Appendix 8).

The greatest advantage of the sequencing approach to de-convoluting mixtures of mitochondrial DNA is its extreme sensitivity. Most importantly, it is insensitive to the blurring and false-priming characteristic of trace-

DNA at the limit of detection. Even if the bulk of any amplification is not the desired product, the sequencing approach allows software to eliminate irrelevant products and abstract from the data as few as 10 to 20 reads per base that are sufficient to obtain a haplotype (genotype). Ten to 20 reads per base is quite a modest minimal requirement given that amplifications frequently yielded 20,000 reads per base (Appendix 8).

The trial reported here was performed with one combined sequencing-batch assembled from 21 sub-samples each representing the molecules from individual preparations made from drug seizures or control samples from a known donor. Each molecule in each sub-sample can be dealt with as a separate entity as it is tagged by sequence labels (the sequence-codes) embedded in the 5' end of the primers. The value of combined sequence batches is twofold—economies of scale and the inclusion of controls of known sequence to permit detection and allowance to be made for any batch-specific sequencing imperfections.

Although only two hyper-variable regions were studied, there is no reason that other mitochondrial or nuclear genome sequences could be treated similarly.

The software developed for this particular study, worked well but there is room for further improvement, particularly for the resolution of SNPs generated by homopolymer runs.

## The economics

The direct costs of sequencing this batch of 23 samples was AUD $1,300 (not including GST or the control-assurance sequencing that it was thought wise to include with this first attempt) and about AUD $56 per seizure or control sample. Of the 291,792 sequences received, 87,621 (30%) had lost or had potentially corrupted sequence-codes and were discarded. Others, uncounted, were discarded because they failed criteria during alignment; mainly because of short read length. Allowing for all these deletions, each potentially useful sequence cost about one cent. So, even after deletions, the methodology is very cheap per sequence and not prohibitive per sample. The major cost is for the labour required to prepare the material. A current serious shortfall is the time currently taken between sample submission and return of the sequence data. However, those delays can be expected to decrease as the use of massively parallel sequencing expands.

The barcoding technique allows for increasing, perhaps massively, the numbers of samples per batch. This is because sequencing technology is evolving rapidly in ways that enable expansion of the number of reads from a single batch. This has the potential to further reduce costs. Read lengths of more than 250 bases, the shortest acceptable read length to cross the loci concerned in two overlapping steps, were routinely obtained and reads were often long enough to cross the loci in a single step. Although the utility of any read depends on its length compared to the length of the target locus, there are usually ample reads to combine to give useful results with currently available 454 sequencing services. Near term developments in 454 sequencing are anticipated to increase read-lengths to about 750 bases.

In summary:

- The technology works well as it stands and is an appropriate alternative to cloning for deconvoluting HVI and HVII mitochondrial haplotypes.
- 454 sequencing makes systemic errors in assessing the length of homo-polymers, and SNPs dependent on this class of error, currently, are probably best ignored.
- At its current state of development, 454 technology frequently gives reads that cross a whole amplimer in a single run. These, together with overlapping shorter reads, allow reliable deconvolution of haplotypes. Expected improvements to 454 sequencing are anticipated to further increase read length.
- Stochastic misreads inherent in 454 sequencing are readily cancelled by contiging multiple reads.

# 3.6 Effect of seizure pH on obtaining a profile

## *Why this was done*

To determine how much human skin flake is detectable in a seizure using the efficient primers for human mitochondrial HVII and to find the pH of solid powders that will preclude the survival of skin-flake-DNA for extended periods of time.

## *Background*

Damage to DNA to be expected in drug seizures can be inferred from a recent review of agents that damage DNA (Gates 2009). Damage to DNA storage is well studied and the process of oxidation and acid hydrolysis in particular has been subject of much study. At pH 7.4, a physiological pH, the phosphodiester bond that forms the backbone of DNA, has a half-life of 30 million years; an unimportant level of instability. Deamination of bases has a half-life of about 30,000 years and is also of minimal significance for forensic purposes. However, the half-life of the purine-sugar bonds is only about 730 years at pH 7.4 and drops precipitously as pH decreases (Lindahl & Nyberg 1972). This makes it increasingly important for seizures having a progressively lower pH. Free radicals promoting oxidation are also important but the effects of these are much more difficult to estimate, particularly as most are likely to have vanished by the time the illicit material has been seized and assayed. The probability of acid-catalysed de-purination is considered the feature most likely to be of interest with respect to survival of human DNA in seizures. This project is not concerned with the details of this relationship of survival against pH but is very much concerned with defining its practical implications for the usefulness of a seizure.

## *What was done*

Artificial solid powder seizures were made by combining a trace of human skin fragment-dust with powdered sucrose—a common cutting agent of illicit materials. The shaver debris used was more, or at least as much, skin fragments as whisker fragments. See Section 5.7 and Appendix 5 for an example of how the mixtures were constructed. The pH of the mix was manipulated by additions of a medium-strength carboxylic acid powder (EDTA free acid) and lower pH values were obtained by additional use of HCL vapour, depending on the pH required. Sodium bicarbonate was the standard base. A small, defined, amount of sodium acetate was always present in the sucrose to ensure that, in the absence of HCl, the model seizure had a 'head' of vapour that could transmit the reigning pH, whatever it was, throughout the mass.

After set periods of time at room temperature the model seizures at different set pHs were processed for DNA by method (see Section 5.2.2) and scored (+-) for the survival of the HVII locus.

## *What was observed*

The survival of the HVII locus of the human mitochondrion in sugar mixtures was scored by observing the presence or absence of a PCR product on a conventional agarose gel. The detection threshold was approximately 11 average-sized skin particles (Figure 4) with a total particle area of 7,270mm$^2$ (0.5mg) per 150 mg of sugar. Below this amount of particles, the signal became unreliable (data not shown). Figure 5 shows the near linear decline in signal from HVII with time at pH 3.7, the pH at the boundary of conditions where template might be recovered with prompt analysis.

## *Conclusions*

The lower limit for exposure to acid pH that allows the production of a detectable amplicon is approximately pH 3.7 over 44 hours. Seizures with this pH or lower are not worth considering unless there is independent information that suggests DNA was deposited only a very few hours beforehand. Bearing in mind the variations introduced by dilution and pH estimations, it is suggested that pH 4.5 is a working lower limit. However, the actual decline in signal with time is only readily measured at pH 3.7 (Figure 5).

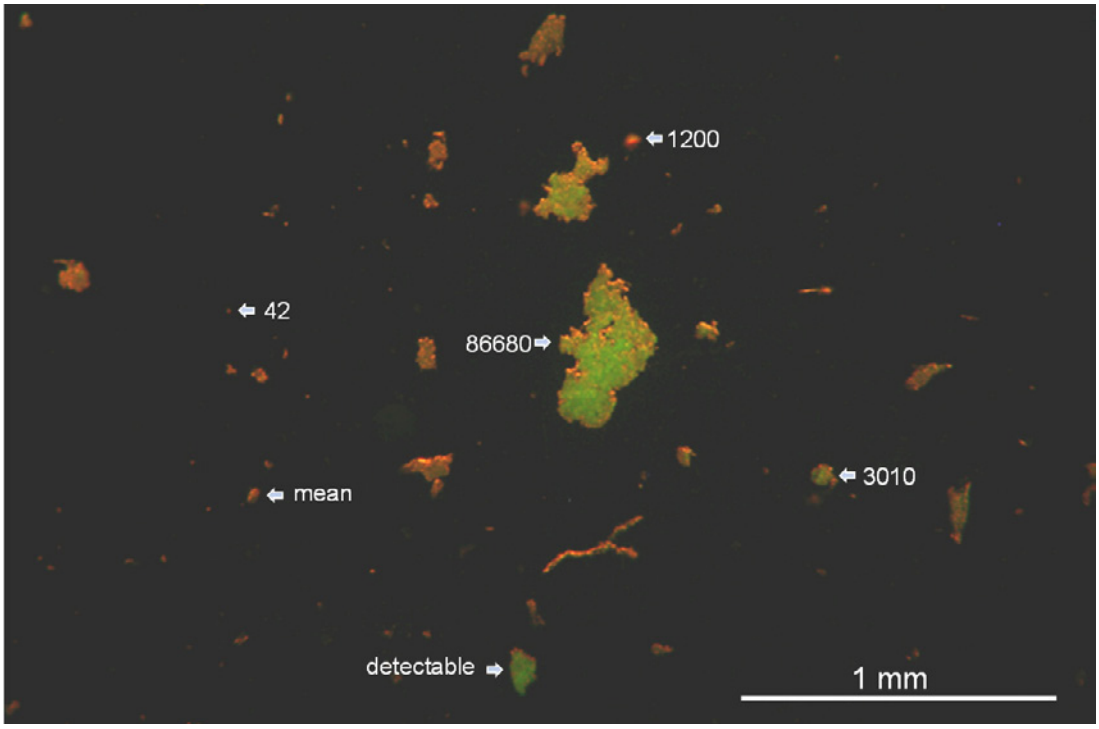


**Figure 4** photomicrograph of a representative sample of skin fragments obtained from electric razor shavings and stained with acridine orange to highlight fragments of any nuclei. Numbers represent the area of individual particles in $mm^2$. A particle of average size is labelled 'mean'. A particle close in area to that required for obtaining a signal from mitochondrial HVII locus when present in 150 mg of sugar processed for DNA extraction and PCR amplification, is labelled 'detectable'. A number of 'mean' sized fragments are required for a sample to reliably yield a detectable signal.

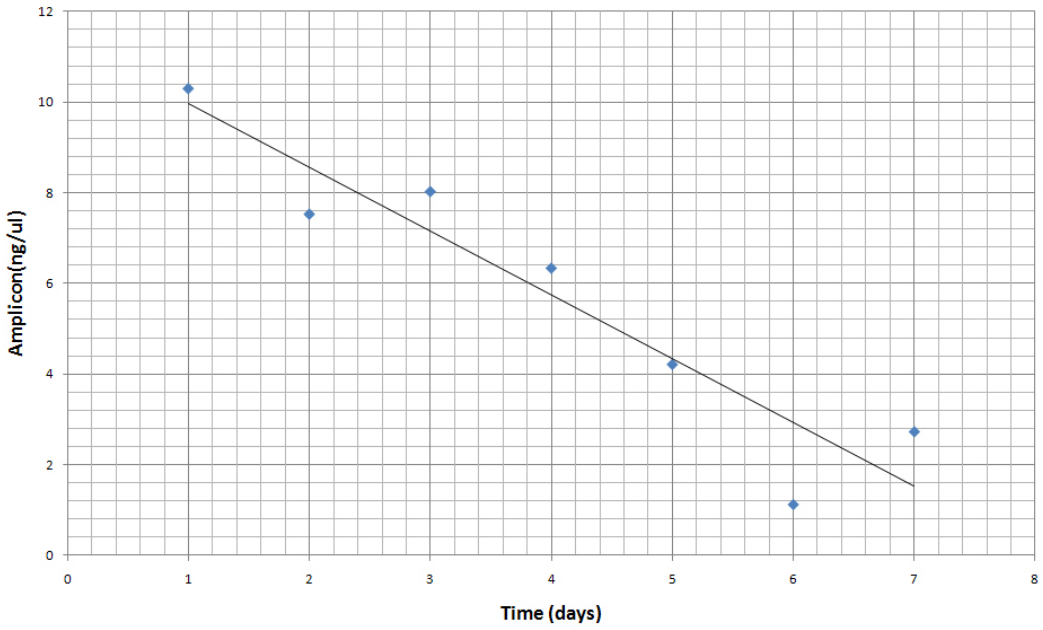


**Figure 5** The decline in amplifiability of mitochondrial DNA (locus HVI) with time. A model seizure containing skin flakes was prepared and acidified to a nominal pH of 3.7 with HCl vapour (see Appendix 5) and incubated at room temperature. Each day (X axis), DNA was prepared (see Section 5.2.2 ) and HVII sequences amplified and the product yield determined (Y axis).

## Discussion

The pH obtained on dilution is not the exact pH of the undiluted powder. However, in a casework situation, the seizure pH also would not be available. Therefore, artificial seizure material was dissolved in a set volume of solution as would probably be done in casework, and the pH taken to be the nominal pH of the powder. This is an arbitrary index of the true seizure pH and should be regarded as a general guide for two reasons. Firstly, the unavoidable reliance on using a solution of the seizure means that the observed pH and the seizure pH are not identical and are different for different acid/base systems. The nominal pH set with carboxylates might be higher relative to the same nominal pH obtained with a mineral acid-like HCl. Secondly, the operationally safe pH is probably above the pH at which amplification failure occurs. This is because when DNA fragmentation has just begun, product can still be formed by the PCR reaction engaging in template-switching, which will produce synthetic and novel products. The nominal pH at which this begins to be a problem is of most professional importance. Without further work studying the effects of fragmentation on template-switching during PCR, it is recommended that amplification not be attempted unless the nominal pH is at least pH4.5. This is a whole pH unit above the lowest nominal pH found that allows detection of amplification product after 44 hours and will ensure that template switching from fragments is not an issue. It is worth noting that template switching from fragments is capable of causing serious difficulties in interpretation. However, a technology that used cloning could almost certainly display this to a competent interpreter and not generate false results.

# 4 General discussion

Working on DNA in drug seizures has been an interesting and enjoyable challenge. However, it is only two years' work in a very broad field and there is a large and increasing number of ways to study the human genome in detritus. Very early in the project it was decided that a large range of compromises were unavoidable and, with one partial-exception, depth of investigation had to be sacrificed to its breadth. The section of moderate depth was the use of STR multiplex analyses, mainly Profiler Plus™ but also, a quintuplex of our own. Neither proved very profitable. Overall, STR amplifications usually gave some sort of result but were not highly rewarding because of the way complexity tends to overwhelm information on the original template-levels for each allele. WGA pre-amplification was disappointing, probably due to the effects of the presence of large amounts of non-human DNA. Other technologies more novel to forensic laboratories, that we did not have time to put as much effort into, do appear to be applicable to drug seizures.

The only non-STR application we had time to investigate to any extent was the hyper-variable mitochondrial loci where cloning of the amplification products provided useful information on the number of donors and sequences of potential use in tracing chains of drug distribution. Cloning is a technology that is not commonly used in forensic laboratories and alternative ways of accessing the sequence data are desirable. We foresee ways to achieve this using next generation sequencing of amplification products of short STR loci which would have the advantage of yielding results from lower DNA amounts than can be achieved from the current, industry standard capillary electrophoresis technology. If mitochondrial loci multiplexes can be made to generate products within the range of 454 sequencing, this will create a favourable environment for development of new technologies. This should strongly reduce the effects of the main source of distortion of results obtained at low levels of DNA, the need for very high cycle amplification.

Overall, a key issue for this project was to develop some basis for making decisions on whether or not to pay the costs of looking at the DNA present in drug seizures. This decision is, of course, a balance of cost versus anticipated benefit and reasonably anticipated benefit is a mix of either or both evidence or criminal intelligence. The one thing that this project has made clear is that *currently* the likelihood of getting information that could be used as evidence while real, is low, compared to the likelihood of getting DNA information worthy of criminal intelligence. It is also clear that probably less than half of seizures will have enough DNA in them for useful amounts of either type of information to be found.

A key outcome of this project is the recommendation that the assessment procedure should begin with examining a mitochondrial locus that amplifies well, such as HVII, perhaps in a 'real time' amplification followed by use of the product to assess donor number. This should use only a small amount of the template and provide sufficient information to make a rational decision on whether or not to use STR multiplexes on the remaining template. It will also directly yield some data on the donors themselves.

The future of DNA in seizures of illicit substances will be dominated by the habits of the persons that produce and cut drugs and the cleanliness of their procedures. Some insights gained here may be helpful to the forensic community.

In a discussion it is appropriate to consider unusual and poorly substantiated implications of results just so long as they may help other users. With that caveat, the following facts and conclusions are worth considering in conjunction with each other *as long as the tenuous nature of the evidence is recognised*.

- Laboratory contamination in STR profiles from relatively clean powdered drugs was tracked down. It came almost entirely from one individual who did not come near open seizure material any more than many other people. This individual was in less proximity to open or closed seizure packets than the actual processing person who was not a source of contamination. This fact is reasonably solid.
- The person routinely producing STR-detectable contamination certainly did not touch the seizures and transfer skin flakes directly to them but did regularly cough in a nearby room. Also reasonably solid fact.

- Deliberate attempts to contaminate the STR profiles with DNA from skin flakes or from drug samples by touching the samples before processing were not noticed to be dramatically efficient. This was only cursorily checked.
- With mitochondrial profiles rather than STR profiles, cloning readily separated multiple users. Inadvertent contamination by the principal contaminator individual (individual#1) was not observed and when seizures were deliberately contaminated with skin flakes from this same individual the contrived contamination-events were detected but did not completely mask the pre-existing contaminations. This is based on only a few observations but seemed clear.

Although this evidence collectively makes for a thin argument, the implications, if taken, are important. The STR profiles from an effective, single donor can come considerable distance carried by micro-drop, respiratory spray that had probably dried and had travelled distances as great as, at least, between rooms. This, if true, has very serious evidence-value implications.

Heavy skin contact as, for example, with tablet handling, will give STR profiles. In seizures, however, we mostly observed these to be from multiple donors and, because of this, of low evidential value but perhaps of not so low intelligence value.

Low levels of skin flakes are expected to be good sources of mitochondrial loci but poor sources of STR loci in accord with basic molecular biology of tissue senescence and cell-death. Skin flakes were not observed to spread as far as putative respiratory spray and so, despite their low informational content, they may have more evidential and intelligence value.

The STR multiplexes commonly give alleles but have been disappointing. While there is certainly a possibility of extracting a single useful multiplex STR profile out of a drug seizure, it will be a low probability event. On the other hand, mitochondrial profiles seem much more common and much better to analyse as they are less corrupted when there are multiple donors. The only unequivocal case observed of a single useful multiplex STR profile out of a drug seizure was an incidence of laboratory contamination. This was still, de-facto, the type of event we were hoping to observe in uncontaminated seizures. So at least this confirms that single STR profiles may occasionally be found.

# 5 Methods

## Both forensic and non-forensic technology was used

The research in this project commonly used techniques that are available to conventional forensic laboratories. Although the project had very strict forensic goals, Ii also took advantage of standard molecular biology techniques that are not standard forensic techniques available in working forensic laboratories and probably never will be. Some of the non-forensic techniques, the most notable being cloning and Sanger-sequencing, are unlikely ever to be used in a practical, forensic context but they have been most important in elucidating what is *actually occurring*, allowing monitoring of the underlying realities of amplification and purification processes at the molecular level during a simulated forensic investigation. Still other currently non-forensic techniques may be adopted for use as forensic techniques in the future; for example, 'next generation' DNA sequencing, such as 454 sequencing or its successors.

# 5.1 DNA extraction

Two extraction methods were developed for seizures and although each had its advantages and disadvantages they were largely equivalent. The 'Microcon Extraction method' (method 1) and the 'modified QIAFilter DNA Investigator Kit method' (method 2) are described below. A third method used in model, extremely dirty samples taken from car interiors was described in the report to NDLERF (Burgoyne et al. 2008). Although there was a lower overall level of contamination in the detritus of seizures it was considered likely to be similar in general properties to that found in vehicle interiors (results not shown). If a seizure is extremely dirty then there may be PCR inhibitors present, a circumstance we have seen previously in soils and car interior dusts. In such cases it is recommended to use the standard procedures appropriate for soils. For extractions of samples described in this report, the soil extraction method was rarely used.

Both methods typically use 100 mg of sample but up to 200 mg has sometimes been used with care. Amounts less than 100 mg are acceptable and there is no lower limit. A seizure with a highly buffered pH, for example a seizure containing a lot of sodium bicarbonate or carbonate as a cutting agent, is likely to give a low yield with method 2 unless the seizure pH is corrected. Method 1 does not suffer from this difficulty but can have physical problems in centrifuging out a fine precipitate that is characteristic of seizures with high heroin content if the imidazole extraction is omitted. If no heroin is expected then the imidazole extraction can be omitted and begun at the sodium carbonate extraction.

## 5.2.1 Custom designed method for extracting DNA from illicit drugs

This method was designed in house and is called the Microcon Extraction Method. It was developed from the previously described carbonate method that gave an extremely fine precipitate with heroin-containing seizures when the seizure came into direct contact with alkaline carbonate. This precipitate caused physical difficulties during centrifugation. To remedy this, a step was introduced in which the first wash employed imidazole, a milder base, which dissolved the heroin and DNA without producing a difficult precipitate, after which the carbonate solution could be used to dissolve remaining DNA.

### *How it was done*

1. Add 600 µl of 150 mM imidazole to each sample of, up to 200 mg, in a 1.5 mL microfuge tube.
2. Vortex vigorously until samples dissolve (approximately five minutes).
3. Centrifuge 14,000 rpm for 10 minutes to pellet any undissolved material. Retain pellet.

4. Transferr supernatant to microcon filter.
5. Centrifuge filter at 500 x g for 20 minutes at room temperature, discard supernatant.
6. Add 400 µl of forensic-grade (Ultraviolet radiation [UV] treated pure water) H2O to filter.
7. Spin at 500 x g for 20 minutes at room temperature and discard supernatant.

The first 'harvest' of DNA is now on the filter. The next steps add the second DNA harvest to the filter, a carbonate extract from the material that did not dissolve imidazole.

1. Add 400 µl of 150 mM sodium carbonate to pellet from step 3.
2. Vortex pellet vigorously and incubate at 60°C for 10 minutes.
3. Centrifuge 14,000 rpm for 10 minutes to pellet any undissolved material.
4. Transfer supernatant to the same microcon filter.
5. Spin filter at 500 x g for 20 minutes at room temp and discard supernatant.
6. Add 400 µl of forensic grade H2O to filter.
7. Spin at 500 x g for 20 minutes at room temp and discard supernatant.
8. Repeat water wash procedure in steps 13 and 14.
9. Add 20 µl of forensic grade H2O to filter and flick a few times to suspend DNA.
10. Invert filter in a clean collection tube and spin at 14,000 rpm for one minute at room temperature to collect DNA.

### 5.2.2 Extracting DNA from drugs using the QIAFilter DNA Investigator Kit

1. Place up to 200 mg of sample in 1.5 mL microfuge tube.
2. Add 20 µl of proteinase K and 600 µl of kit-buffer ATL. Pulse vortex for 10 seconds.
3. Incubate at 56°C for one hour, rotating at 10 rpm.
4. Briefly spin tube to remove drops from the lid.
5. Add 600 µl of kit-buffer AL. Pulse vortex for 15 seconds.
6. Incubate in water bath at 70°C for 10 minutes. Remove tubes and vortex for 10 seconds every three minutes.
7. Briefly spin to remove drops from the lid.
8. Add 300 µl of 99 percent ethanol and pulse vortex for 15 seconds.
9. Briefly spin tube to remove drops from the lid.
10. Transfer contents (700 µl at a time) to a QIAfilter mini elute column placed in a 2 mL collection tube, avoiding any debris and the last few drops at the bottom of the tube.
11. Close the lid and spin at 6000 x g for one minute. Empty collection tube and load remaining lysate and repeat spin step. Transfer the filter unit to a clean collection tube.
12. Add 500 µl of kit-buffer AWI and spin at 6000 x g for one minute. Transfer filter unit to a clean collection tube.
13. Add 700 µl of buffer AW2 and spin at 6000 x g for one minute. Transfer filter unit to a clean collection tube.
14. Add 700 µl of 99 percent ethanol and spin at 6000 x g for one minute. Transfer filter unit to a clean collection tube.
15. Centrifuge at 14,000 rpm for three minutes to dry membrane completely.
16. Place filter unit in a clean 1.5 mL collection tube and incubate in 56°C oven with lid open for three minutes.
17. Add 20 µl of 0.2 x TE to the centre of the filter.(TE buffer is 10mM Tris, 1mM EDTA adjusted to pH 7.4 with HCl.)
18. Close the lid and incubate at room temperature for one minute.
19. Spin at 14,000 rpm for one minute to elute DNA.

## 5.3 Array construction and methods

Detail of the primary version of this technique was provided in a previous report to NDLERF (Burgoyne 2008). The array 'spots' were those used in this report plus additional ones as noted. Although the array spots used in this project were mainly derived from single sequences cloned out of seizure material, some of the control-spots, for example, the human positive control, C2, were just made from crude, unsorted amplification products.

The names of the spots in Figure 1 can be obtained from Appendix 1 of this report and what the names actually denote can be obtained from the database from a previous report (Burgoyne 2008). For example, in the database of the previous report, a spot labelled C2 (a human mixed control) is summarized in C2.htm and a spot S5, a single sequence derived from soil, is described in SC5.htm with a HTML tag to lead to its actual sequence. The origin of the prefix NX for seizure material has been described previously (Burgoyne 2008).

The crude amplification products (profiles) from seizure DNA were the products of a three-stage amplification using antiseq05 [1] as the primer for all stages. The first two stages were to generate the products and the third stage was to make the products fluorescent as described in Burgoyne (2008). The profiles were used as-is after amplification products were removed.

At Flinders University equal amounts of each DNA profile and appropriate control samples (including spotting solution alone, PCR blanks from each amplification round and purified human DNA profiles) were printed to microarray slides (GAPS II amino-silane coated slides, Corning). Either profiles (single-profile probes) or individual sequences (single-sequence probes) cloned from profiles, were ethanol precipitated and resuspended in Pronto Universal Spotting Solution (Corning). The spots were printed to UltraGAPS coated slides (Corning) by a BioOdyssey Calligrapher miniarrayer (BioRad) with a quill type pin, such that the spots were approximately 140 microns in diameter and the spacing, spot centre to spot centre, was 400 microns. The array printing used BioOdyssey Calligrapher software. The spots were fixed by four hours drying in a vacuum at room temperature with ten second exposure to 20 mJ UV in the Biorad Genelinker machine. For each array, fluorescence values were normalised, relative to the most fluorescent spot on the array image, to give relative fluorescence units (RFU), before further data analysis

### Challenging the array probes (the spots)

Fluorescently labelled challenge mixtures were produced by re-amplification of a profile, using high stringency conditions, and containing 2 nmole of Cy3-dCTP or Cy5-dCTP and 80 µM unlabelled dCTP. Reaction mix residues were removed using an UltraClean™ PCR purification DNA clean-up kit (MoBio Laboratories) according to the manufacturer`s instructions.

One volume of 2X hybridisation buffer (50% formamide, 5X SSC and 0.2% SDS) was added to the labelled profile and denatured by heating at 100°C for 10 minutes followed by two minutes on ice. After 45 minutes incubation with pre-hybridisation buffer (10 mg/ml BSA, 25% formamide, 5X SSC and 0.1% SDS) at 45°C, the microarray was hybridised with denatured probe at 45°C for six to eight hours. The array was then washed at 45°C for five minutes in 1X SSC, 0.2% SDS, five minutes in 0.1X SSC, 0.2% SDS then twice in 0.1X SSC, air dried and scanned in a GenePix 4000A (Axon Instruments).

Reaction mix residues were removed using a PCR DNA clean-up kit (AdBiotec) according to the manufacturer's instructions and the profile was eluted in 50 µl of H2O prior to adding the following reagents in preparation for hybridisation; 250 µl formamide, 62.5 µl of 20X SSC, 50 µl of 1% SDS, 1 µl of 2-hydroxyethyl disulphide and 87.5 µl H2O. The labelled profile/hybridisation mixture was denatured by heating at 100°C for 10 minutes followed by two minutes on ice.

After 45 minutes incubation with pre-hybridisation buffer (5x SSC, 0.1% SDS, 0.1 mg/ml BSA) at 42°C, the microarray was twice transferred to 0.1 x SSC and incubated at 25°C for five minutes before rinsing with H2O and drying by centrifugation at 1,500 rpm for five minutes. The microarray was hybridised with denatured

probe at 58°C for 12–16 hours then washed once in 2 x SSC, 0.1% SDS at 58°C for five minutes, twice in 0.1 x SSC, 0.1% SDS at 25°C for five minutes, four times in 0.1 x SSC at 25°C for one minute, and finally once in 0.01 x SSC for 10 seconds. It was then air-dried and scanned in a GenePix 4000A (Axon Instruments).

# 5.4 Other amplification systems

## 5.4.1 Amplification for the Profiler Plus$^{tm}$ system

This system was the commercial AmpF/STR® Profiler Plus® (Applied Biosystems, CA, USA) with nine loci. Forensic Science SA carried out the amplification and analysis using the kit manufacturer`s instructions and materials.

## 5.4.2 Amplification with the quintuplex PCR system

Analyses using amelogenin primers or the quintuplex primer set were performed in 25 µl reactions comprising Phusion GC buffer (750 mM Tris-HCl, pH 8.8, 200 mM (NH4)2SO4, 0.1% (v/v) Tween® 20), 200 µM of each dNTP (New England Biolabs, Inc, MA, USA), 0.5 U of Phusion™ high-fidelity DNA polymerase (Finnzymes, Inc, Massachusetts, USA), 0.14 µM of each AMG primer, 0.2 µM of each D5S818 primer, 0.4 µM of each D21S11 primer, 0.16 µM of each D18S51 primer and 0.14 µM of each FIBRA primer. A hot start at 98°C for 30 seconds was followed by 34 cycles of 10 seconds at 98°C, 13 seconds at 59°C, and 11 seconds at 72°C with a final five minute extension at 72°C.

**Table 4** Primers used for the quintuplex.

| Locus | Product length (bp) | Primer sequences (5'–3') |
|---|---|---|
| AMGx/y | 106 and 112 | F: CCCTGGGCTCTGTAAAGAATAGT |
| | | R: ATCAGAGCTTAAACTGGGAAGCTG |
| D5S818 | 123–155 | F: GGTGATTTTCCTCCTTGGTATCC |
| | | R: AGCCACAGTTTACAACATTTGTATCT |
| D21S11 | 205–249 | F: ATATGTGAGTCAATTCCCCAAG |
| | | R: TGTATTAAGTCAATGTTCTCCAG |
| D18S51 | 275–323 | F: CAAACCCGACTACCAGCAAC |
| | | R: GAGCCATGTTCATGCCAATG |
| FIBRA | 350–400 | F: GGACAATCTTAACTGGCATTCA |
| | | R: TGCGCTTCAAGGACTTCA |

## 5.4.3 Human mitochondria loci typing

### 5.4.3.1 Primers for amplification of HVI and HVII from mtDNA

The HVI and HVII primer sites are underlined in the hypervariable sequence included in Appendix 4. The HVI and HVII primers amplify approximately 440 bp and 415 bp fragments respectively.

HVI F15971 5'-TTAACTCCACCATTAGCACC-3' (Tm 59.58°C)

HVI R16410 5'-GAGGATGGTGGTCAAGGGAC-3' (Tm 66.14°C)

HVII F15 5'-CACCCTATTAACCACTCACG-3' (Tm 60.1°C)

HVII R429 5'-CTGTTAAAAGTGCATACCGCCA-3' (Tm 66.02°C)

A high fidelity polymerase (Phusion by Finzymme) was chosen for amplification due to its low error rate and rapid extension rate. To test the efficacy of the PCR, the hypervariable regions from control samples (purified DNA) were amplified and sent for sequencing at the Australian Genome Research Facility.

*Amplification of two mitochondrial loci from DNA extracted from blood or skin flakes*

Human DNA, 1 µl (5 ng), purified from whole blood from a single donor (donor #1), was used as template in a 50 µl reaction. Human skin was also tested as a template by gently scraping a sterile pipette tip on the forearm and placing the lifted cells into the PCR mix. Reaction mixtures contained primers at a final concentration of 0.4 µM, dNTPs at 0.2 µM, 1.5 mM MgCl2, and one unit of 'Phusion' polymerase. Cycling was performed with an initial denaturation at 98°C for three minutes, then 35 cycles at 98°C for 15s, 63°C for 15s, and 72°C for 15s. A final extension was carried out at 72°C for five minutes.

For each PCR product, 5 µl was electrophoresed on one percent agarose gel stained with ethidium bromide.

### 5.4.3.2 T-vector cloning and sequencing: T-Vector cloning

The PCR products from GMQ36/HVII were purified using 'Adbiotec' PCR clean-up kit, and eluted DNA in 50 µl of forensic grade H2O.

Ligation reactions were set up using the 'invitrogen' PCR 2.1 cloning kit, which contained:

| | |
|---|---|
| PCR product | 1 µl |
| 10x ligation buffer | 1 µl |
| pCR 2.1 vector (25 ng/µl) | 2 µl |
| T4 ligase | 1 µl |
| Sterile water | 5 µl |
| TOTAL | 10 µl |

Incubated overnight in a 1.5 mL microfuge tube at 14°C. Control ligations had no added DNA.

'One shot' TOP 10F competent cells were transformed with 1 µl of the ligation mixture as per the manufacturer's instructions with the following exceptions. The 50 µl cell aliquots were divided between two transformations (25 µl each). Only 200 µl of SOC medium (Hanahan, D 1983) was added to the vial prior to the one hour incubation at 37°C. Following incubation, cells were spread plated onto S-Gal/LB agar medium (Sigma) containing 100µg/mL ampicillin and incubated at 37°C overnight. This medium enables identification of colonies containing transformants with an insert in the vector by black/white selection.

Control plates (no DNA added to the ligation) generated one white colony, compared to >200 white colonies on the GMQ36 plates. Twelve white colonies were picked and used to inoculate 5 mL aliquots of LB media containing 100µg/mL ampicillin. Inoculated vials were incubated at 37°C with shaking at 200 opm overnight.

### 5.4.3.3 Purification of plasmid DNA

Plasmid DNA was purified from 2.5 mL of culture taken from the 12 samples using 'Adbiotec mini plasmid kit'. DNA was eluted in 50 mL forensic grade H2O and presence of the insert was verified by PCR (using phusion polymerase and the HVII primer set). The PCR conditions were as per the re-amplification reactions, however amplification was carried out in a volume of 25 µl. The resulting products were examined by electrophoresing 5 µl of each PCR on a one percent agarose gel and staining with ethidium bromide.

## 5.4.4 WGA amplification

Whole genome amplification (WGA) was performed using the Sigma WGA1 GenomePlex®) kit using the manufacturer's instructions. The resulting amplified product was used as template in a multiplex PCR reaction. Initially 1 µl was used as template but 5 µl led to better results.

# 5.5 Amplification product analysis separation and detection systems

## 5.5.1 Capillary electrophoresis (CE) systems

Two capillary electrophoresis systems were used in this study: an ABI 3130xl Genetic Analyzer (Applied Biosystems, CA, USA) with the 3130xl Genetic Analyzer Data Collection software v3.0 (Applied Biosystems, CA, USA) and an Agilent 2100 Bioanalyser™ system (Agilent Technologies, Waldbronn, Germany) with the DNA 1000 kit (Agilent Technologies, Waldbronn, Germany). For both systems, all operations were performed according to manufacturers' instructions.

## 5.5.2 Agarose gel systems

Agarose gel electrophoresis of 5 µl of PCR amplification products at 5V/cm for 50 minutes in 2.5 percent agarose gel (Certified™ low range ultra agarose, Bio-Rad Laboratories, CA, USA) in TBE buffer (89 mM Tris, 89 mM boric acid, 2mM EDTA, pH8.4) was used for low-resolution applications. Gels were stained in 0.025 percent ethidium bromide for 30 minutes, followed by washing in water for 10 minutes. DNA bands were visualised using ultraviolet irradiation.

## 5.5.3 DNA size standards

The DNA size standards used for the proprietary separation systems are specified in the appropriate supplier's manual. The standards used for the non-proprietary separation systems (Figure 6) were those in item N3231s from New England Biolabs.

| Fragment | Base Pairs | DNA Mass |
|---|---|---|
| 1 | 1,517 | 45 ng |
| 2 | 1,200 | 35 ng |
| 3 | **1,000** | **95 ng** |
| 4 | 900 | 27 ng |
| 5 | 800 | 24 ng |
| 6 | 700 | 21 ng |
| 7 | 600 | 18 ng |
| 8 | **500,517** | **97 ng** |
| 9 | 400 | 38 ng |
| 10 | 300 | 29 ng |
| 11 | 200 | 25 ng |
| 12 | 100 | 48 ng |

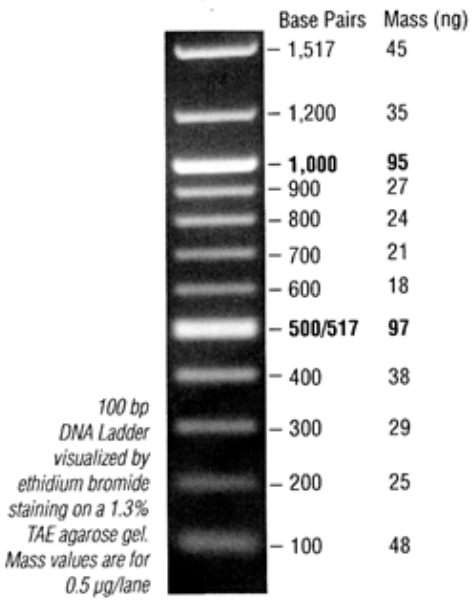


**Figure 6** Size standards used for non-proprietory separation systems

## 5.6 Artefacts and contamination issues

Equipment and water was decontaminated with UV as per the previous report (Burgoyne 2008) except that the UV chamber was kept at nearly 100 percent relative humidity

Contamination is the most important issue in processing illicit substances. Contamination by the putative offender must be the source of DNA profiles and contamination by the personnel processing the seizures is the most serious source of confusion. It is almost impossible to plan rational studies of contamination because to plan presupposes knowledge of the process of contamination. However it is quite appropriate to take advantage of fortuitous information that arises during the monitoring of contamination.

In the case of this project, the fortuitous information on contamination appeared to be informative. Despite a number of attempts to intentionally contaminate seizures by the personnel processing the DNA deliberately handling seizures, it was observed that touching and handling was surprisingly inefficient. Moreover, examining the profiles in seizures or the filters that were used to process them had clearly been accidentally contaminated during processing. This showed that it came from one single individual who was working in a nearby room, only connected by an intermittently open doorway, who hardly ever came near the exposed seizures or processing equipment much less touched them. This person's profile or depleted versions of it was involved in multiple separate incidents of contamination. Moreover there were many other persons in the same room as and nearer to the processing than the identified individual. Thus the DNA was moving considerable distances between rooms and was being picked up efficiently from that individual alone. There was no possibility that the person was maliciously or otherwise improperly contaminating samples so why was this so? The person concerned was suffering intermittent severe respiratory distress and coughing which strongly indicates that aerosols were at least the beginning of the problem. The efficiency of the materials acquiring these presumably air-dried aerosols was impressive compared with the inefficiency of contamination by handling and very strongly suggested some mechanism involving attraction of fine materials with a charge suspended in air by electrostatic attraction to clean plastic ware and filters.

The main artefact was probably generated from the effects of template diversity on multiplex amplification systems. This is discussed in Section 3.4.

### *The profile that Profiler Plus™ most often synthesises from high diversity template*

| | |
|---|---|
| D3S1358 | 16, 15, 17 |
| VWA | 17, 16, 15, 18 |
| FGA | 20, 21, 22 |
| D8S1179 | 13,, 12, 14 |
| D21S11 | 29, 28, 30 |
| D18S51 | 12, 16, 15 |
| D5S818 | 11, 12, 13 |
| D13S317 | 11, 12 |
| D7S820 | 10, 8, 11 |

The principal identified laboratory contamination profile.

| | |
|---|---|
| D3S1358 | 15 (homozygous) |
| VWA | 15, 16 |
| FGA | 19, 24 |
| D8S1179 | 13, 14 |
| D21S11 | 30, 30.2 |

| | |
|---|---|
| D18S51 | 12, 13 |
| D5S818 | 12 (homozygous) |
| D13S317 | 11, 12 |
| D7S820 | 11, 9 |

## 5.7 Constructing model seizure mixtures for pH studies

When constructing model seizure mixtures containing human skin for pH studies for pH values down to about 4.0, basic sugar, acid sugar and mock unbuffered drug sugar mix were prepared in a Sunbeam AutoGrinder (model EM0415) set on 'FINE/ESPRESSO' number 15. The sugar was Woolworths Homebrand cane sugar from a freshly opened 3 kg pack. Shavings were taken from a male face using a Remington electric shaver two hours after a wet shave.

- Basic sugar = 10 g sugar + 84 mg NaHCO3
- Acid sugar = 10 g sugar + 292 mg EDTA
- Mock unbuffered mix = 10 g sugar, 4 mg sodium acetate and 10 mg shavings.

Aliquots of 400 mg of various basic/acid sugar mixtures were combined with 400 mg mock drug in a 50 ml Falcon tube and mixed as described for sugar mixes. Tubes were incubated at 25°C in a hybridisation chamber with continuous rotation. Duplicate samples of 50 mg were removed, processed with a Qiagen Investigator kit and PCR with HVII primers after various periods of days. This gave pH values down to a little below 4.0. pH. Values below this were obtained by taking 100 mg 'mock drug' into duplicate 50 ml tubes with an atmosphere made acid by a strip of Whatman 3MM paper impregnated with 20 ml of HCl being placed in each tube and the tubes sealed and incubated at 25°C in the dark (for example, 20 µl of 4.0M HCl gave pH 3.7 after dilution.)

The nominal pH of different ratios of basic to acid sugar was obtained by diluting 50 mg of the final powder-mix, containing the sucrose and buffers, as described, in 10 ml MilliQ water which then had its pH measured with a glass electrode in the usual way.

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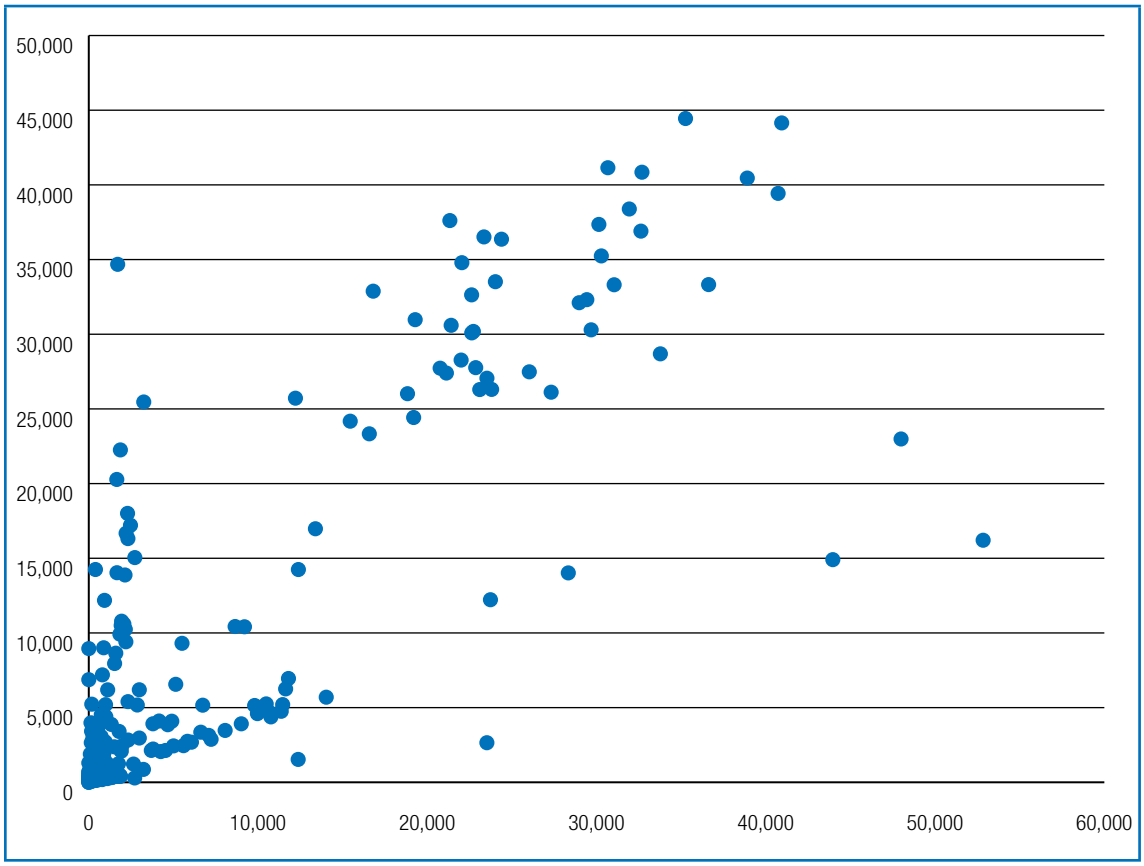
# Appendix 1: Signals from array spots hybridised with GR170 (green) and human DNA (red)

Refer to Figure 1




*Provenance of DNA in spots on the array*

| Name | Description |
|---|---|
| SA1 | soil FUSA single clone insert 9-1 (flanking primers M13F-M13R) |
| SA2 | soil FUSA single clone insert 9-2 (flanking primers M13F-M13R) |
| SA3 | soil FUSA single clone insert 9-3 (flanking primers M13F-M13R) |
| SA4 | soil FUSA single clone insert 9-4 (flanking primers M13F-M13R) |
| SA5 | soil FUSA single clone insert 9-5 (flanking primers M13F-M13R) |
| SA6 | soil FUSA single clone insert 9-6 (flanking primers M13F-M13R) |
| SA7 | soil FUSA single clone insert 9-8 (flanking primers M13F-M13R) |
| SA8 | soil FUSA single clone insert 9-11 (flanking primers M13F-M13R) |
| SA9 | soil FUSA single clone insert 4-1 (flanking primers M13F-M13R) |
| SA10 | soil FUSA single clone insert 4-2 (flanking primers M13F-M13R) |

| Name | Description |
|---|---|
| HA1 | heroin microcon S1-2 (flanking primers M13F-M13R) |
| HA2 | heroin microcon S1-3 (flanking primers M13F-M13R) |
| HA3 | heroin microcon S1-6 (flanking primers M13F-M13R) |
| HA4 | heroin microcon S1-7 (flanking primers M13F-M13R) |
| HA5 | heroin microcon S1-9 (flanking primers M13F-M13R) |
| HA6 | heroin microcon S1-10 (flanking primers M13F-M13R) |
| HA7 | heroin microcon S1-13 (flanking primers M13F-M13R) |
| HA8 | heroin microcon S1-14 (flanking primers M13F-M13R) |
| HA9 | heroin microcon S1-15 (flanking primers M13F-M13R) |
| HA10 | heroin microcon S1-18 (flanking primers M13F-M13R) |
| HA11 | heroin microcon S1-20 (flanking primers M13F-M13R) |
| HA12 | heroin microcon S1-21 (flanking primers M13F-M13R) |
| HB1 | heroin microcon S2-1 (flanking primers M13F-M13R) |
| HB2 | heroin microcon S2-2 (flanking primers M13F-M13R) |
| HB3 | heroin microcon S2-4 (flanking primers M13F-M13R) |
| HB4 | heroin microcon S2-5 (flanking primers M13F-M13R) |
| HB5 | heroin microcon S2-7 (flanking primers M13F-M13R) |
| HB6 | heroin microcon S2-8 (flanking primers M13F-M13R) |
| HB7 | heroin microcon S2-10 (flanking primers M13F-M13R) |
| HB8 | heroin microcon S2-13 (flanking primers M13F-M13R) |
| HB9 | heroin microcon S2-14 (flanking primers M13F-M13R) |
| HB10 | heroin microcon S2-15 (flanking primers M13F-M13R) |
| HB11 | heroin microcon S2-19 (flanking primers M13F-M13R) |
| HB12 | heroin microcon S2-20 (flanking primers M13F-M13R) |
| HC1 | heroin microcon S3-5 (flanking primers M13F-M13R) |
| HC2 | heroin microcon S3-6 (flanking primers M13F-M13R) |
| HC3 | heroin microcon S3-12 (flanking primers M13F-M13R) |
| HC4 | heroin microcon S3-14 (flanking primers M13F-M13R) |
| HC5 | heroin microcon S3-17 (flanking primers M13F-M13R) |
| HC6 | heroin microcon S3-19 (flanking primers M13F-M13R) |
| HC7 | heroin microcon S3-20 (flanking primers M13F-M13R) |
| HC8 | heroin microcon S3-21 (flanking primers M13F-M13R) |
| HC9 | heroin microcon S3-22 (flanking primers M13F-M13R) |
| HC10 | heroin microcon S3-24 (flanking primers M13F-M13R) |
| HC11 | heroin microcon S3-26 (flanking primers M13F-M13R) |
| HC12 | heroin microcon S3-27 (flanking primers M13F-M13R) |
| HD1 | heroin microcon 886-1 (flanking primers M13F-M13R) |
| HD2 | heroin microcon 886-2 (flanking primers M13F-M13R) |
| HD3 | heroin microcon 886-3 (flanking primers M13F-M13R) |
| HD4 | heroin microcon 886-4 (flanking primers M13F-M13R) |
| HD5 | heroin microcon 886-5 (flanking primers M13F-M13R) |
| HD6 | heroin microcon 886-6 (flanking primers M13F-M13R) |

| Name | Description |
|---|---|
| HD7 | heroin microcon 886-7 (flanking primers M13F-M13R) |
| HD8 | heroin microcon 886-8 (flanking primers M13F-M13R) |
| HD9 | heroin microcon 886-11 (flanking primers M13F-M13R) |
| HD10 | heroin microcon 886-13 (flanking primers M13F-M13R) |
| HD11 | heroin microcon 886-14(flanking primers M13F-M13R) |
| HD12 | heroin microcon 886-15 (flanking primers M13F-M13R) |
| MA1 | methamphetamine microcon 925seq2 REC: 04/B77758-8 bag 0401925-8.35 |
| MA2 | methamphetamine microcon 925seq3 REC: 04/B77758-8 bag 0401925-8.35 |
| MA3 | methamphetamine microcon 925seq4 REC: 04/B77758-8 bag 0401925-8.35 |
| MA4 | methamphetamine microcon 925seq9 REC: 04/B77758-8 bag 0401925-8.35 |
| MA5 | methamphetamine microcon 925seq10 REC: 04/B77758-8 bag 0401925-8.35 |
| MA6 | methamphetamine microcon 925seq13 REC: 04/B77758-8 bag 0401925-8.35 |
| MA7 | methamphetamine microcon 925antiseq2 REC: 04/B77758-8 bag 0401925-8.35 |
| MA8 | methamphetamine microcon 925antiseq3 REC: 04/B77758-8 bag 0401925-8.35 |
| MA9 | methamphetamine microcon 925antiseq8 REC: 04/B77758-8 bag 0401925-8.35 |
| MA10 | methamphetamine microcon 925antiseq9 REC: 04/B77758-8 bag 0401925-8.35 |
| MA11 | methamphetamine microcon 925antiseq12 REC: 04/B77758-8 bag 0401925-8.35 |
| BA1 | Anonymous organism isolated from- methamphetamine 73-1 REC:05/A26721-14 case# 0502425 bag14-4 |
| BA2 | Anonymous organism isolated from- methamphetamine 73-2 REC:05/A26721-14 case# 0502425 bag14-4 |
| BA3 | Anonymous organism isolated from- methamphetamine 73-5 REC:05/A26721-14 case# 0502425 bag14-4 |
| BA4 | Anonymous organism isolated from- methamphetamine 73-11 REC:05/A26721-14 case# 0502425 bag14-4 |
| BA5 | Anonymous organism isolated from- methamphetamine 73-13 REC:05/A26721-14 case# 0502425 bag14-4 |
| BA6 | Anonymous organism isolated from- methamphetamine 73-14 REC:05/A26721-14 case# 0502425 bag14-4 |
| BB1 | Anonymous organism isolated from- methamphetamine 75-2 REC:05/A26721-14 case# 0502425 bag14-5 |
| BB2 | Anonymous organism isolated from- methamphetamine 75-5 REC:05/A26721-14 case# 0502425 bag14-5 |
| BB3 | Anonymous organism isolated from- methamphetamine 75-6 REC:05/A26721-14 case# 0502425 bag14-5 |
| BB4 | Anonymous organism isolated from- methamphetamine 75-15 REC:05/A26721-14 case# 0502425 bag14-5 |
| BB5 | Anonymous organism isolated from- methamphetamine 75-18 REC:05/A26721-14 case# 0502425 bag14-5 |
| XA1 | ecstasy pellet 80-1 REC: 06/A48078-2 bag 0504648-1.2 |
| XA2 | ecstasy pellet 80-5 REC: 06/A48078-2 bag 0504648-1.2 |
| XA3 | ecstasy pellet 80-7 REC: 06/A48078-2 bag 0504648-1.2 |
| XA4 | ecstasy pellet 80-17 REC: 06/A48078-2 bag 0504648-1.2 |
| GR170 | drug sample from previous array which gave erroneous results |
| C1 | seq05-human DNA control |
| C2 | antiseq05-human DNA control |
| C3 | seq05-Neurospora DNA control |
| C4 | antiseq05-Neurospora DNA control |
| C5 | Blank spot (pronto Universal spotting solution) |
| EA1 | environmental FTA paper (sample 10) (clone 1) (flanking primers M13F-M13R) |
| EA2 | environmental FTA paper (sample 10) (clone 2) (flanking primers M13F-M13R) |
| EA4 | environmental FTA paper (sample 10) (clone 4) (flanking primers M13F-M13R) |
| EA7 | environmental FTA paper (sample 10) (clone 8) (flanking primers M13F-M13R) |

| Name | Description |
|---|---|
| EA10 | environmental FTA paper (sample 10) (clone 19) (flanking primers M13F-M13R) |
| EA11 | environmental FTA paper (sample 10) (clone 20) (flanking primers M13F-M13R) |
| EB1 | environmental FTA paper (sample 11) (clone 1) (flanking primers M13F-M13R) |
| EB4 | environmental FTA paper (sample 11) (clone 8) (flanking primers M13F-M13R) |
| EB5 | environmental FTA paper (sample 11) (clone 9) (flanking primers M13F-M13R) |
| EB7 | environmental FTA paper (sample 11) (clone 11) (flanking primers M13F-M13R) |
| EB12 | environmental FTA paper (sample 11) (clone 23) (flanking primers M13F-M13R) |
| HE4 | heroin microcon (clone 5) REC: 0E/353051-2 bag 992801 (flanking primers M13F-M13R) |
| HE7 | heroin microcon (clone 12) REC: 0E/353051-2 bag 992801 (flanking primers M13F-M13R) |
| HF1 | heroin microcon pellet (clone 1) REC: 0E/353051-2 bag 992801 (flanking primers M13F-M13R) |
| HF3 | heroin microcon pellet (clone 5) REC: 0E/353051-2 bag 992801 (flanking primers M13F-M13R) |
| HF6 | heroin microcon pellet (clone 12) REC: 0E/353051-2 bag 992801 (flanking primers M13F-M13R) |
| HF7 | heroin microcon pellet (clone 15) REC: 0E/353051-2 bag 992801 (flanking primers M13F-M13R) |
| HF8 | heroin microcon pellet (clone 17) REC: 0E/353051-2 bag 992801 (flanking primers M13F-M13R) |
| HF9 | heroin microcon pellet (clone 18) REC: 0E/353051-2 bag 992801 (flanking primers M13F-M13R) |
| HF12 | heroin microcon pellet (clone 23) REC: 0E/353051-2 bag 992801 (flanking primers M13F-M13R) |
| MC1 | GR176 (clone 1) microcon (pellet) methamphetamine REC: 05/A26721-52 bag 0502425-52 (flanking primers M13F-M13R) |
| MD1 | GR142 (clone 1) microcon (pellet) methamphetamine REC: 04/B77758-8 bag 0401925-8.27 (flanking primers M13F-M13R) |
| MD4 | GR142 (clone 4) microcon (pellet) methamphetamine REC: 04/B77758-8 bag 0401925-8.27 (flanking primers M13F-M13R) |
| MD16 | GR142 (clone 18) microcon (pellet) methamphetamine REC: 04/B77758-8 bag 0401925-8.27 (flanking primers M13F-M13R) |
| MD18 | GR142 (clone 20) microcon (pellet) methamphetamine REC: 04/B77758-8 bag 0401925-8.27 (flanking primers M13F-M13R) |
| MD19 | GR142 (clone 21) microcon (pellet) methamphetamine REC: 04/B77758-8 bag 0401925-8.27 (flanking primers M13F-M13R) |
| ME1 | methamphetamine microcon (clone 4) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| ME2 | methamphetamine microcon (clone 5) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| ME3 | methamphetamine microcon (clone 6) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| ME4 | methamphetamine microcon (clone 8) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| ME5 | methamphetamine microcon (clone 10) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| ME6 | methamphetamine microcon (clone 13) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| ME9 | methamphetamine microcon (clone 20) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| ME10 | methamphetamine microcon (clone 22) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| ME11 | methamphetamine microcon (clone 23) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| MF1 | methamphetamine microcon (clone 8) REC: 06/A40153-9 bag 0503564-9 (flanking primers M13F-M13R) |
| MF2 | methamphetamine microcon (clone 9) REC: 06/A40153-9 bag 0503564-9 (flanking primers M13F-M13R) |
| MF3 | methamphetamine microcon (clone 11) REC: 06/A40153-9 bag 0503564-9 (flanking primers M13F-M13R) |
| MF4 | methamphetamine microcon (clone 12) REC: 06/A40153-9 bag 0503564-9 (flanking primers M13F-M13R) |
| MF7 | methamphetamine microcon (clone 15) REC: 06/A40153-9 bag 0503564-9 (flanking primers M13F-M13R) |
| MF8 | methamphetamine microcon (clone 18) REC: 06/A40153-9 bag 0503564-9 (flanking primers M13F-M13R) |
| MF9 | methamphetamine microcon (clone 19) REC: 06/A40153-9 bag 0503564-9 (flanking primers M13F-M13R) |
| MF12 | methamphetamine microcon (clone 22) REC: 06/A40153-9 bag 0503564-9 (flanking primers M13F-M13R) |
| MG1 | methamphetamine microcon (clone 1) REC: 07/B01458-3 bag 060481-3F (flanking primers M13F-M13R) |
| MG2 | methamphetamine microcon (clone 2) REC: 07/B01458-3 bag 060481-3F (flanking primers M13F-M13R) |
| MG3 | methamphetamine microcon (clone 3) REC: 07/B01458-3 bag 060481-3F (flanking primers M13F-M13R) |

| Name | Description |
|---|---|
| MG4 | methamphetamine microcon (clone 3) REC: 07/B01458-3 bag 060481-3F (flanking primers M13F-M13R) |
| MG5 | methamphetamine microcon (clone 4) REC: 07/B01458-3 bag 060481-3F (flanking primers M13F-M13R) |
| MG6 | methamphetamine microcon (clone 5) REC: 07/B01458-3 bag 060481-3F (flanking primers M13F-M13R) |
| MG7 | methamphetamine microcon (clone 6) REC: 07/B01458-3 bag 060481-3F (flanking primers M13F-M13R) |
| MG8 | methamphetamine microcon (clone 12) REC: 07/B01458-3 bag 060481-3F (flanking primers M13F-M13R) |
| MG9 | methamphetamine microcon (clone 13) REC: 07/B01458-3 bag 060481-3F (flanking primers M13F-M13R) |
| MG10 | methamphetamine microcon (clone 14) REC: 07/B01458-3 bag 060481-3F (flanking primers M13F-M13R) |
| MG11 | methamphetamine microcon (clone 15) REC: 07/B01458-3 bag 060481-3F (flanking primers M13F-M13R) |
| MG12 | methamphetamine microcon (clone 23) REC: 07/B01458-3 bag 060481-3F (flanking primers M13F-M13R) |
| MH1 | methamphetamine microcon pellet (clone 6) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| MH3 | methamphetamine microcon pellet (clone 9) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| MH5 | methamphetamine microcon pellet (clone14) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| MH9 | methamphetamine microcon pellet (clone18) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| MH10 | methamphetamine microcon pellet (clone19) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| MH12 | methamphetamine microcon pellet (clone 24) REC: 06/A40153-4 bag 0503564-4 (flanking primers M13F-M13R) |
| MI3 | methamphetamine microcon pellet (clone 3) REC: 06/A40153-9 bag 0503564-9 (flanking primers M13F-M13R) |
| MI5 | methamphetamine microcon pellet (clone 5) REC: 06/A40153-9 bag 0503564-9 (flanking primers M13F-M13R) |
| MI6 | methamphetamine microcon pellet (clone 6) REC: 06/A40153-9 bag 0503564-9 (flanking primers M13F-M13R) |
| MI8 | methamphetamine microcon pellet (clone 8) REC: 06/A40153-9 bag 0503564-9 (flanking primers M13F-M13R) |
| MI9 | methamphetamine microcon pellet (clone 11) REC: 06/A40153-9 bag 0503564-9 (flanking primers M13F-M13R) |
| SB1 | Soil sample 1 (clone 1) S 34.72728 E 138.67374 (flanking primers M13F-M13R) |
| SB2 | Soil sample 1 (clone 2) S 34.72728 E 138.67374 (flanking primers M13F-M13R) |
| SB3 | Soil sample 1 (clone 3) S 34.72728 E 138.67374 (flanking primers M13F-M13R) |
| SB6 | Soil sample 1 (clone 6) S 34.72728 E 138.67374 (flanking primers M13F-M13R) |
| SB8 | Soil sample 1 (clone 8) S 34.72728 E 138.67374 (flanking primers M13F-M13R) |
| SB9 | Soil sample 1 (clone 9) S 34.72728 E 138.67374 (flanking primers M13F-M13R) |
| SB10 | Soil sample 1 (clone 10) S 34.72728 E 138.67374 (flanking primers M13F-M13R) |
| SB12 | Soil sample 1 (clone 12) S 34.72728 E 138.67374 (flanking primers M13F-M13R) |
| SC1 | Soil sample 2 (clone 1) S 34.70920 E 138.65593 (flanking primers M13F-M13R) |
| SC2 | Soil sample 2 (clone 3) S 34.70920 E 138.65593 (flanking primers M13F-M13R) |
| SC3 | Soil sample 2 (clone 4) S 34.70920 E 138.65593 (flanking primers M13F-M13R) |
| SC4 | Soil sample 2 (clone 5) S 34.70920 E 138.65593 (flanking primers M13F-M13R) |
| SC5 | Soil sample 2 (clone 6) S 34.70920 E 138.65593 (flanking primers M13F-M13R) |
| SC6 | Soil sample 2 (clone 7) S 34.70920 E 138.65593 (flanking primers M13F-M13R) |
| SC8 | Soil sample 2 (clone 9) S 34.70920 E 138.65593 (flanking primers M13F-M13R) |
| SC10 | Soil sample 2 (clone 11) S 34.70920 E 138.65593 (flanking primers M13F-M13R) |
| SC11 | Soil sample 2 (clone 12) S 34.70920 E 138.65593 (flanking primers M13F-M13R) |
| SD1 | Soil sample 3 (clone 1) S 35.11947 E 138.52400 (flanking primers M13F-M13R) |
| SD4 | Soil sample 3 (clone 4) S 35.11947 E 138.52400 (flanking primers M13F-M13R) |
| SD5 | Soil sample 3 (clone 5) S 35.11947 E 138.52400 (flanking primers M13F-M13R) |
| SD7 | Soil sample 3 (clone 7) S 35.11947 E 138.52400 (flanking primers M13F-M13R) |
| SD9 | Soil sample 3 (clone 9) S 35.11947 E 138.52400 (flanking primers M13F-M13R) |

| Name | Description |
|---|---|
| SD12 | Soil sample 3 (clone 12) S 35.11947 E 138.52400 (flanking primers M13F-M13R) |
| SE1 | Soil sample 4 (clone 1) S 35.11852 E 138.52701 (flanking primers M13F-M13R) |
| SE3 | Soil sample 4 (clone 3) S 35.11852 E 138.52701 (flanking primers M13F-M13R) |
| SE5 | Soil sample 4 (clone 5) S 35.11852 E 138.52701 (flanking primers M13F-M13R) |
| SE7 | Soil sample 4 (clone 8) S 35.11852 E 138.52701 (flanking primers M13F-M13R) |
| SE8 | Soil sample 4 (clone 9) S 35.11852 E 138.52701 (flanking primers M13F-M13R) |
| SE9 | Soil sample 4 (clone 10) S 35.11852 E 138.52701 (flanking primers M13F-M13R) |
| SF7 | Soil sample 3 (clone 3) S 35.11955 E 138.52420 (flanking primers M13F-M13R) |
| SF9 | Soil sample 3 (clone 5) S 35.11955 E 138.52420 (flanking primers M13F-M13R) |
| SF11 | Soil sample 3 (clone 10) S 35.11955 E 138.52420 (flanking primers M13F-M13R) |
| SG6 | Soil sample 4 (clone 14) S 35.11862 E 138.52719 (flanking primers M13F-M13R) |
| SG7 | Soil sample 4 (clone 15) S 35.11862 E 138.52719 (flanking primers M13F-M13R) |
| SG8 | Soil sample 4 (clone 16) S 35.11862 E 138.52719 (flanking primers M13F-M13R) |
| SG11 | Soil sample 4 (clone 22) S 35.11862 E 138.52719 (flanking primers M13F-M13R) |
| SG12 | Soil sample 4 (clone 23) S 35.11862 E 138.52719 (flanking primers M13F-M13R) |
| SH1 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH2 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH3 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH4 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH5 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH6 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH7 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH8 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH9 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH10 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH11 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH12 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH13 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH14 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH15 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH16 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH17 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH18 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH19 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH20 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH21 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH22 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| SH23 | Soil sample (mixed soils) (flanking primers M13F-M13R) |
| XB1 | ecstasy microcon sn 648-16/7 REC: ? bag 0504648 |
| XB2 | ecstasy microcon sn 648-16/7 REC: ? bag 0504648 |
| XB3 | ecstasy microcon sn 648-16/7 REC: ? bag 0504648 |
| XB4 | ecstasy microcon sn 648-16/7 REC: ? bag 0504648 |

| Name | Description |
|---|---|
| XB5 | ecstasy microcon sn 648-16/7 REC: ? bag 0504648 |
| XB6 | ecstasy microcon sn 648-16/7 REC: ? bag 0504648 |
| XB7 | ecstasy microcon sn 648-16/7 REC: ? bag 0504648 |
| XB8 | ecstasy microcon sn 648-16/7 REC: ? bag 0504648 |
| XB9 | ecstasy microcon sn 648-16/7 REC: ? bag 0504648 |
| XB10 | ecstasy microcon sn 648-16/7 REC: ? bag 0504648 |
| XB11 | ecstasy microcon sn 648-16/7 REC: ? bag 0504648 |
| XB12 | ecstasy microcon sn 648-16/7 REC: ? bag 0504648 |
| MB1 | methamphetamine microcon sn 707-16/7 REC: 02/A43507-1 bag 013707 |
| MB2 | methamphetamine microcon sn 707-16/7 REC: 02/A43507-1 bag 013707 |
| MB3 | methamphetamine microcon sn 707-16/7 REC: 02/A43507-1 bag 013707 |
| MB4 | methamphetamine microcon sn 707-16/7 REC: 02/A43507-1 bag 013707 |
| MB5 | methamphetamine microcon sn 707-16/7 REC: 02/A43507-1 bag 013707 |
| MB6 | methamphetamine microcon sn 707-16/7 REC: 02/A43507-1 bag 013707 |
| MB7 | methamphetamine microcon sn 707-16/7 REC: 02/A43507-1 bag 013707 |
| MB8 | methamphetamine microcon sn 707-16/7 REC: 02/A43507-1 bag 013707 |
| MB9 | methamphetamine microcon sn 707-16/7 REC: 02/A43507-1 bag 013707 |
| MB10 | methamphetamine microcon sn 707-16/7 REC: 02/A43507-1 bag 013707 |
| MB11 | methamphetamine microcon sn 707-19/7 REC: 02/A43507-1 bag 013707 |
| MB12 | methamphetamine microcon sn 707-19/7 REC: 02/A43507-1 bag 013707 |
| MB13 | methamphetamine microcon sn 707-19/7 REC: 02/A43507-1 bag 013707 |
| MB14 | methamphetamine microcon sn 707-19/7 REC: 02/A43507-1 bag 013707 |
| MB15 | methamphetamine microcon sn 707-19/7 REC: 02/A43507-1 bag 013707 |
| MB16 | methamphetamine microcon sn 707-19/7 REC: 02/A43507-1 bag 013707 |
| MB17 | methamphetamine microcon sn 707-19/7 REC: 02/A43507-1 bag 013707 |
| MB18 | methamphetamine microcon sn 707-19/7 REC: 02/A43507-1 bag 013707 |
| MB19 | methamphetamine microcon sn 707-19/7 REC: 02/A43507-1 bag 013707 |
| MB20 | methamphetamine microcon sn 707#1-24/7 REC: 02/A43507-1 bag 013707 |
| MB21 | methamphetamine microcon sn 707#1-24/7 REC: 02/A43507-1 bag 013707 |
| MB22 | methamphetamine microcon sn 707#1-24/7 REC: 02/A43507-1 bag 013707 |
| MB23 | methamphetamine microcon sn 707#1-24/7 REC: 02/A43507-1 bag 013707 |
| MB24 | methamphetamine microcon sn 707#1-24/7 REC: 02/A43507-1 bag 013707 |
| MB25 | methamphetamine microcon sn 707#1-24/7 REC: 02/A43507-1 bag 013707 |
| MB26 | methamphetamine microcon sn 707#1-24/7 REC: 02/A43507-1 bag 013707 |
| MK1 | methamphetamine microcon (soluble pellet) clone 1 REC: 04/B64627-2 bag 0402460 2e,2f,2g,2h |
| MK2 | methamphetamine microcon (soluble pellet) clone 2 REC: 04/B64627-2 bag 0402460 2e,2f,2g,2h |
| MK3 | methamphetamine microcon (soluble pellet) clone 3 REC: 04/B64627-2 bag 0402460 2e,2f,2g,2h |
| MK4 | methamphetamine microcon (soluble pellet) clone 5 REC: 04/B64627-2 bag 0402460 2e,2f,2g,2h |
| MK5 | methamphetamine microcon (soluble pellet) clone 6 REC: 04/B64627-2 bag 0402460 2e,2f,2g,2h |
| MK6 | methamphetamine microcon (soluble pellet) clone 13 REC: 04/B64627-2 bag 0402460 2e,2f,2g,2h |
| MK7 | methamphetamine microcon (soluble pellet) clone 16 REC: 04/B64627-2 bag 0402460 2e,2f,2g,2h |
| MK8 | methamphetamine microcon (soluble pellet) clone 18 REC: 04/B64627-2 bag 0402460 2e,2f,2g,2h |

| Name | Description |
|---|---|
| MK9 | methamphetamine microcon (soluble pellet) clone 19 REC: 04/B64627-2 bag 0402460 2e,2f,2g,2h |
| MK10 | methamphetamine microcon (soluble pellet) clone 20 REC: 04/B64627-2 bag 0402460 2e,2f,2g,2h |
| MK11 | methamphetamine microcon (soluble pellet) clone 23 REC: 04/B64627-2 bag 0402460 2e,2f,2g,2h |
| MK12 | methamphetamine microcon (soluble pellet) clone 24 REC: 04/B64627-2 bag 0402460 2e,2f,2g,2h |
| MJ1 | methamphetamine microcon (soluble pellet) clone 1 REC: 07/B10176-2 bag 0605455-2a,2b,2c,2d |
| MJ2 | methamphetamine microcon (soluble pellet) clone 2 REC: 07/B10176-2 bag 0605455-2a,2b,2c,2d |
| MJ3 | methamphetamine microcon (soluble pellet) clone 6 REC: 07/B10176-2 bag 0605455-2a,2b,2c,2d |
| MJ4 | methamphetamine microcon (soluble pellet) clone 8 REC: 07/B10176-2 bag 0605455-2a,2b,2c,2d |
| MJ5 | methamphetamine microcon (soluble pellet) clone 10 REC: 07/B10176-2 bag 0605455-2a,2b,2c,2d |
| MJ6 | methamphetamine microcon (soluble pellet) clone 13 REC: 07/B10176-2 bag 0605455-2a,2b,2c,2d |
| MJ7 | methamphetamine microcon (soluble pellet) clone 15 REC: 07/B10176-2 bag 0605455-2a,2b,2c,2d |
| MJ8 | methamphetamine microcon (soluble pellet) clone 16 REC: 07/B10176-2 bag 0605455-2a,2b,2c,2d |
| MJ9 | methamphetamine microcon (soluble pellet) clone 17 REC: 07/B10176-2 bag 0605455-2a,2b,2c,2d |
| MJ10 | methamphetamine microcon (soluble pellet) clone 18 REC: 07/B10176-2 bag 0605455-2a,2b,2c,2d |
| MJ11 | methamphetamine microcon (soluble pellet) clone 20 REC: 07/B10176-2 bag 0605455-2a,2b,2c,2d |
| MJ12 | methamphetamine microcon (soluble pellet) clone 23 REC: 07/B10176-2 bag 0605455-2a,2b,2c,2d |
| GR170.2 | single sequence cloned from GR170 (clone 2) |
| GR170.4 | single sequence cloned from GR170 (clone 4) |
| GR170.5 | single sequence cloned from GR170 (clone 5) |
| GR170.7 | single sequence cloned from GR170 (clone 7) |
| TVEC | short fragment of DNA from the T-vector PCR.2.1 which flanks insert amplified with M13.1 primers |
| TVEC.1 | short fragment of DNA from the T-vector PCR.2.1 which flanks insert amplified with M13 primers |
| NX1 | ecstasy tablet 1 surface microcon REC: 06/A48078-2 |
| NX2 | ecstasy tablet 2 surface microcon REC: 06/A48078-2 |
| NX3 | ecstasy tablet 3 surface microcon REC: 06/A48078-2 |
| NX4 | ecstasy tablet 4 surface microcon REC: 06/A48078-2 |
| NX5 | ecstasy tablet 5 surface microcon REC: 014131 |
| NX6 | ecstasy tablet 6 surface microcon REC: 014131 |
| NX7 | ecstasy tablet 7 surface microcon REC: 014131 |
| NX8 | ecstasy tablet 8 surface microcon REC: 014131 |
| NX9 | ecstasy capsule 9 surface microcon REC: A215200 |
| NX10 | ecstasy capsule 10 surface microcon REC: A215200 |
| NX11 | ecstasy capsule 11 surface microcon REC: A215200 |
| NX12 | ecstasy capsule 12 surface microcon REC: A215200 |
| NX13 | ecstasy tablet 1 to 12 PCR 1st round blank |
| NX14 | ecstasy tablet 1 to 12 PCR 2nd round blank |
| NX15 | ecstasy tablet 15 surface microcon REC: 06/A48078-2 |
| NX16 | ecstasy tablet 16 surface microcon REC: 06/A48078-2 |
| NX17 | ecstasy tablet 17 surface microcon REC: 06/A48078-2 |
| NX18 | ecstasy tablet 18 surface microcon REC: 06/A48078-2 |
| NX19 | ecstasy tablet 19 surface microcon REC: 014131 |
| NX20 | ecstasy tablet 20 surface microcon REC: 014131 |

| Name | Description |
|---|---|
| NX21 | ecstasy tablet 21 surface microcon REC: 014131 |
| NX22 | ecstasy tablet 22 surface microcon REC: 014131 |
| NX23 | ecstasy capsule 23 surface microcon REC: A215200 |
| NX24 | ecstasy capsule 24 surface microcon REC: A215200 |
| NX25 | ecstasy capsule 25 surface microcon REC: A215200 |
| NX26 | ecstasy capsule 26 surface microcon REC: A215200 |
| NX27 | ecstasy tablet (15 to 26) microcon blank 1 |
| NX28 | ecstasy tablet (15 to 26) microcon blank 2 |
| NX29 | ecstasy tablet 15 to 26 PCR 1st round blank |
| NX30 | ecstasy tablet 15 to 26 PCR 2nd round blank |
| NX31 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 1 |
| NX32 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 2 |
| NX33 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 3 |
| NX34 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 4 |
| NX35 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 5 |
| NX36 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 6 |
| NX37 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 7 |
| NX38 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 8 |
| NX39 | ecstasy (2nd batch 1 to 22) surface microcon REC: 014131 tablet 9 |
| NX40 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 10 |
| NX41 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 11 |
| NX42 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 12 |
| NX43 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 13 |
| NX44 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 14 |
| NX45 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 15 |
| NX46 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 16 |
| NX47 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 17 |
| NX48 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 18 |
| NX49 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 19 |
| NX50 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 20 |
| NX51 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 21 |
| NX52 | ecstasy (2nd batch) surface microcon REC: 014131 tablet 22 |
| NX53 | ecstasy tablet (2nd batch 1 to 22) microcon blank 1 |
| NX54 | ecstasy tablet (2nd batch 1 to 22) microcon blank 2 |
| NX55 | ecstasy tablet (2nd batch 1 to 22) PCR 2nd round blank |

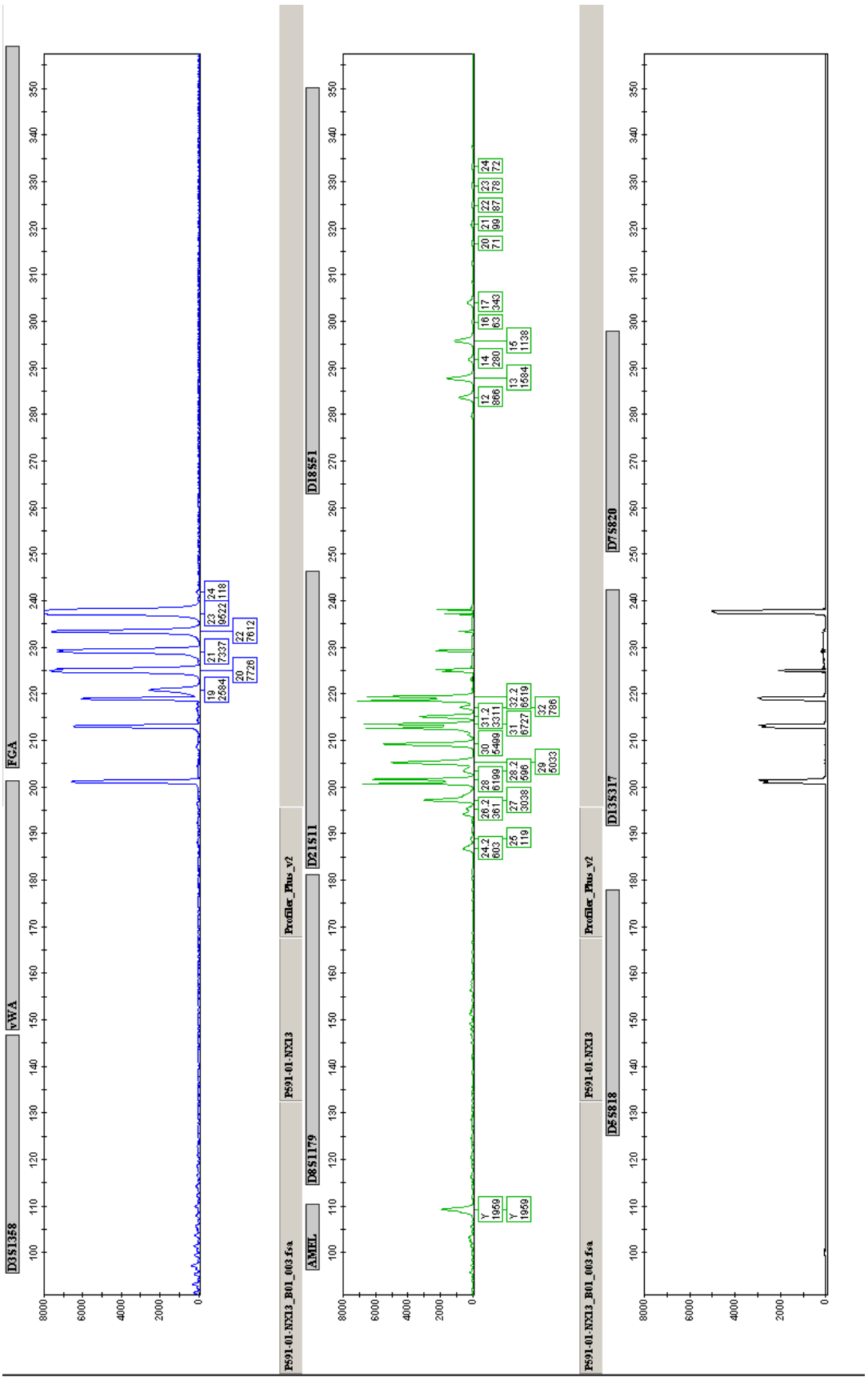
# Appendix 2: Profiler plus$^{tm}$ Profiles of NX series samples.

For reference the high-diversity “spurious” profile is-

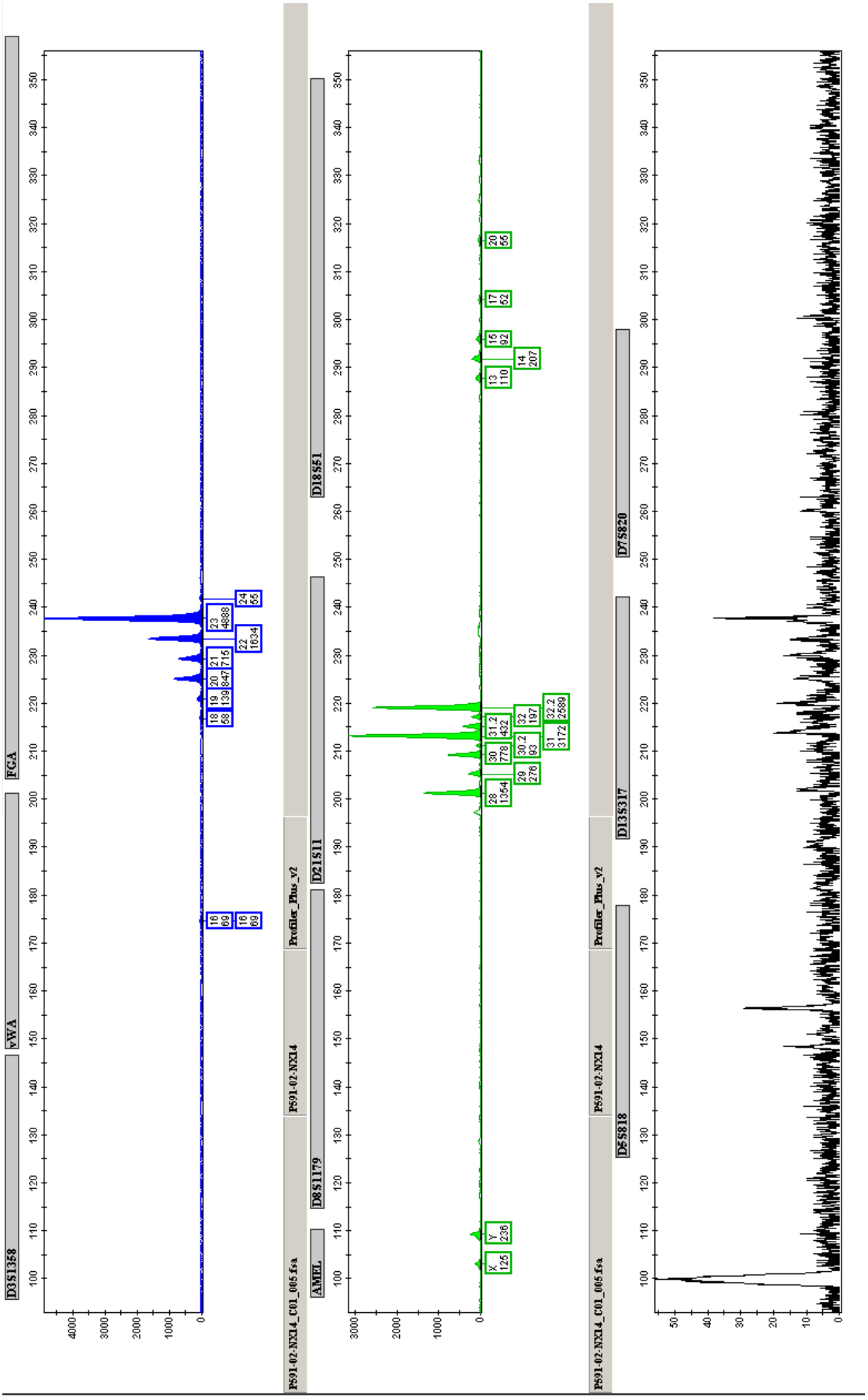
- D3S1358 16,15,17
- Vwa 17, 16,15,18
- FGA 20,21,22
- D8S1179 13, 12,14
- D21S11 29, 28,30
- D18S51 12,16,15
- D5S818 11,12,13
- D13S317 11,12
- D7S820 10,8,11

See images listed on following pages-

NX15


D3S1358
vWA
FGA
P591-01-NX13_B01_003.fsa
Profiler_Plus_v2
AMEL
D8S1179
D21S11
D18S51
D5S818
D13S317
D7S820

NX16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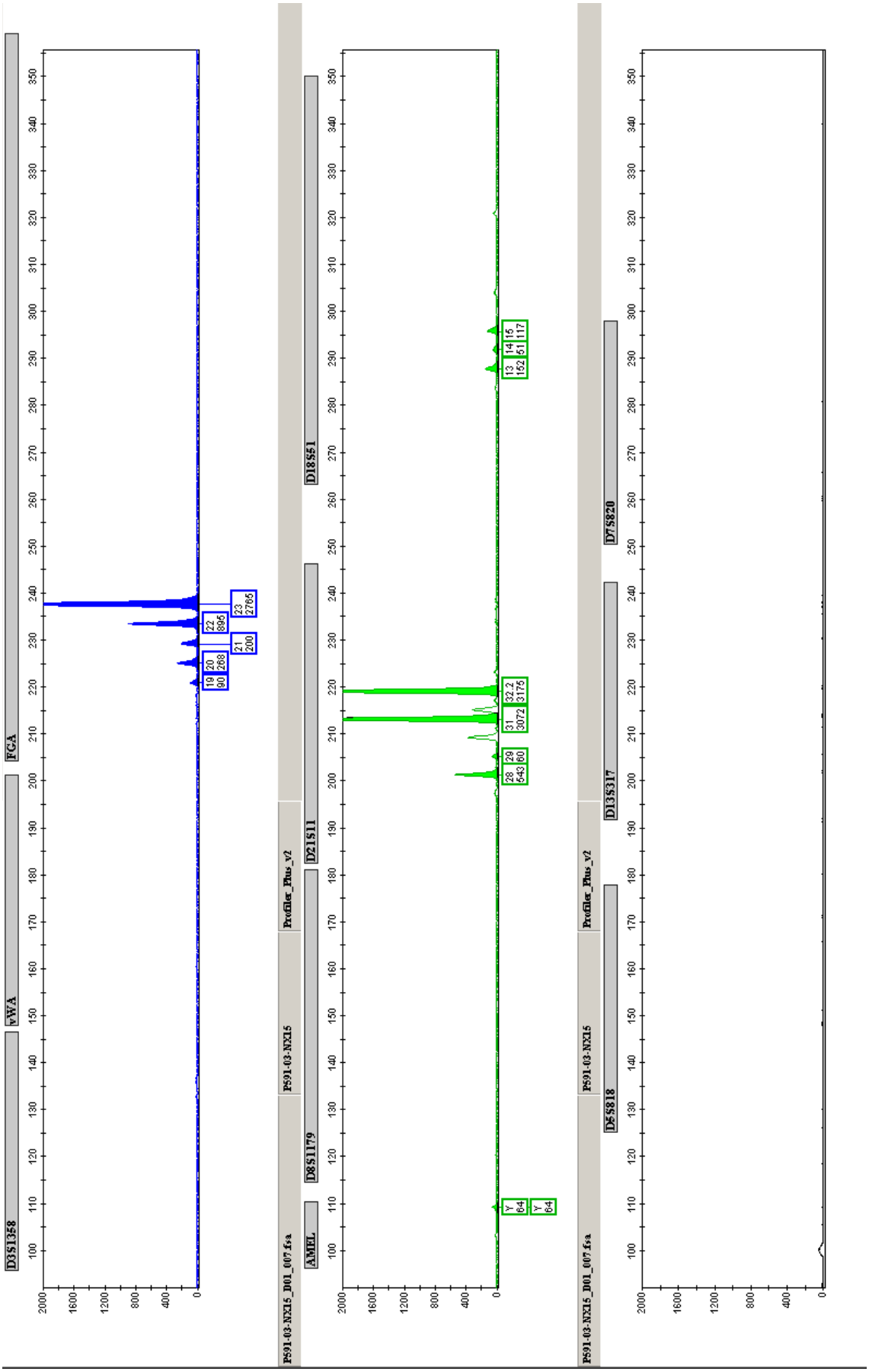
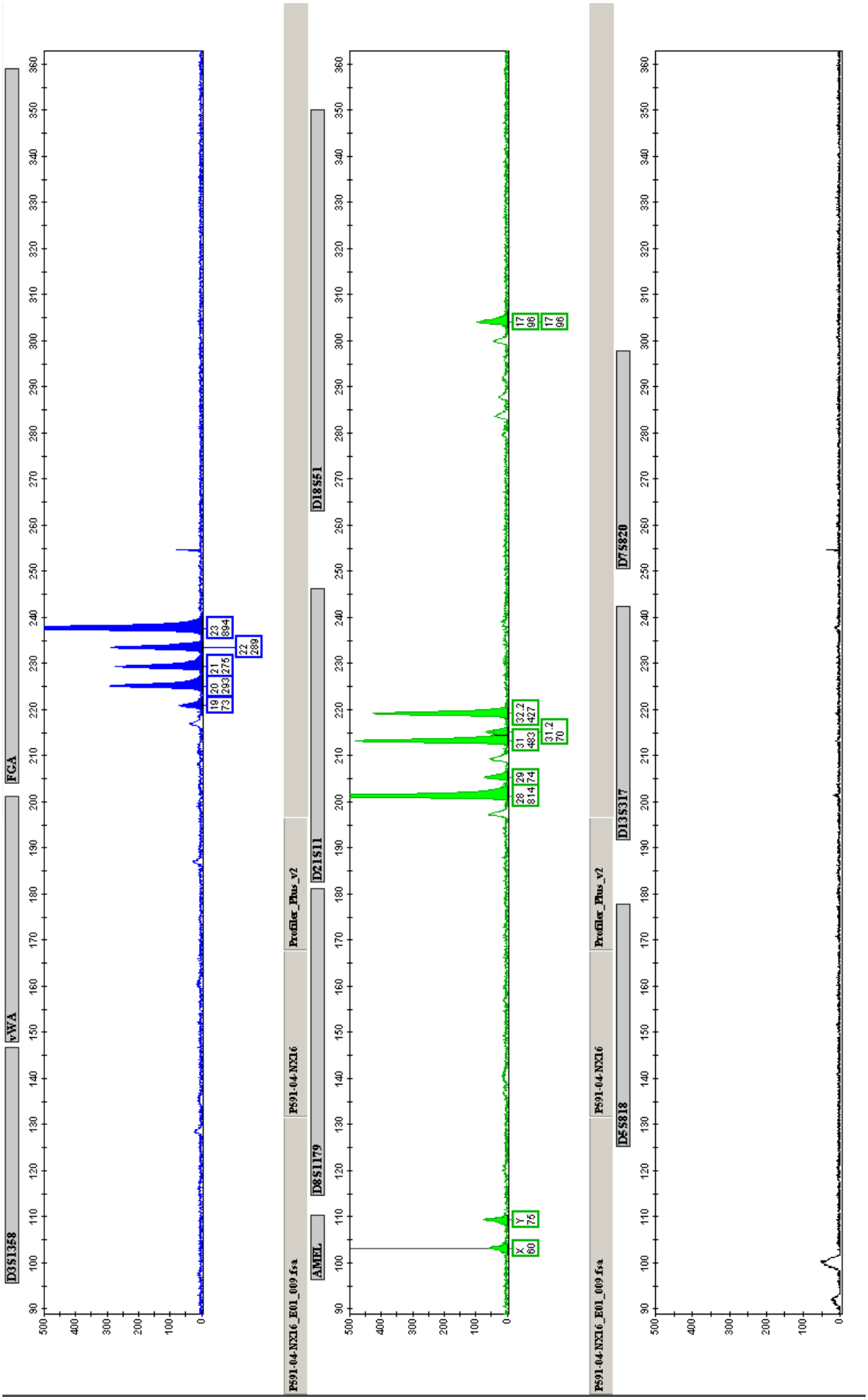
vWA
FGA
P591-02-NX14_C01_005.fsa
P591-02-NX14
ProFiler_Plus_v2
AMEL
D8S1179
D21S11
D18S51
D6S818
D13S317
D7S820

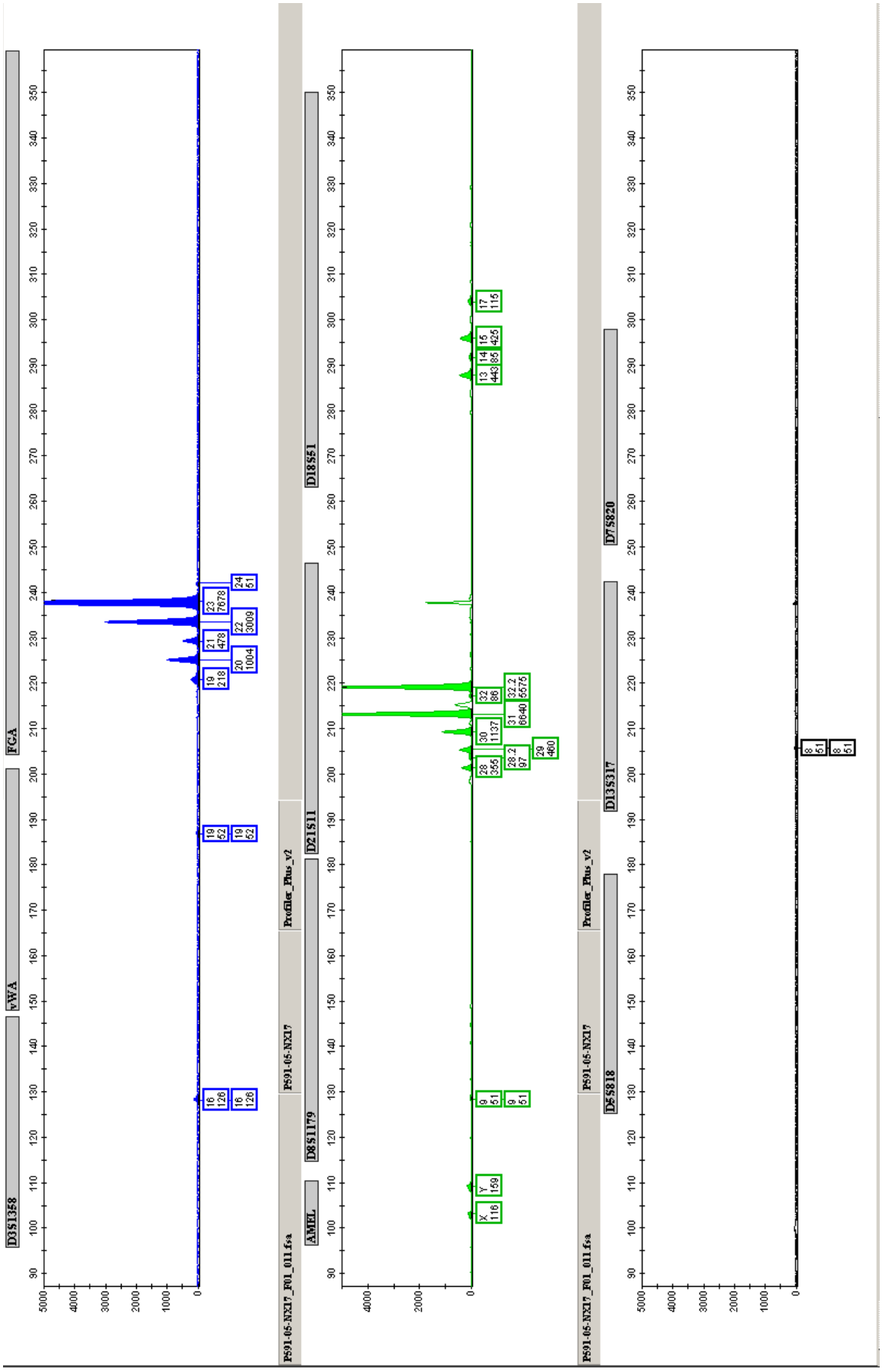
NX17


D3S1358
vWA
FCA
P591-03-NX15
Profiler_Plus_v2
AMEL
D8S1179
D21S11
D18S51
D5S818
D13S317
D7S820
P591-03-NX15_D01_007.fsa

NX18


D3S1358
vWA
FCA
P591-04-NX16_E01_009.fsa
P591-04-NX16
Profiler_Plus_v2
AMEL
D8S1179
D21S11
D18S51
D5S818
D13S317
D7S820

NX19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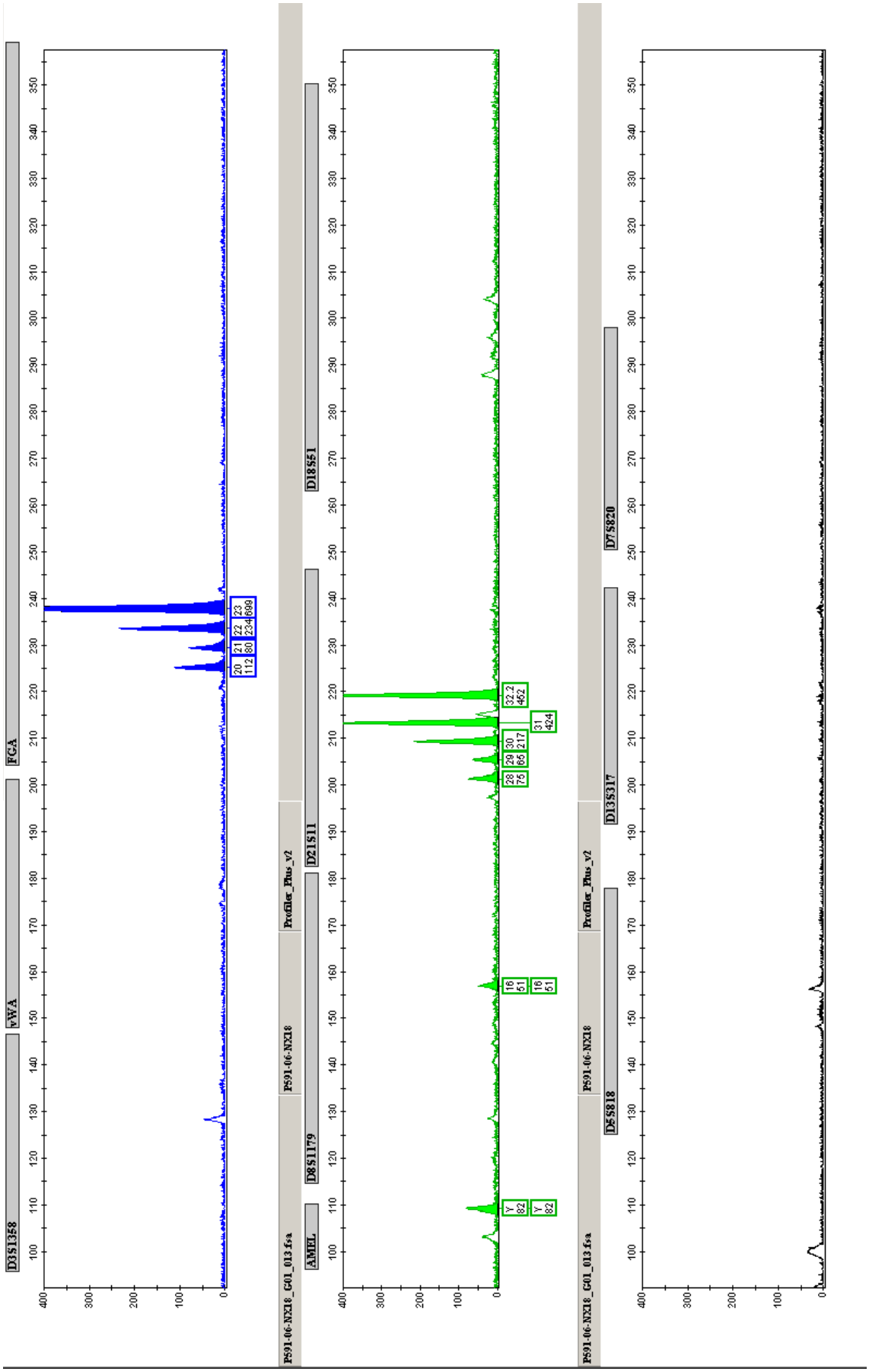
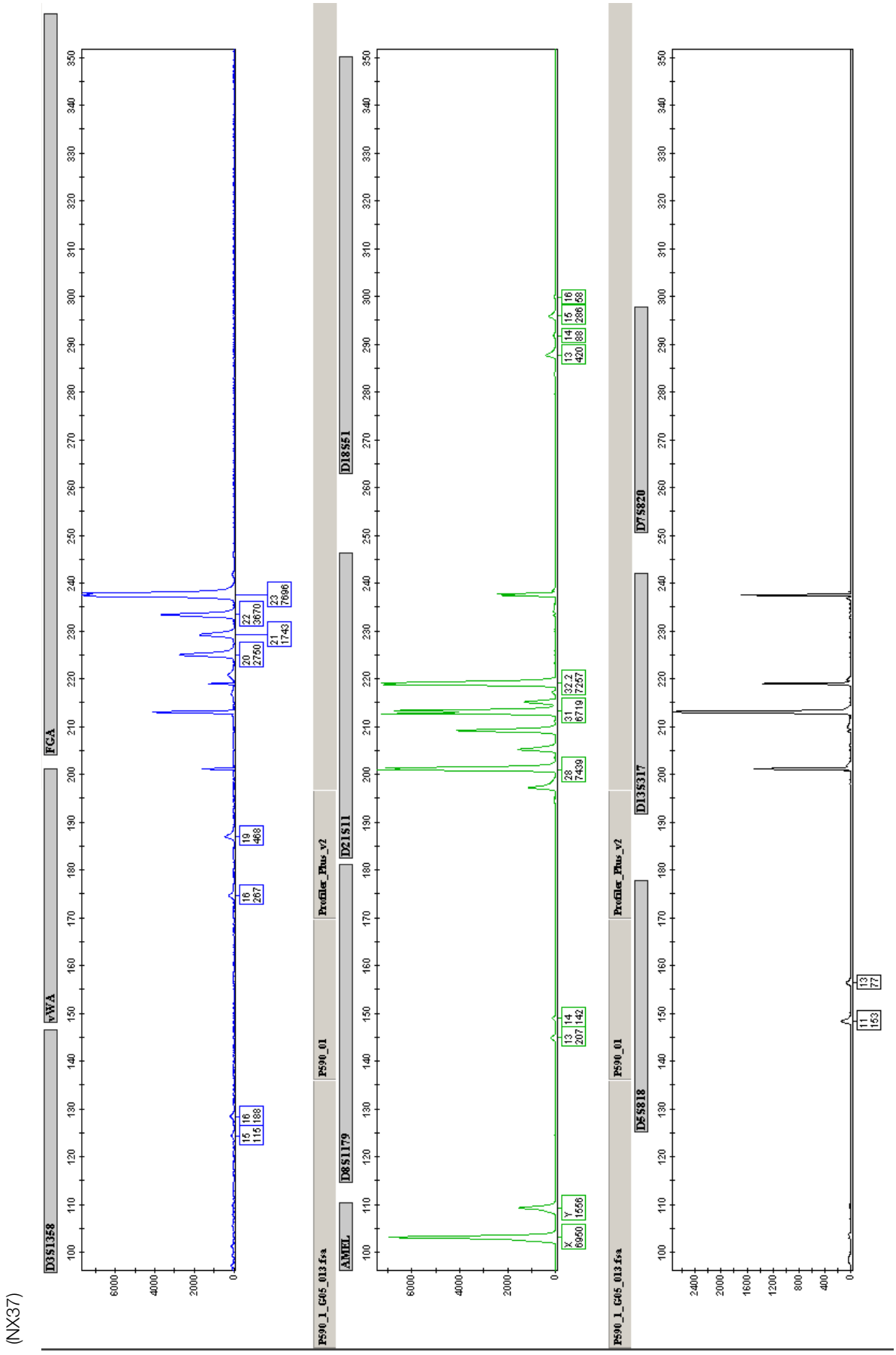
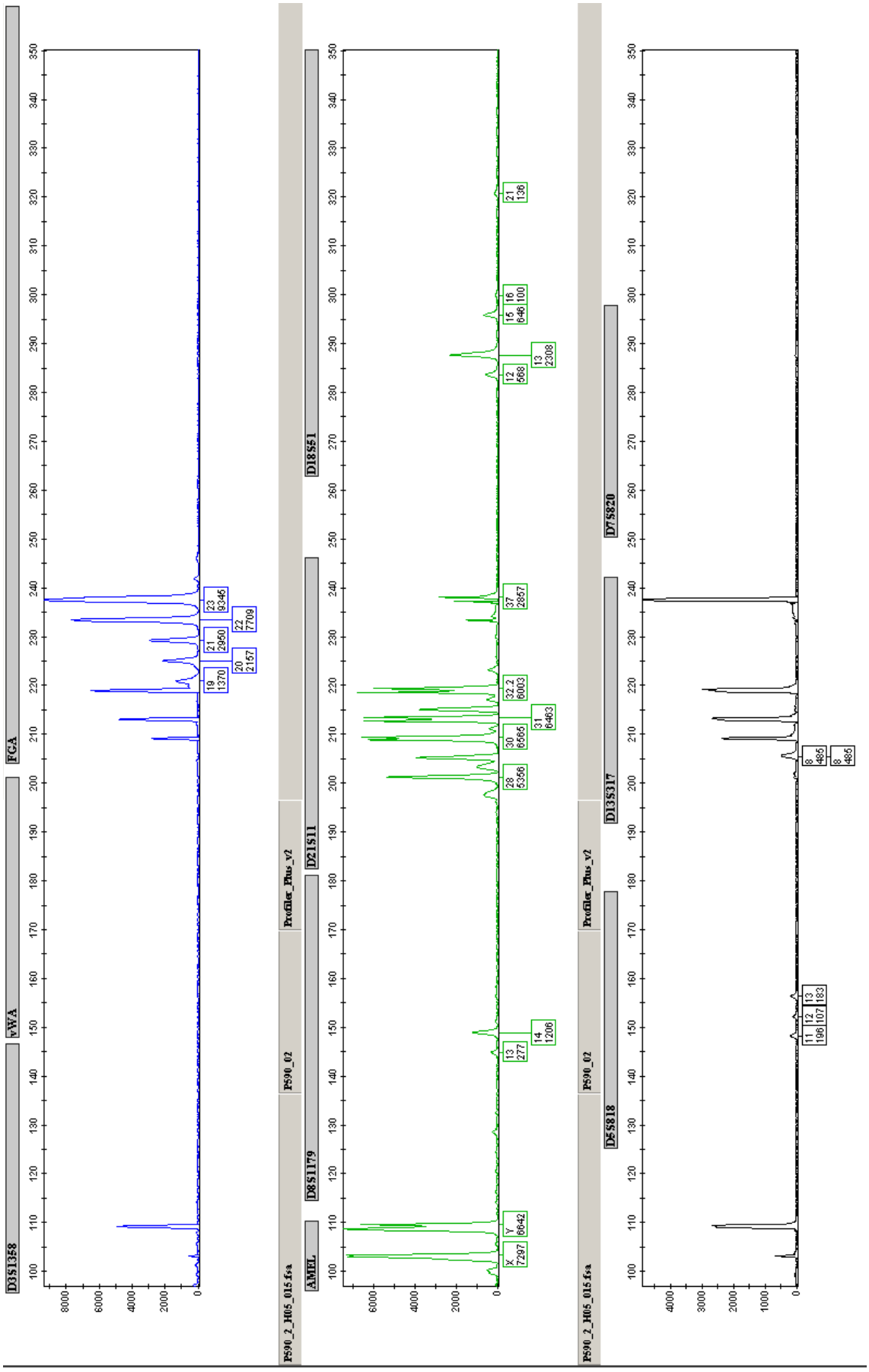
vWA
FGA
P591-05-NX17_F01_011.fsa
P591-05-NX17
Profiler_Plus_v2
AMEL
D8S1179
D21S11
D18S51
D5S818
D13S317
D7S820

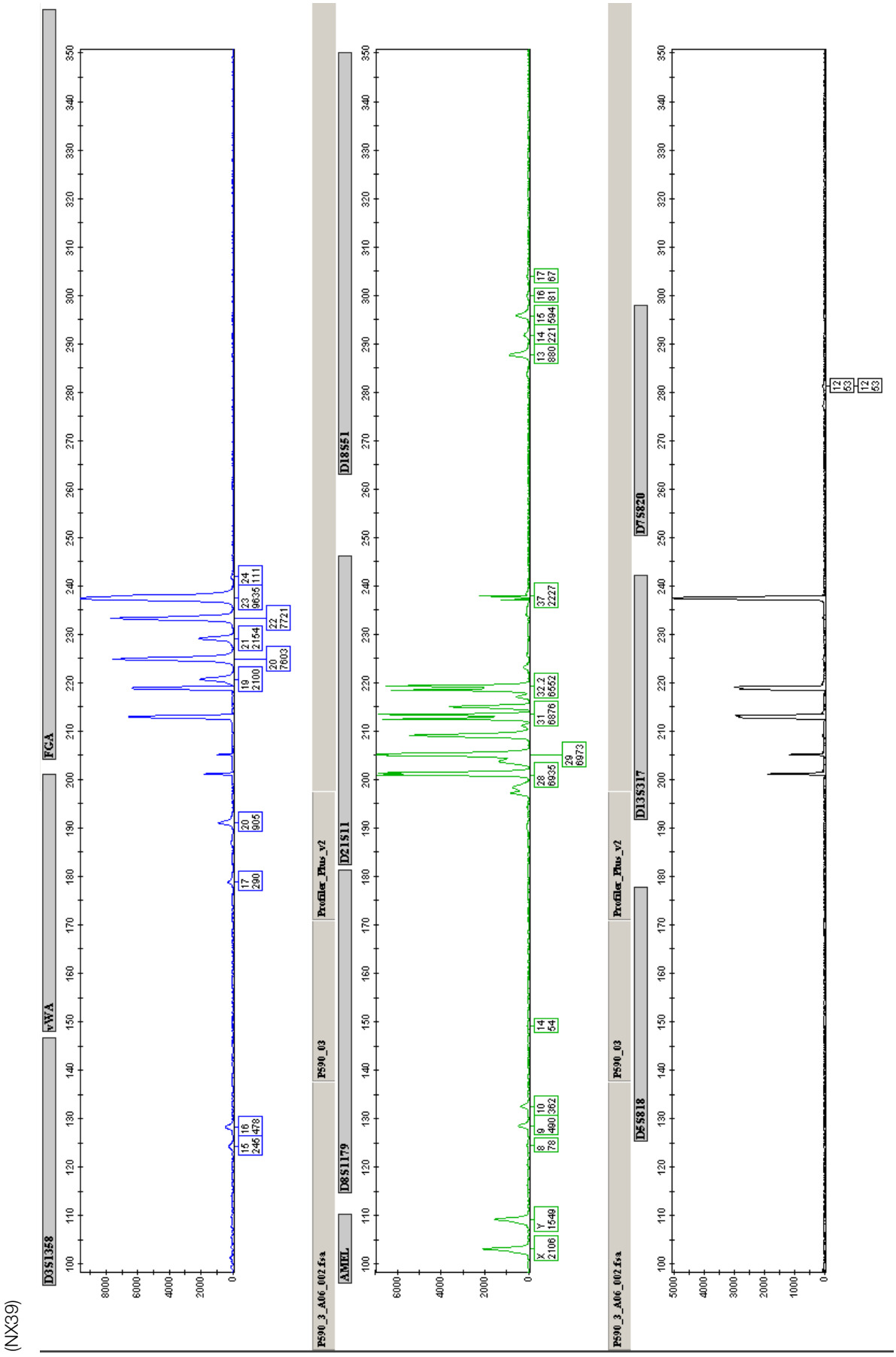
NX20


D3S1358
vWA
FGA
P591-06-NX18_G01_013.fsa
Profiler_Plus_v2
AMEL
D8S1179
D21S11
D18S51
D5S818
D13S317
D7S820


(NX37)
D3S1358
vWA
FGA
P590_1_G05_013.fsa
P590_01
Profiler_Plus_v2
AMEL
D8S1179
D21S11
D18S51
D5S818
D13S317
D7S820

(NX38)


D3S1358
vWA
FGA
AMEL
D8S1179
D21S11
D18S51
D5S818
D13S317
D7S820
P590_02
Profiler_Plus_v2
P590_2_H05_015.fsa


D3S1358
vWA
FGA
P590_03
Profiler_Plus_v2
AMEL
D8S1179
D21S11
D18S51
D5S818
D13S317
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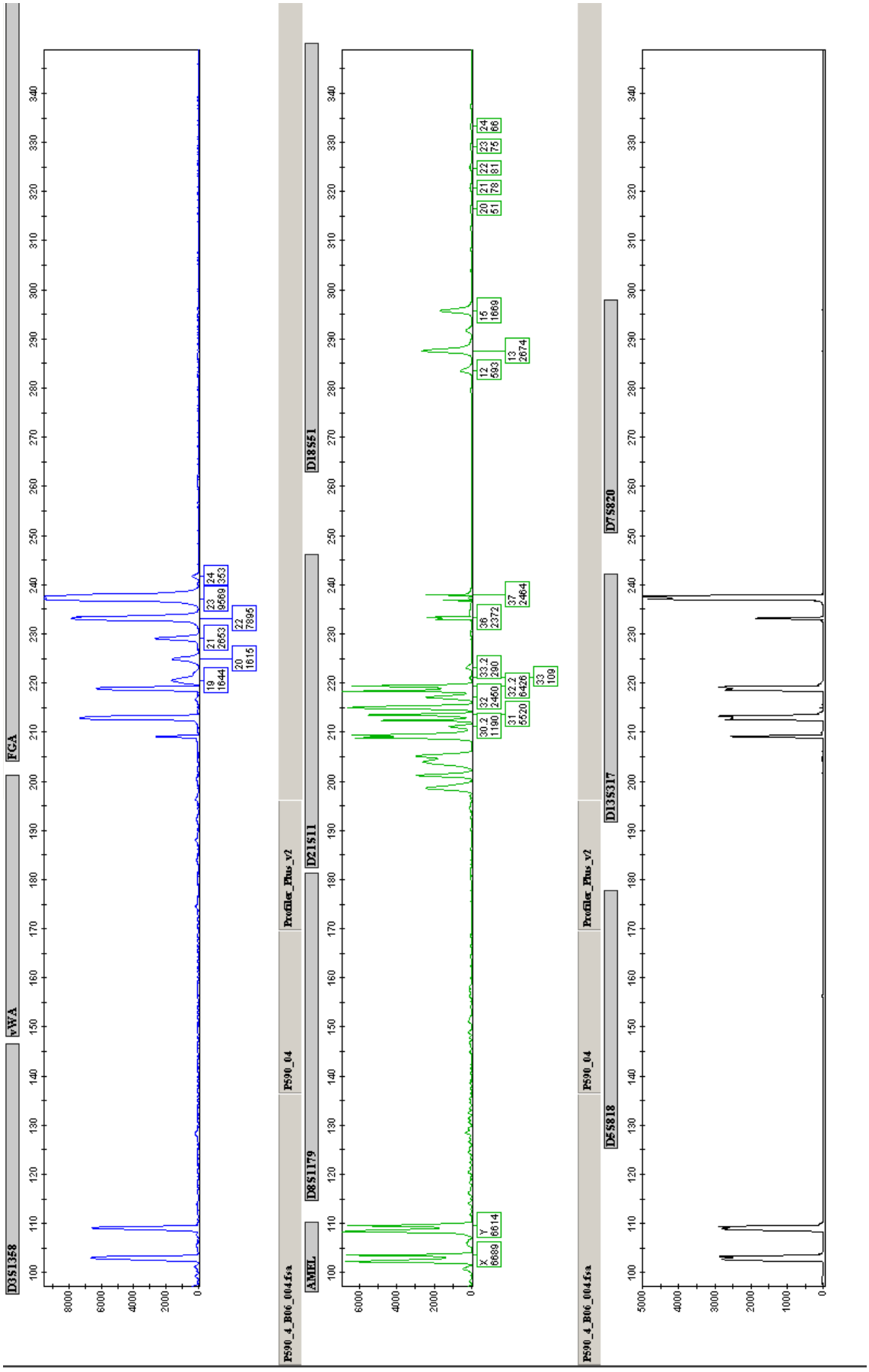
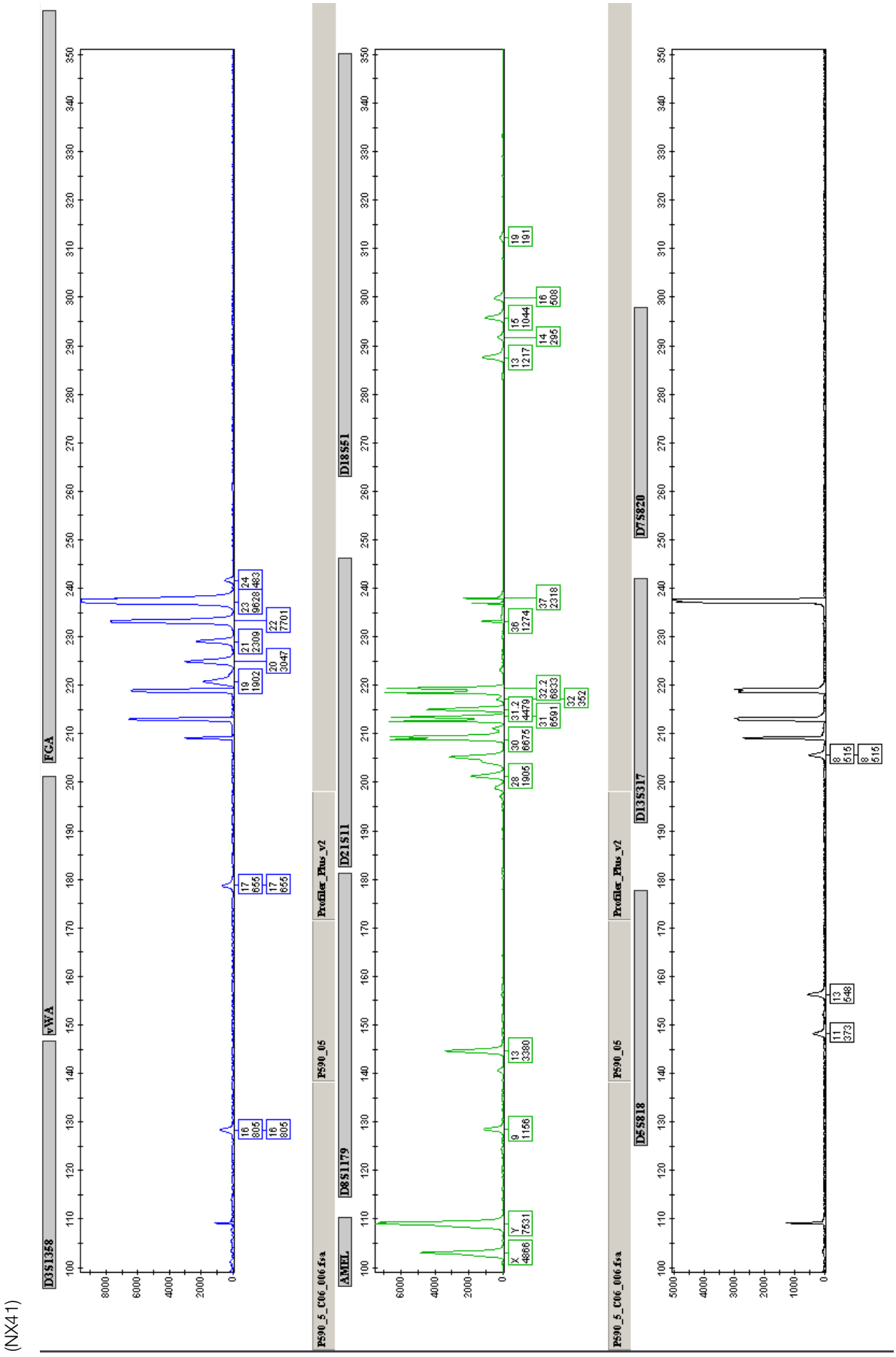
P590_3_A06_002.fsa

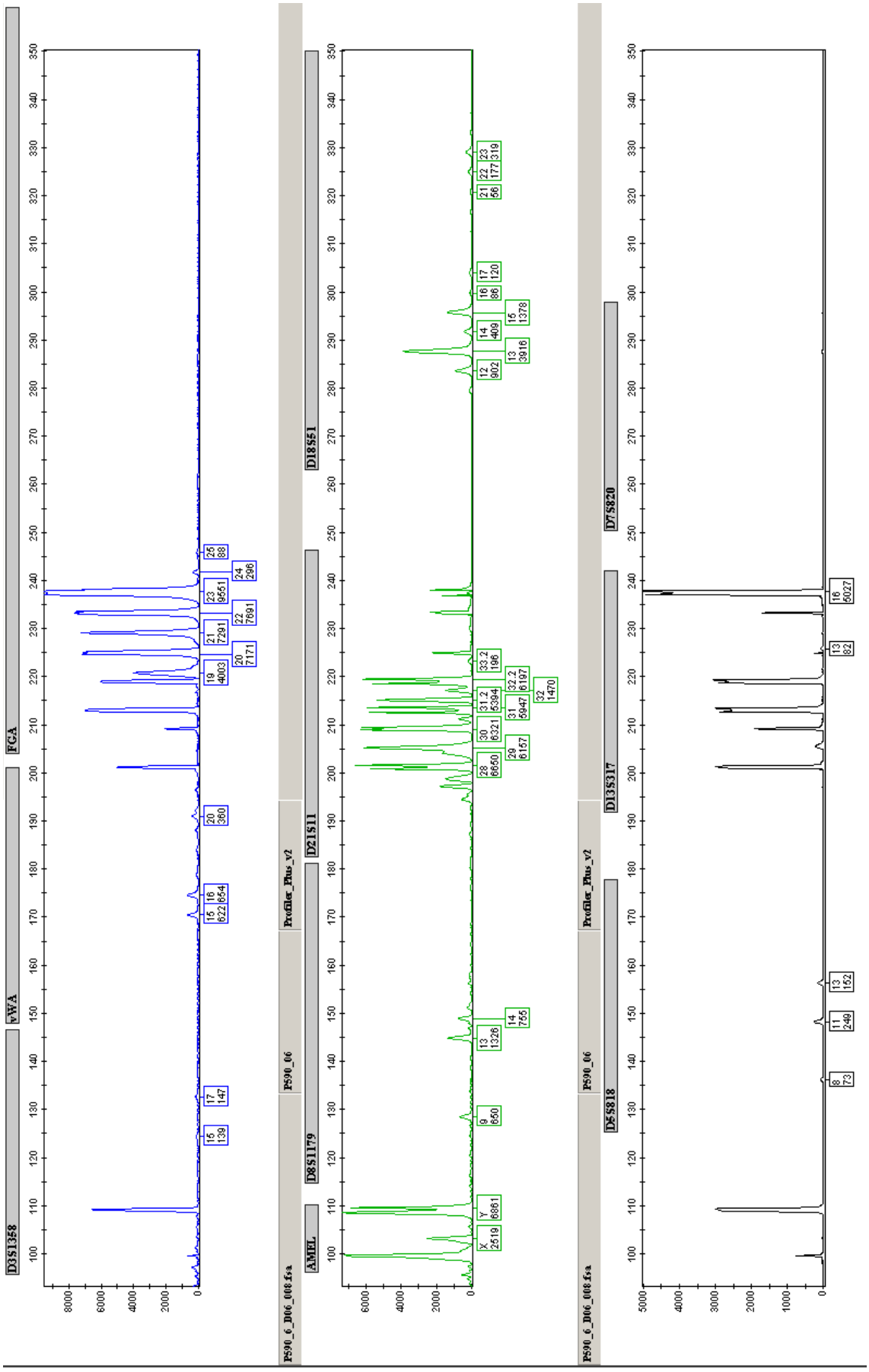
(NX39)

(NX40)

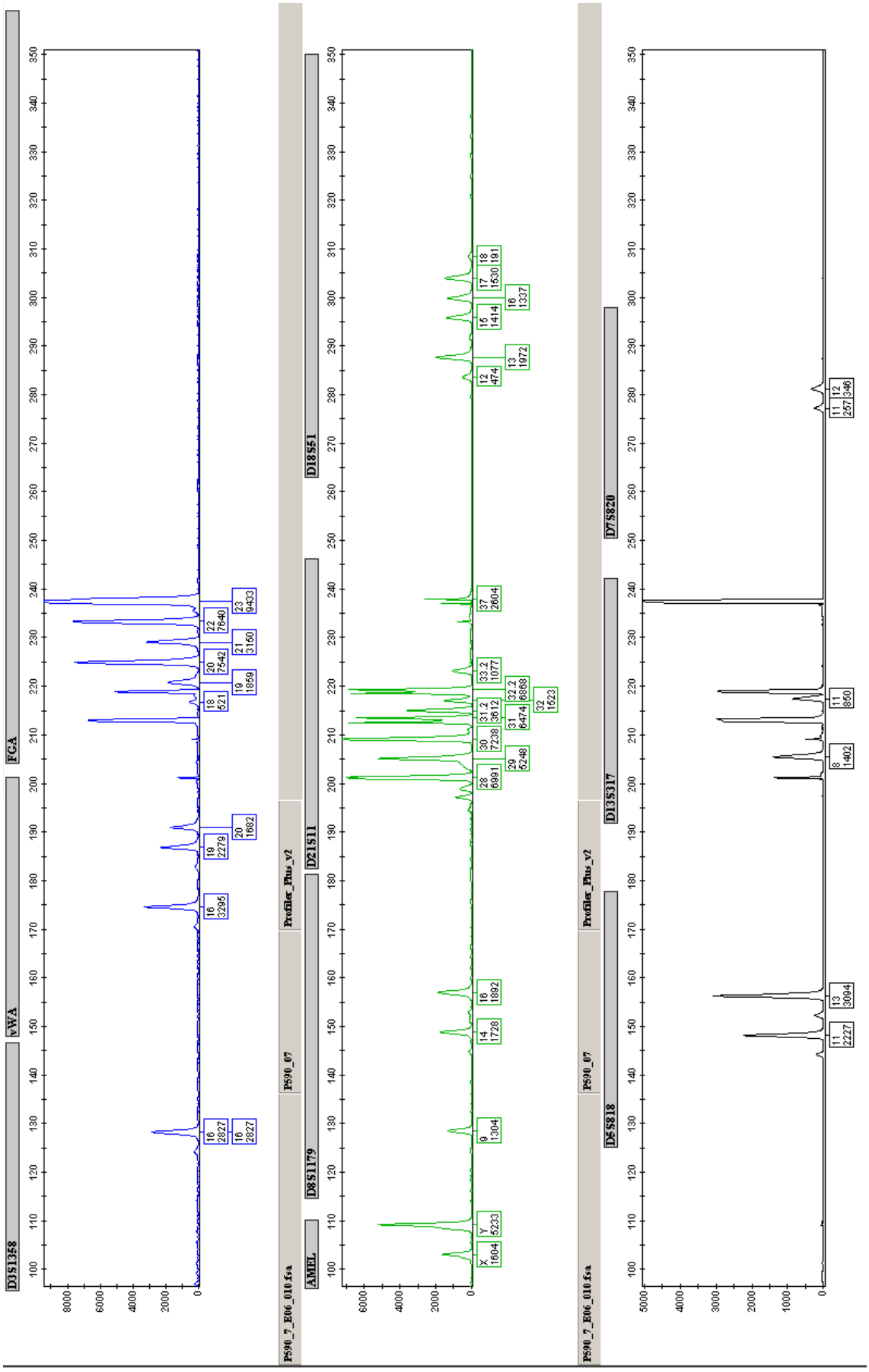

D3S1358
vWA
FGA
P590_4_B06_004.fsa
P590_04
Profiler_Plus_v2
AMEL
D8S1179
D21S11
D18S51
D5S818
D13S317
D7S820


(NX41)
P590_5_C06_006.fsa
Profiler_Plus_v2
P590_05
D3S1358
vWA
FGA
AMEL
D8S1179
D21S11
D18S51
D5S818
D13S317
D7S820

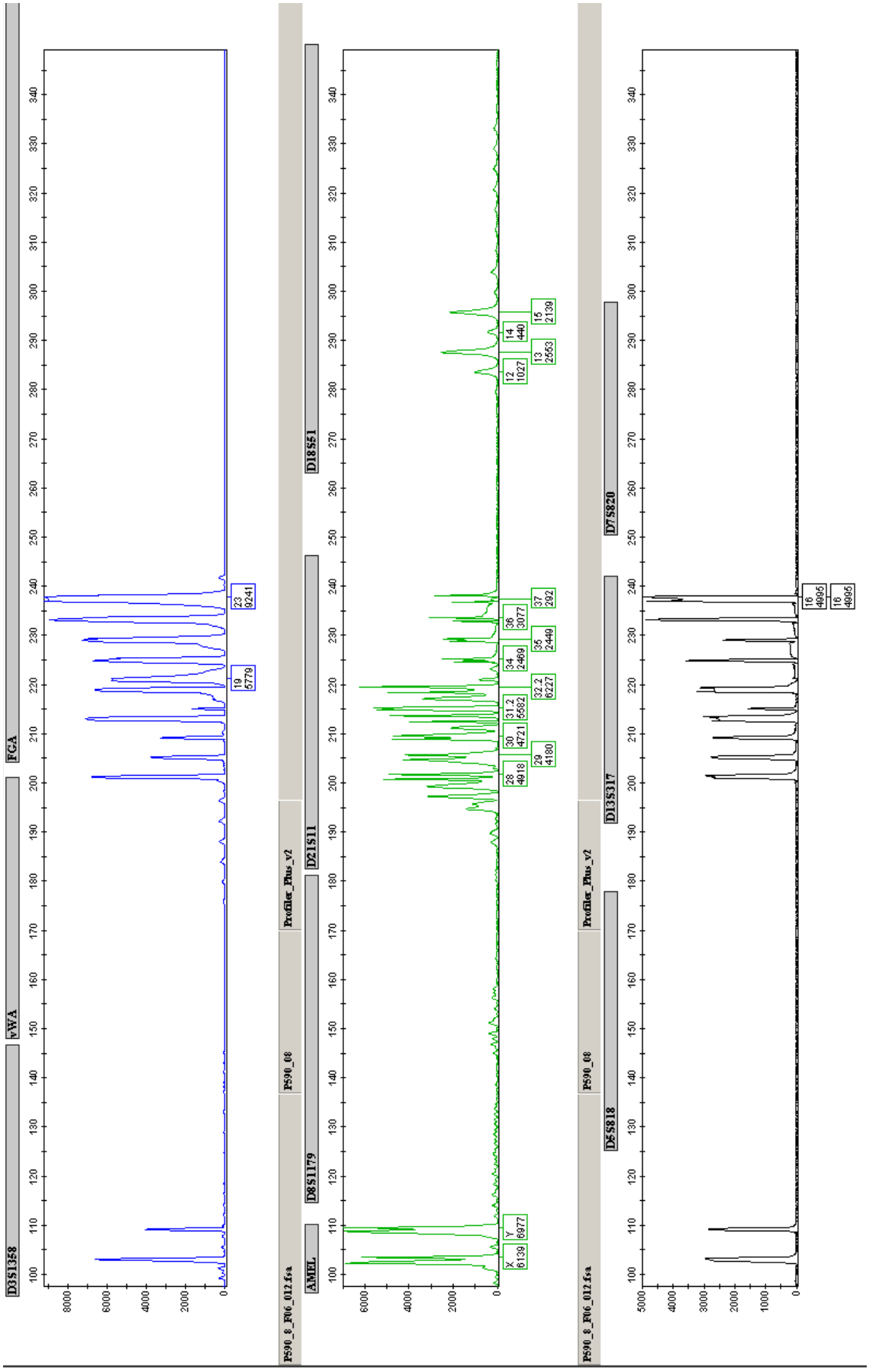
(NX42) The primary reader suggested that it possibly includes animal DNA such as dog, pig, cow, horse?



(NX43)


D3S1358
vWA
FGA
P590_7_E06_010.fsa
P590_07
Profiler_Plus_v2
AMEL
D8S1179
D21S11
D18S51
D5S818
D13S317
D7S820

(NX44)


D3S1358
vWA
FGA
P590_8_F06_012.fsa
P590_08
Profiler_Plus_v2
AMEL
D8S1179
D21S11
D18S51
D5S818
D13S317
D7S820

# Appendix 3: Provenance of NX series of seizures.

NX series of samples are on lab note book page 105 dated 12/05/08 in book 1.

These were all ecstasy tablets. (MDMA)

The original extractions are on pages 95-99 in same book. Extraction for these seizures was the standard sodium carbonate with microcon concentrations.

- NX-NX4 Rec  06/A48078-2
- NX5-NX8 Rec 014131
- NX9-NX12 Rec A215200
- NX13- blank NX14 second round blank
- NX15-NX18 Rec 06/A48078-2
- NX19-NX22 Rec 014131
- NX23-NX26 Rec A215200
- NX27 and NX28 are blank microcons.
- NX29 is a first round PCR blank NX30 is second round PCR blanks.
- NX31-NX35 Rec 014131
- NX36-NX52 Rec 014131
- NX53- NX54 Blank microcons
- NX55 is a second round PCR blank.

Further information on provenance can be found in appendix 1.

# Appendix 4: Mitochondrial profiles of significant individuals

The mitochondrial profiles of two individuals were considered important. Individual#1 was the person whose DNA most frequently contaminated STR profiles as discussed elsewhere in this report. Individual#2 was the person who actually performed the laboratory operations and was in most frequent close proximity to samples and solutions.

In the HVI locus we observed the expected sequences with all but one polymorphism at conventional sites. There were thus two differences between Individual#1 and Individual#2

Below is a table showing the polymorphisms detected the position in the mtDNA genome and comparison to the 'reference' sequence. mtDNA reference sequence and SNP database by Ingman et al. 2006.

| Position | Reference seq. | Individual#1 | Individual#2 |
|---|---|---|---|
| **HVII** | | | |
| 64 | T | C | T |
| 152 | T | C | C |
| 263 | A | G | G |
| 310 | T | TC | TC |
| 317 | C | C | CC |
| 315 | C | CC | C |
| 430 | T | A | T |
| **HVI** | | | |
| 16288 | T | T | C |
| 16311 | T | C | T |

Note: the SNP at position 430 is a novel observation, not reported in the published literature.

### HVI sequence Individual 1.

CATTAGCACCCAAAGCTAAGATTCTAATTTAAACTATTCTCTGTTCTTTCATGGGGAAGCAGATTTGGGTACCAC
CCAAGTATTGACTCACCCATCAACAACCGCTATGTATTTCGTACATTACTGCCAGCCACCATGAATATTGTACG
GTACCATAAATACTTGACCACCTGTAGTACATAAAAACCCAATCCACATCAAAACCCCCTCCCCATGCTTACAA
GCAAGTACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGA
TACCAACAAACCTACCCACCCTTAACAGTACATAGCACATAAAGCCATTTACCGTACATAGCACATTACAGTCA
AATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGA

### HVI sequence Individual 2.

ACCATTAGCACCCAAAGCTAAGATTCTAATTTAAACTATTCTCTGTTCTTTCATGGGGAAGCAGATTTGGGTACC
ACCCAAGTATTGACTCACCCATCAACAACCGCTATGTATTTCGTACATTACTGCCAGCCACCATGAATATTGTAC
GGTACCATAAATACTTGACCACCTGTAGTACATAAAAACCCAATCCACATCAAAACCCCCTCCCCATGCTTACA
AGCAAGTACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGG
ATACCAACAAACCCACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTC
AAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAC

**HVII sequence Individual 1.**

CACCCTATTAACCACTCACGGGAGCTCTCCATGCATTTGGTATTTTCGTTTGGGGGGTATGCACGCGATAGCA
TTGCGAGACGCTGGAGCCGGAGCACCCTATGTCGCAGTATCTGTCTTTGATTCCTGCCTCATCCCATTATTTA
TCGCACCTACGTTCAATATTACAGGCGAACATACTTACTAAAGTGTGTTAATTAATTAATGCTTGTAGGACATAAT
AATAACAATTGAATGTCTGCACAGCCGCTTTCCACACAGACATCATAACAAAAAATTTCCACCAAACCCCCCC
CTCCCCCCGCTTCTGGCCACAGCACTTAAACACATCTCTGCCAAACCCCAAAAACAAAGAACCCTAACACC
AGCCTAACCAGATTTCAAATTTTATCTTTTGGCGGTATGCACTTTTAACAGA

**The standard mitochondrial genome: HVII region to be amplified is highlighted in red. HVI region is highlighted in blue.**

GATCACAGGTCTATCACCCTATTAACCACTCACGGGAGCTCTCCATGCATTTGGTATTTTCGTCTGGGGGGTA
TGCACGCGATAGCATTGCGAGACGCTGGAGCCGGAGCACCCTATGTCGCAGTATCTGTCTTTGATTCCTGCC
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TTGTAGGACATAATAATAACAATTGAATGTCTGCACAGCCACTTTCCACACAGACATCATAACAAAAAATTTCCA
CCAAACCCCCCCTCCCCCGCTTCTGGCCACAGCACTTAAACACATCTCTGCCAAACCCCAAAAACAAAGAA
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CTACTCACCAGACGCCTCAACCGCCTTTTCATCAATCGCCCACATCACTCGAGACGTAAATTATGGCTGAATC
ATCCGCTACCTTCACGCCAATGGCGCCTCAATATTCTTTATCTGCCTCTTCCTACACATCGGGCGAGGCCTATA
TTACGGATCATTTCTCTACTCAGAAACCTGAAACATCGGCATTATCCTCCTGCTTGCAACTATAGCAACAGCCT
TCATAGGCTATGTCCTCCCGTGAGGCCAAATATCATTCTGAGGGGCCACAGTAATTACAAACTTACTATCCGCC
ATCCCATACATTGGGACAGACCTAGTTCAATGAATCTGAGGAGGCTACTCAGTAGACAGTCCCACCCTCACAC
GATTCTTTACCTTTCACTTCATCTTGCCCTTCATTATTGCAGCCCTAGCAACACTCCACCTCCTATTCTTGCACG
AAACGGGATCAAACAACCCCCTAGGAATCACCTCCCATTCCGATAAAATCACCTTCCACCCTTACTACACAAT
CAAAGACGCCCTCGGCTTACTTCTCTTCCTTCTCTCCTTAATGACATTAACACTATTCTCACCAGACCTCCTAG
GCGACCCAGACAATTATACCCTAGCCAACCCCTTAAACACCCCTCCCCACATCAAGCCCGAATGATATTTCCT
ATTCGCCTACACAATTCTCCGATCCGTCCCTAACAAACTAGGAGGCGTCCTTGCCCTATTACTATCCATCCTCA
TCCTAGCAATAATCCCCATCCTCCATATATCCAAACAACAAAGCATAATATTTCGCCCACTAAGCCAATCACTTT
ATTGACTCCTAGCCGCAGACCTCCTCATTCTAACCTGAATCGGAGGACAACCAGTAAGCTACCCTTTTACCAT
CATTGGACAAGTAGCATCCGTACTATACTTCACAACAATCCTAATCCTAATACCAACTATCTCCCTAATTGAAAA
CAAAATACTCAAATGGGCCTGTCCTTGTAGTATAAACTAATACACCAGTCTTGTAAACCGGAGATGAAAACCTT
TTTCCAAGGACAAATCAGAGAAAAAGTCTTTAACTCCACCATTAGCACCCAAAGCTAAGATTCTAATTTAAACTA
TTCTCTGTTCTTTCATGGGGAAGCAGATTTGGGTACCACCCAAGTATTGACTCACCCATCAACAACCGCTATG
TATTTCGTACATTACTGCCAGCCACCATGAATATTGTACGGTACCATAAATACTTGACCACCTGTAGTACATAAAA
ACCCAATCCACATCAAAACCCCCTCCCCATGCTTACAAGCAAGTACAGCAATCAACCCTCAACTATCACACAT
CAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTTAACAGTACATAG
TACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAG
ATAGGGGTCCCTTGACCACCATCCTCCGTGAAATCAATATCCCGCACAAGAGTGCTACTCTCCTCGCTCCGG
GCCCATAACACTTGGGGGTAGCTAAAGTGAACTGTATCCGACATCTGGTTCCTACTTCAGGGTCATAAAGCCT
AAATAGCCCACACGTTCCCCTTAAATAAGACATCACGATG

# Appendix 5: Mock drug pH and DNA stability

Basic sugar, acid sugar and mock, unbuffered drug sugar mix were prepared in a Sunbeam AutoGrinder (model EM0415) set on “FINE/ESPRESSO” number 15 . “Sugar” is Woolworths homebrand cane sugar from a freshly opened 3kg pack. “Shavings” is material taken from a male face using a Remington electric shaver two hours after a wet shave.

Basic sugar = 10g sugar + 84mg NaHCO3.

Acid sugar = 10g sugar + 292mg EDTA

Mock, unbuffered drug sugar mix = 10g sugar, 4mg sodium acetate and 10mg shavings.

Choosing a pH range

The effect of sugar concentration on pH was determined by diluting varying amounts of acid or basic sugar in 10ml MilliQ water (figs. 1 & 2).

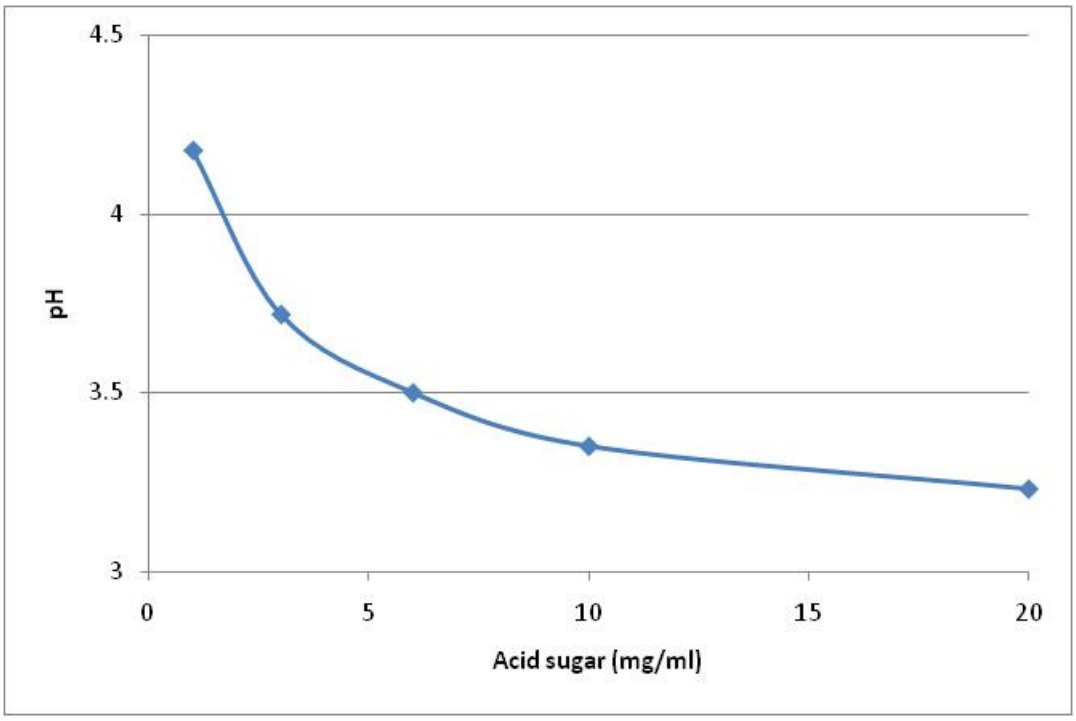



**Figure 1** The effect of acid sugar concentration on pH.

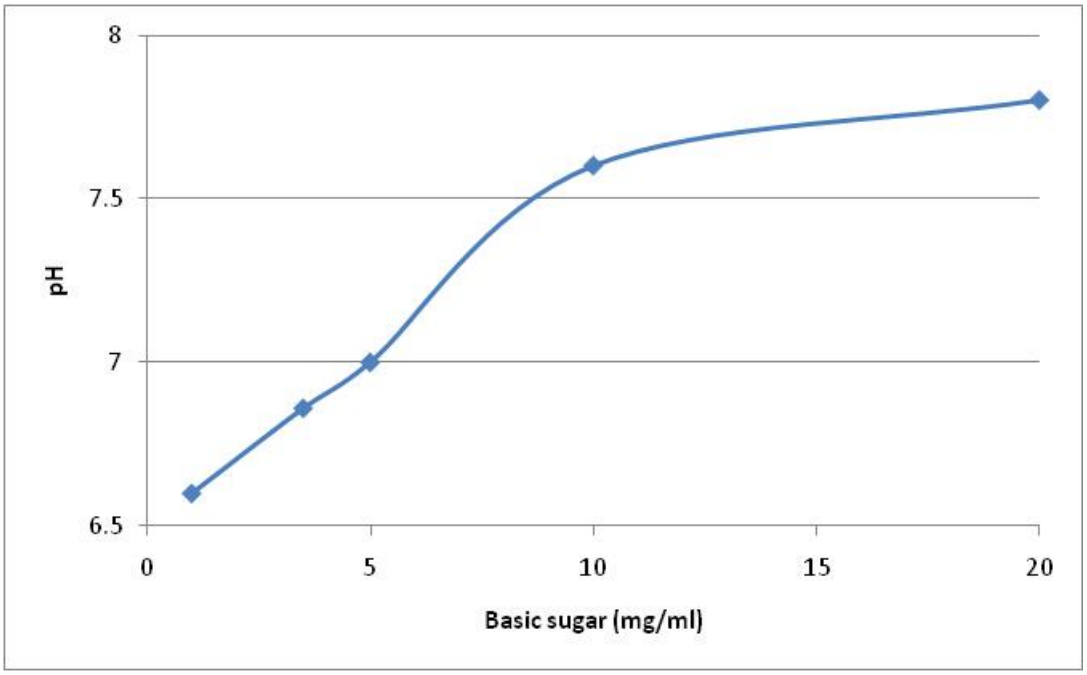



**Figure 2** The effect of basic sugar concentration on pH.

The pH of different ratios of basic to acid sugar was determined. 50mg sugar was dissolved in 10ml MilliQ water (Figure 3).

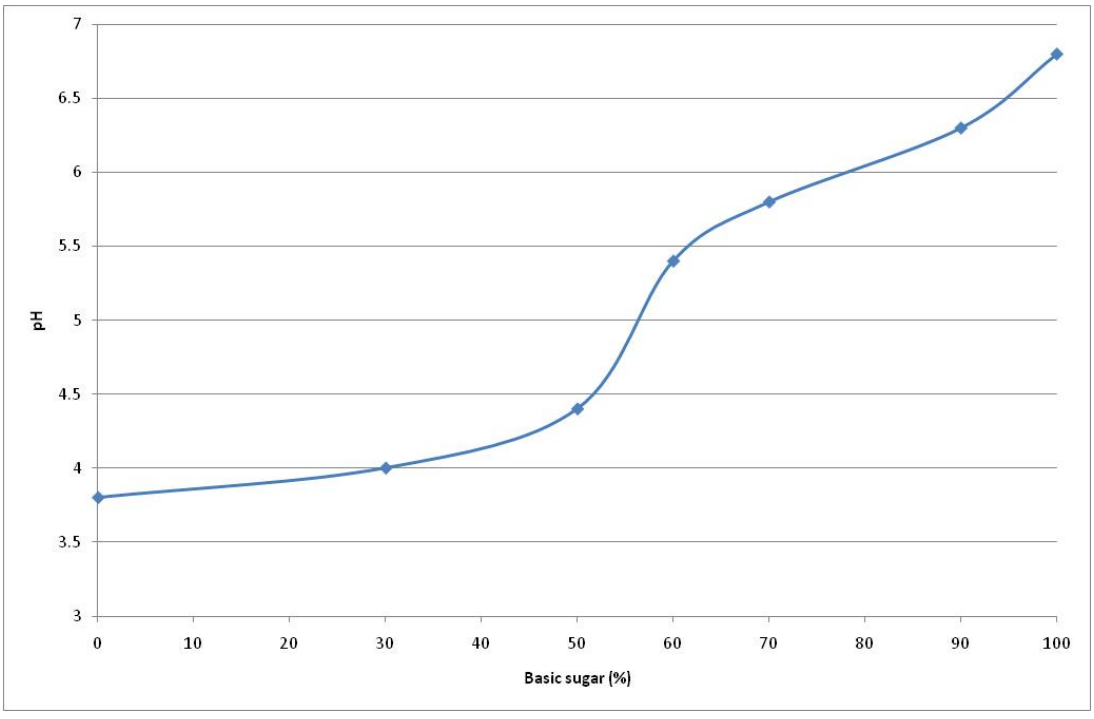



**Figure 3** pH of various basic acid sugar mixtures. Only percentage basic sugar is shown but mixtures were made up to 100% with acid sugar (e.g. 50% basic sugar also includes 50% acid sugar).

**Table 1** The following ratios were chosen. Mixing was done by vigorous shaking and stirring with a UV treated pipette tip.

| Basic sugar (mg) | Acid sugar (mg) | Nominal pH |
|---|---|---|
| 1000 | 0 | 6.9 |
| 900 | 100 | 6.3 |
| 700 | 300 | 5.8 |
| 600 | 400 | 5.5 |
| 500 | 500 | 4.4 |
| 300 | 700 | 4.1 |
| 0 | 1000 | 3.8 |

400mg of each sugar mix was combined with 400mg mock drug in a 50ml Falcon tube and mixed as described for sugar mixes. Tubes were incubated at 25C in a hybridization chamber with continuous rotation. 50mg duplicate samples were removed, processed with a Qiagen Investigator kit and PCR with HVI primers at the following times: 44 hours, 1 week, 3 weeks, 6 weeks.

# Appendix 6: An examination of the Sequence coding of mitochondrial loci amplimers from seizure material.

Primary template from seizures: Graham Eariss; Reference - Graham's Lab Book 6 page 137). Amplified the hypervariable regions in the human mtDNA genome (HVI/HVII)

Sequence coded primers were then used to re-amplify the hypervariable regions in the human mtDNA genome (HVI/HVII) for 454 sequencing. So multiple samples can then be profiled simultaneously.

Using the Stitch Sequence Code Maker Program, 9mer sequence codes were embedded into the 5' end of the primer sequences for HVI (F15971, R16410) and HVII (F15, R429). The term 'embedding' means that the original primer is truncated to allow the bar-code to be added in such a way that all composite primers still have similar and reasonable amplification properties.

The composite primer sequences are listed in Table 1.

(STITCH BARCODEMAKER and primer.bas)

**Table 1** Composite primer sequences for amplification of HVI and HVII

| Primer Name | Composite Primer Sequence |
|---|---|
| AGATCATTC-HVIF(1) | AGATCATTCCCATTAGCACC |
| GAATGATCT-HVIR(1) | GAATGATCTGTGGTCAAGGGAC |
| AGATCATTC-HV2F(1) | AGATCATTCTAACCACTCACG |
| GAATGATCT-HV2R(1) | GAATGATCTGTGCATACCGCCA |
| | |
| AGTAATCTC-HVIF(2) | AGTAATCTCCCATTAGCACC |
| GAGATTACT-HVIR(2) | GAGATTACTTGGTCAAGGGAC |
| AGTAATCTC-HV2F(2) | AGTAATCTCTAACCACTCACG |
| GAGATTACT-HV2R(2) | GAGATTACTTGCATACCGCCA |
| | |
| AGCTTCATA-HVIF(3) | AGCTTCATACCATTAGCACC |
| TATGAAGCT-HVIR(3) | TATGAAGCTTGGTCAAGGGAC |
| AGCTTCATA-HV2F(3) | AGCTTCATATAACCACTCACG |
| TATGAAGCT-HV2R(3) | TATGAAGCTTGCATACCGCCA |
| | |
| AGCACTATT-HVIF(4) | AGCACTATTCCATTAGCACC |
| AATAGTGCT-HVIR(4) | AATAGTGCTTGGTCAAGGGAC |
| AGCACTATT-HV2F(4) | AGCACTATTTAACCACTCACG |
| AATAGTGCT-HV2R(4) | AATAGTGCTTGCATACCGCCA |
| | |
| AGTCTACAT-HVIF(5) | AGTCTACATCCATTAGCACC |
| ATGTAGACT-HVIR(5) | ATGTAGACTTGGTCAAGGGAC |
| AGTCTACAT-HV2F(5) | AGTCTACATTAACCACTCACG |
| ATGTAGACT HV2R(5) | ATGTAGACTTGCATACCGCCA |

**Table 1** (Continued)

| Primer Name | Composite Primer Sequence |
|---|---|
| | |
| AGACTTCTA- HVIF(6) | AGACTTCTACCATTAGCACC |
| TAGAAGTCT-HVIR(6) | TAGAAGTCTTGGTCAAGGGAC |
| AGACTTCTA- HV2F(6) | AGACTTCTATAACCACTCACG |
| TAGAAGTCT-HV2R(6) | TAGAAGTCTTGCATACCGCCA |

- HVI and HVII templates from 5 different batches of drug seizures were used to re-amplify HVI and HVII using a set of complementary sequence codes in the forward and reverse primers. The templates were amplifications using mtHVI and mtHVII primers without sequence codes. A different set of sequence coded primers was used for templates from each batch (Table 2).
- The PCR was repeated with primers containing non-complementary sequence codes in the forward and reverse primers (Table 2)

**Table 2A** Complementary sequence codes in forward and reverse primers used to re-amplify HVI and HVII from 5 drug batches and a positive control . Highlighting marks actual sequence code on truncated (non-highlighted) original primer.

| Drug Batch ID | Composite Primer Sequence HVI | Composite Primer Sequence HVII |
|---|---|---|
| GH3A5 | F- AGTAATCTCCCATTAGCACC<br>R- GAGATTACTTGGTCAAGGGAC | F- AGTAATCTCTAACCACTCACG<br>R- GAGATTACTTGCATACCGCCA |
| GH3 POOL | F- AGCTTCATACCATTAGCACC<br>R- TATGAAGCTTGGTCAAGGGAC | F- AGCTTCATATAACCACTCACG<br>R- TATGAAGCTTGCATACCGCCA |
| GM3A1 | F- AGCACTATTCCATTAGCACC<br>R- AATAGTGCTTGGTCAAGGGAC | F- AGCACTATTTAACCACTCACG<br>R- AATAGTGCTTGCATACCGCCA |
| GM3A2 | F- AGTCTACATCCATTAGCACC<br>R- ATGTAGACTTGGTCAAGGGAC | F- AGTCTACATTAACCACTCACG<br>R- ATGTAGACTTGCATACCGCCA |
| GM3A3 | F- AGACTTCTACCATTAGCACC<br>R- TAGAAGTCTTGGTCAAGGGAC | F- AGACTTCTATAACCACTCACG<br>R- TAGAAGTCTTGCATACCGCCA |
| + CONTROL | F- AGATCATTCCCATTAGCACC<br>R- GAATGATCTGTGGTCAAGGGAC | F- AGATCATTCTAACCACTCACG<br>R- GAATGATCTGTGCATACCGCCA |
| | | |

- The PCR reaction mix contained primers at a final concentration of 0.2µM, dNTPs at 0.2µM, 1.5mM MgCl2 and 1 unit Phusion DNA polymerase.
- PCR conditions were: an initial denaturation at 98°C for 5 min, followed by 35 cycles of (98°C for 20 sec, 58°C for 20 sec, 72°C for 20 sec) and a final extension at 72°C for 7 min.
- 5µl of PCR products were electrophoresed on 1% agarose containing 0.001% ethidium bromide with 100bp DNA ladder at 100 volts for 60 mins

## Results

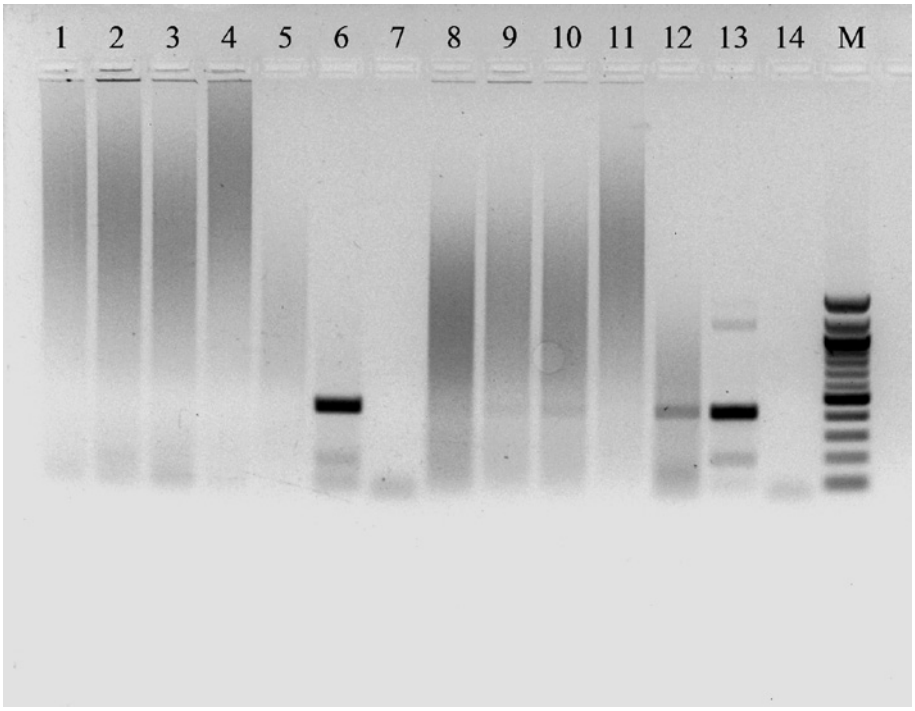



**Figure 1** Agarose gel electrophoresis of PCR products from mtHVI and mtHVII using forward and reverse primers with complementary sequence codes.

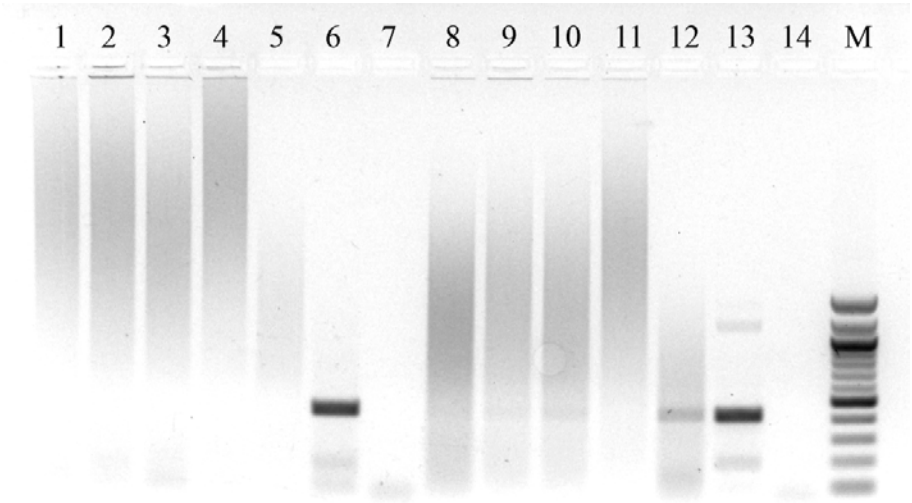



**Figure 2** Agarose gel electrophoresis of PCR products from mtHVI and mtHVII using forward and reverse primers with non-complementary sequence codes.

| Lane | Template | HVI/HVII |
|---|---|---|
| 1 | GH3A5 | HVI |
| 2 | GH3 POOL | HVI |
| 3 | GM3A1 | HVI |
| 4 | GM3A2 | HVI |
| 5 | GM3A3 | HVI |
| 6 | Positive control | HVI |
| 7 | Blank | HVI |
| 8 | GH3A5 | HVII |
| 9 | GH3 POOL | HVII |
| 10 | GM3A1 | HVII |
| 11 | GM3A2 | HVII |
| 12 | GM3A3 | HVII |
| 13 | Positive control | HVII |
| 14 | Blank | HVII |
| M | 100bp DNA ladder | |

## Summary.

- Complementary or non-complementary sequence codes in the forward and reverse primers did not affect the sizes of the PCR products.
- For HVI - the expected mtHVI fragment of approximately 440bp was not observed in all 5 samples. There were smears which indicate the presence of considerably larger sized products (Lanes 1-5, Figures 1 & 2).
- For HVII – a faint band of the expected-sized fragment of 415bp was observed in the GH3 pool, GM3A1 and GM3A3 (Lanes 9, 10, 12, Figures 1 & 2). As in HVI, smears indicating the presence of larger sized products were present.

## Sequencing of PCR products.

The PCR products from GH3A5, GH3 POOL, GM3A1, GM3A2, GM3A3 and the positive control using the set of complementary barcoded primers were pooled for sequencing.

For 454 sequencing, 1ug DNA is required and the PCR amplicons have to be purified. A gel photo of the electrophoresis of 1µl of sample on 2% agarose with 1kb DNA ladder is required by the sequencing facility.

## Procedure

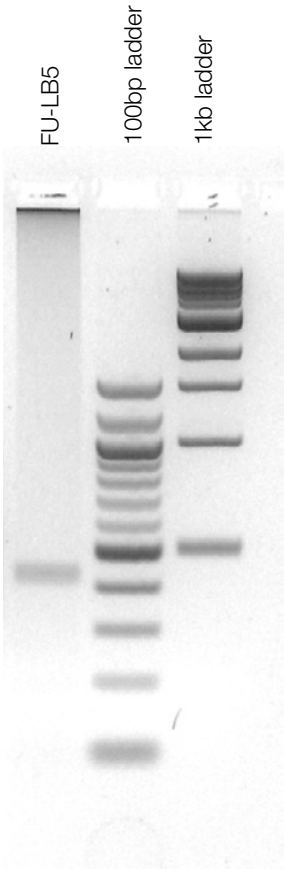
- Sample FU-LB5 was prepared by pooling 20µl of the separately and distinctively bar coded HVI/HVII products from each of 11 separately labeled batches of drug seizures and a positive control
- The sample was purified using AdBiotec PCR DNA purification columns (Catalogue number PCR-1) and the DNA eluted in 50µl TE.
- DNA concentrations was measured using a NanoDrop Spectrophotometer.
- 1µl of each sample was electrophoresed on 2% agarose containing 0.001% ethidium bromide with 100bp and 1kb DNA ladders at 100 volts for 90 min.

## Results

(1) DNA concentrations and spectrophotometric data for FU-LB5

| Sample | $A_{260}/A_{280}$ | [DNA] ng/ul | Volume DNA provided (µl) | Total DNA provided (µg) |
|---|---|---|---|---|
| FU-LB5 | 1.91 | 171 | 30 | 5.1 |

(2) Agarose gel electrophoresis of FU-LB5




**Ethidium bromide stained gel of 1ul samples of FU-LB3, FU-LB4 and FU-LB5 with 100bp DNA ladder and 1kb DNA ladder on 2% agarose**

## Summary

FU-LB5 – the pool of HVI/HVII amps from 11 batches of drug seizures and a positive control, were placed in sterile screw capped tubes, sealed with parafilm and handed to Mike Gardner on 06-01-2011 to be sent off for 454 sequencing.

# Appendix 7: Summary of the data obtained from 454 sequencing of mitochondrial HV1 and HV2 loci obtained from seizure and control DNA.

**Table 1** Sequence reads obtained

| SAMPLE NAME | Sequence reads obtained | Average read length | Maximum read length |
|---|---|---|---|
| GH3A5HV1 | 5430 | 129 | 526 |
| GH3A5HV2 | 12155 | 138 | 523 |
| GH3POOLHV1 | 5576 | 137 | 528 |
| GH3POOLHV2 | 5432 | 156 | 521 |
| GM3A1HV1 | 7757 | 135 | 537 |
| GM3A1HV2 | 12701 | 149 | 537 |
| GM3A2HV1 | 4263 | 125 | 527 |
| GM3A3HV1 | 1551 | 141 | 521 |
| GM3A3HV2 | 3795 | 201 | 523 |
| CONTROLHV1 | 56150 | 395 | 556 |
| CONTROLHV2 | 41860 | 330 | 520 |
| GM4A1HV1 | 2809 | 113 | 490 |
| GM4A1HV2 | 7431 | 121 | 521 |
| GM4A2HV1 | 1459 | 107 | 410 |
| GM4A2HV2 | 2758 | 101 | 492 |
| GM4A3HV1 | 2048 | 112 | 446 |
| GM4A3HV2 | 5969 | 110 | 516 |
| GM5A1HV1 | 2022 | 104 | 446 |
| GM5A1HV2 | 7376 | 104 | 500 |
| GM5A2HV1 | 1181 | 106 | 376 |
| GM5A2HV2 | 5491 | 108 | 469 |
| GM5A3HV1 | 1692 | 111 | 439 |
| GM5A3HV2 | 7265 | 106 | 490 |

Unprocessed data. A page of the sequence data obtained in this trial. The whole data set contains 291,791 reads. Long reads are truncated here due to the limited page width. Sequences from each sample are distinguished one from another by a unique sequence code added by incorporating them into primers.

A page of the 12,702 sequences from sample GM3A1 amplicons for HVII extracted from the whole data set using its unique sequence code.

# Appendix 8: Deducing sequences from reads and comparing them to data-bases.

Alignment settings: alignment stringency was set so that there had to be at least 300 base pairs overlap in any acceptable alignment and within that 300 base pairs at least 95% homology. The alignments were examined visually for the validity of the consensus. (Table 2.1 shows a fragment of an alignment-table. )

Example 1- HVI sequence deduced from the control subject, based on alignment of 56150 reads (Figure 2.1). For example, for the yellow highlighted sequence based on a subset of these reads, the individual base calls for the first base are 4A; 20,202T; 0G; 9C (Table 2.1). The massive predominance of over 20202 reads indicates clearly that "T" is the true base at this particular position. A similar low frequency of miscalls, typical for this technology, occur for each of the other positions in this randomly chosen sequence (Also table 2.1). However, the true sequence is not in doubt. The red highlight includes sequences of the bar code and PCR overrun beyond the second primer, which is usually detected when large numbers of reads are available. The best match for the deduced HVI sequence in the NCBI data-base (Figure 2.2) is a perfect match with the exception of an example of the likely unreliability of length polymorphisms in runs of G (discussed).

**Figure 2.1 Deduced sequence.**

GGTAACAGCTGTCCTAGACTCACCTAGGCCAGGATAATAATCTGAAGAAAAGGG
ACAGTGGTGGTGGTAACGTTCCGTCTCGGAATGATCTGTGGTCAAGGGACCCCTA
TCTGAGGGGGGTCATCCATGGGGACGAGAAGGGATTTGACTGTAATGTGCTATG
TACGGTAAATGGCTTTATGTGCTATGTACTGTTAAGGGTGGGTAGGTTTGTTGGT
ATCCTAGTGGGTGAGGGGTGGCTTTGGAGTTGCAGTTGATGTGTGATAGTTGAGG
GTTGATTGCTGTACTTGCTTGTAAGCATGGGGAGGGGTTTTGATGTGGATTGGGT
TTTTATGTACTACAGGTGGTCAAGTATTTATGGTACCGTACAATATTCATGGTGG
CTGGCAGTAATGTACGAAATACATAGCGGTTGTTGATGGGTGAGTCAATACTTGG
GTGGTACCCAAATCTGCTTCCCCATGAAAGAACAGAGAATAGTTTAAATTAGAA
TCTTAGCTTTGGGTGCTAATGGGAATGATCTAGAGACGGAGTGGTCTGGTTGTGG
TGGTAAGAGGGAGGTAGAGGGAGTGGATAAGTGGACCGTGAGTGGTTAATAGG
GTG

**Table 2.1 sequencing data for the yellow highlighted sequence in Figure 2.1**

| A | T | G | C | CONSENSUS BASE |
|---|---|---|---|---|
| 4 | 20202 | 0 | 9 | T |
| 20204 | 7 | 5 | 0 | A |
| 4 | 20211 | 5 | 0 | T |
| 3 | 9 | 20208 | 0 | G |
| 2 | 20208 | 6 | 4 | T |
| 20208 | 7 | 3 | 2 | A |
| 7 | 4 | 1 | 20209 | C |
| 0 | 20215 | 0 | 9 | T |
| 5 | 8 | 20212 | 0 | G |
| 1 | 20218 | 7 | 0 | T |
| 3 | 20223 | 1 | 0 | T |
| 20218 | 7 | 2 | 0 | A |

**Figure 2.2 Best match to the NCBI data-base of the deduced HVI sequence obtained from the control subject.**

```
>gb|HM852892.1|  Homo sapiens isolate Georgian38 mitochondrion, complete genome
Length=16568

 Score =  776 bits (420),  Expect = 0.0
 Identities = 423/424 (99%), Gaps = 1/424 (0%)
 Strand=Plus/Minus

Query  92     GTGGTCAAGGGACCCCTATCTGAGGGGGGTCATCCATGGGGACGAGAAGGGATTTGACTG  151
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16402  GTGGTCAAGGGACCCCTATCTGAGGGGGGTCATCCATGGGGACGAGAAGGGATTTGACTG  16343

Query  152    TAATGTGCTATGTACGGTAAATGGCTTTATGTGCTATGTACTGTTAAGGGTGGGTAGGTT  211
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16342  TAATGTGCTATGTACGGTAAATGGCTTTATGTGCTATGTACTGTTAAGGGTGGGTAGGTT  16283

Query  212    TGTTGGTATCCTAGTGGGTGAGGGGTGGCTTTGGAGTTGCAGTTGATGTGTGATAGTTGA  271
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16282  TGTTGGTATCCTAGTGGGTGAGGGGTGGCTTTGGAGTTGCAGTTGATGTGTGATAGTTGA  16223

Query  272    GGGTTGATTGCTGTACTTGCTTGTAAGCATGGGGA-GGGGTTTTGATGTGGATTGGGTTT  330
              ||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  16222  GGGTTGATTGCTGTACTTGCTTGTAAGCATGGGGAGGGGGTTTTGATGTGGATTGGGTTT  16163

Query  331    TTATGTACTACAGGTGGTCAAGTATTTATGGTACCGTACAATATTCATGGTGGCTGGCAG  390
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16162  TTATGTACTACAGGTGGTCAAGTATTTATGGTACCGTACAATATTCATGGTGGCTGGCAG  16103

Query  391    TAATGTACGAAATACATAGCGGTTGTTGATGGGTGAGTCAATACTTGGGTGGTACCCAAA  450
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16102  TAATGTACGAAATACATAGCGGTTGTTGATGGGTGAGTCAATACTTGGGTGGTACCCAAA  16043

Query  451    TCTGCTTCCCCATGAAAGAACAGAGAATAGTTTAAATTAGAATCTTAGCTTTGGGTGCTA  510
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16042  TCTGCTTCCCCATGAAAGAACAGAGAATAGTTTAAATTAGAATCTTAGCTTTGGGTGCTA  15983

Query  511    ATGG  514
              ||||
Sbjct  15982  ATGG  15979
```

Example 2. HVII sequence deduced from seizure sample GH3A5HV2, based on alignment of 21 reads (Figure 2.3). This subset was selected as more or less typical of the data from a seizure batch that had a low but usable number of useful reads.

**Figure 2.3: The full length of the deduced sequence.**

AGTAATTTGTAAACAATAACGCACTAAATTAACCACTAACTCTATACCCTAACCA
AGAACGCTTTAAGCACTCACAGTAATCTCTAACCACTCACGGGAGCTCTCCATGC
ATTTGGTATTTTCGTCTGGGGGGTATGCACGCGATAGCATTGCGAGACGCTGGAG
CCGGAGCACCCTATGTCGCAGTATCTGTCTTTGATTCCTGCCTCATCCCATTATTT
ATCGCACCTACGTTCAATATTACAGGCGAACATACTTACTAAAGTGTGTTAATTA
ATTAATGCTTGTAGGACATAATAATAACAATTGAATGTCTGCACAGCCGCTTTCC
ACACAGACATCATAACAAAAAATTTCCACCAAACCCCCCCCCCCCCGCTTCTGGC
CACAGCACTTAAACACATCTCTGCCAAACCCCAAAAACAAAGAACCCTAACACC
AGCCTAACCAGATTTCAAATTTTATCTTTTGGCGGTATGCAAGTAATCTCCAGCG
GTATGCACTTTTAACATGGCGGTATGCACTTTTAACAGTGGCGGTATGCACTTTT
AACATGGCGGTATGCAAGTAATCTC

Figure 2.4 Best match to the NCBI data-base of the deduced HVII sequence from seizure sample GH3A5HV2.

```
>gb|HM852878.1|  Homo sapiens isolate Georgian10 mitochondrion, complete genome
Length=16568

 Score =  734 bits (397),  Expect = 0.0
 Identities = 397/397 (100%), Gaps = 0/397 (0%)
 Strand=Plus/Plus

Query  85   TAACCACTCACGGGAGCTCTCCATGCATTTGGTATTTTCGTCTGGGGGGTATGCACGCGA  144
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  23   TAACCACTCACGGGAGCTCTCCATGCATTTGGTATTTTCGTCTGGGGGGTATGCACGCGA  82

Query  145  TAGCATTGCGAGACGCTGGAGCCGGAGCACCCTATGTCGCAGTATCTGTCTTTGATTCCT  204
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  83   TAGCATTGCGAGACGCTGGAGCCGGAGCACCCTATGTCGCAGTATCTGTCTTTGATTCCT  142

Query  205  GCCTCATCCCATTATTTATCGCACCTACGTTCAATATTACAGGCGAACATACTTACTAAA  264
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  143  GCCTCATCCCATTATTTATCGCACCTACGTTCAATATTACAGGCGAACATACTTACTAAA  202

Query  265  GTGTGTTAATTAATTAATGCTTGTAGGACATAATAATAACAATTGAATGTCTGCACAGCC  324
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  203  GTGTGTTAATTAATTAATGCTTGTAGGACATAATAATAACAATTGAATGTCTGCACAGCC  262

Query  325  GCTTTCCACACAGACATCATAACAAAAAATTTCCACCAAACCCCCCCCCCCCCGCTTCTG  384
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  263  GCTTTCCACACAGACATCATAACAAAAAATTTCCACCAAACCCCCCCCCCCCCGCTTCTG  322

Query  385  GCCACAGCACTTAAACACATCTCTGCCAAACCCCAAAAACAAAGAACCCTAACACCAGCC  444
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  323  GCCACAGCACTTAAACACATCTCTGCCAAACCCCAAAAACAAAGAACCCTAACACCAGCC  382

Query  445  TAACCAGATTTCAAATTTTATCTTTTGGCGGTATGCA  481
            |||||||||||||||||||||||||||||||||||||
Sbjct  383  TAACCAGATTTCAAATTTTATCTTTTGGCGGTATGCA  419
```

Table 2.2: sequencing data for the yellow highlighted sequence in Figure 2.3.

| A | T | G | C | CONSENSUS BASE |
|---|---|---|---|---|
| 0 | 0 | 0 | 21 | C |
| 0 | 0 | 21 | 0 | G |
| 21 | 0 | 0 | 0 | A |
| 0 | 0 | 21 | 0 | G |
| 21 | 0 | 0 | 0 | A |
| 0 | 0 | 0 | 21 | C |
| 0 | 0 | 21 | 0 | G |
| 0 | 0 | 0 | 21 | C |
| 0 | 21 | 0 | 0 | T |

# Appendix 9. The haplotypes observed in seizures. (*Omitting those SNPs that are a part of homopolymer runs*.)

| Seizure | | SNP position | | SNP position | | |
|---|---|---|---|---|---|---|
| | | 16311 | | 64 | 152 | 263 |
| GH3POOL | HVI | T - C ; T | HVII | T-C | T-C | A - G |
| GH3A5 | HVI | T - C | HVII | T-C | T-C | A - G |
| GM3A1 | HVI | T - C; T | HVII | T | T-C | A - G |
| GM4A3 | HVI | T - C | HVII | T-C | T-C | A |
| GM5A2 | HVI No Matches | HVII | T-C | T-C | A | |
| GM4A2 | HVI No Matches | HVII | T | T-C | A | |
| GM5A1 | HVI No Matches | HVII | T-C | T-C | A | |
| GM5A3 | HVI No Matches | HVII | T-C | T-C | A | |
| GM4A1 | HVI No Matches | HVII | T-C | T-C | A | |
| GM3A3 | HVI No matches | HVII | T-C | T-C | A - G | |
| | | | T-C | T-C | A | |
| GM3A2 | HVI | T - C ; T | | | | |

GH3POOL is from seizure P5311965 (Item 2)

GH3A5 is from seizure P53111965 (Item1)

GM3, GM4 and GM5 are different samples from seizure P53061764 (Sample B)

| Seizure | Minimum # of individuals present | Reasoning |
|---|---|---|
| P53111965 (Item 2) | 2 | Two different HVI alleles. |
| P53111965 (Item1) | 1 | Single HVI allele and single HVII haplotype. |
| P53061764 (Sample B) | 4 | Four different HVII haplotypes. |