**Final Report**

Further Development and Validation of DNA-Based Methods for Species of Origin Determination and Human DNA Quantitation in Forensic Casework Specimens

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## Part I. Comparison of DNA Quantitation Techniques for Forensic Specimens

Current techniques used for forensic DNA profiling are STR amplification assays such as AmpFℓSTR® Profiler Plus™ and COfiler® kits (ABI). These kits are representative of those used within the field of forensic DNA analysis to give a DNA profile at the 13 CODIS loci (1). These kits are designed to perform optimally within a narrow range of input (template) nuclear DNA – the recommended range is 1.0-1.5 ng DNA for amplification. Too little input DNA may case allele dropout or peak imbalance. Too much DNA can lead to stutter or other artifact peaks and may interfere with the identification of a correct profile (2). These problems can cause specimens to be re-amplified and analyzed, wasting not only the DNA, but also laboratory time and money. Because the optimum template for these STR kits is narrow, DNA quantitation is a critical step in forensic DNA analysis.

Most forensic laboratories quantitate nuclear DNA by a preliminary yield gel, followed by the QuantiBlot (ABI) slot blot hybridization assay. The yield gel is a simple agarose gel containing ethidium bromide. Aliquots of specimen are loaded into the gel and electrophoresed alongside standards of known quantity (typically 5 to 125 ng). Under UV illumination, the finished gel provides a rough estimation of total DNA, as well as an idea of how much high molecular weight DNA is present in the specimen. This estimate is useful as a guideline for estimating the quantity of input specimen for QuantiBlot. The assessment of degradation (HMW DNA content) from the gel was more important in the era preceding PCR, when HMW template was required for successful typing. The quantity of human DNA in a specimen is estimated using QuantiBlot, a slot/dot blot hybridization assay. Standard DNA (K562 HMW) in quantities ranging from 0.075 to 10 ng are also blotted onto a nylon membrane, that is next hybridized to a 50 nt probe for the D17Z1 locus. D17Z1 is an alphoid satellite repeat region on chromosome 17, and there are some 500,000 copies per diploid genome. Visualization is either colorimetric or chemiluminescent. The quantity of human DNA is estimated by judging the intensity of the standards compared with that of the specimens. This evaluation is subjective, and there may be variation among analysts or laboratories (3,4). The analysis can also be done using a CCDBIO (MiraiBio) imaging system. Here, a cooled CCD camera "reads" the chemiluminescence from the membrane, and is attached to a computer which can extrapolate the intensities of the standards onto a standard curve. The standard curve is then used to calculate the DNA quantity in specimens from chemiluminescence intensities. This method is more objective than the analysis based on visual evaluation alone (5). QuantiBlot is a time-consuming and tedious procedure. A few alternative quantitation techniques have been developed and are being validated or used in forensic laboratories. One of these was developed and is in current use by the Bode Technology Group (2). This technique – BodeQuant - uses a combination of amplification of DNA by PCR followed by incubation of the amplicon with a dsDNA-binding dye (resulting in fluorescence) to estimate human/primate nuclear DNA quantity. Another technique, using real-time PCR (RT-PCR), is rapidly expanding into widespread use in forensic laboratories.

In this project, we proposed to optimize and validate BodeQuant, for potential incorporation into casework in the ISP laboratory system. Specimens were run to compare quantitation results between the QuantiBlot and BodeQuant assays.

Typical of the speed with which DNA procedures may change, RT-PCR came on the scene as the most likely replacement for QuantiBlot after our proposal had been accepted. Thus, although we were successful, it is not likely that BodeQuant will be widely used once commercial and peer laboratory pressure succeed in convincing most laboratories to implement RT-PCR.

Methods and Materials:

For preliminary validation of the BodeQuant assay for use, the procedure released by the Bode Technology Group (6) was followed, using K562 HMW DNA to obtain a usable standard curve. K562 is a human female cell line DNA available commercially. The reagents used in the assay were 10X GoldST*R buffer (Promega), AmpliTaq Gold polymerase (ABI, 5U/μL), TH01 Primer set (Qiagen, diluted to 10μM), K562 HMW DNA (Promega), and PicoGreen® dye (Molecular Probes). The Bode Technology Group used a "10X" TH01 primer set from Promega for their assay. Promega refused to reveal the absolute concentration of this "10X" primer set (typical of the lack of cooperation and transparency of the biotechnology companies that sell most of the products used in forensic laboratories). Accordingly, we were forced to determine the proper primer set concentration by running a range of trial-and-error PCR protocols: 40, 20, 10, and 1 ng of K562 HMW DNA, and 10μM, 1μM, and 0.1μM primer sets. A positive PCR control, consisting of 1kb amplified segment of λ DNA, was run with the K562 samples. Each sample was run with 0.75μL of primer set (at varying concentrations), 2.5 μL Gold ST*R Buffer, 0.15 μL of TaqGold polymerase, and 20.6 μL of SNDW. To the PCR reaction mix, 1 μL of sample was added to give a 25 μL final sample volume. For the positive λ control, 5 μL of DNA was added, and the SNDW was reduced to 16.6 μL. The samples were then amplified on a PE 2400 thermal cycler using the following parameters: 95°C x 11 minutes, 96°C x 2 minutes; 94°C x 1 minute, 64°C x 1 minute, 70°C x 1.5 minutes (10 cycles); 90°C x 1 minute, 64°C x 1 minute, 70°C x 1.5 minutes; 4°C hold. The PCR products (7 μL of each) were then run on a 4% agarose gel containing EtBr for approximately 1 hour at 110V. From this experiment, it was determined that at least a 10μM concentration of primer set was needed to be equivalent to the Promega "10X" primer set.

The BodeQuant assay procedure is as follows (6): For the standard curve, eleven dilutions of known DNA ranging from 0.1 ng/μL to 40 ng/μL (0.1, 0.2, 0.4, 0.7, 1.0, 2.0, 4.0, 7.0. 10.0, 20.0, and 40.0) are made, along with one blank ($TE^{-4}$). One μL of each standard curve sample is added to the 24 μl PCR reaction mix (2.5 μL of GoldST*R buffer, 0.15 μL of AmpliTaq Gold polymerase, 0.75 μL of the TH01 primer set (10 μM), and 20.6 μL of autoclaved ddi $H_2O$). The total reaction volume of 25μL is brought up in a 0.2 mL thin-walled PCR tube. These are run on a thermal cycler with the following parameters: 95°C x 11 minutes; 96°C x 1 minute; 10 cycles: 94°C x 30 seconds, 60°C x 30 seconds (33% ramp), 70°C x 45 seconds (13% ramp); 20 cycles: 90°C x 30 seconds, 60°C x 30 seconds (33% ramp), 70°C x 45 seconds (13% ramp); 60°C x 30 minutes; 4°C hold. When the amplification is complete, samples can be frozen at –20°C or used immediately for the microplate portion of the assay.

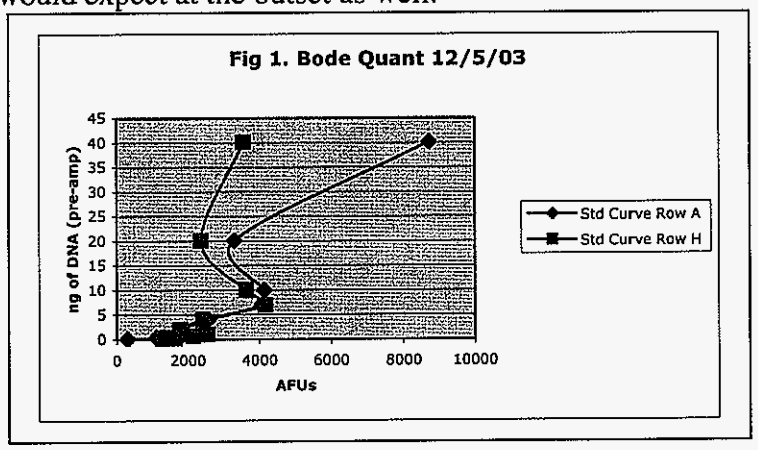
Two μL of each sample are added to 98 μL of $TE^{-4}$ in the wells of a 96-well microplate. For this procedure, we used ABI 96-well Cytoplates. The PicoGreen® dye is diluted to 1:1000 in $TE^{-4}$, and then 100 μL of this diluted dye is added to each well. The plate is incubated at room temperature for approximately 5 minutes, and then placed on the CytoFluor 4000 plate reader for scanning.

The fluorometer is set at a gain of 80, with an excitation filter at 485 nm and emission filter at 530 nm. Absolute fluorescence units (AFUs) are collected by the attached computer into the CytoFluor software, and can then be exported into an Excel spreadsheet. The standard curve AFUs are then plotted vs. starting input of genomic DNA, and a 3rd-order polynomial trend line is created from the graphs for both 0-40 ng and 0-20 ng ranges. AFU output values for any samples of unknown concentration are then inserted into the appropriate polynomial equation to determine the concentration of DNA for the sample. The Bode Technology Group provides a spreadsheet, which can automatically establish the trendline polynomial equation and output sample values.

Results and Discussion

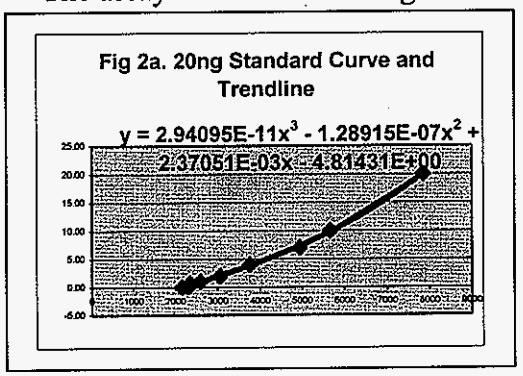
Assay Optimization:

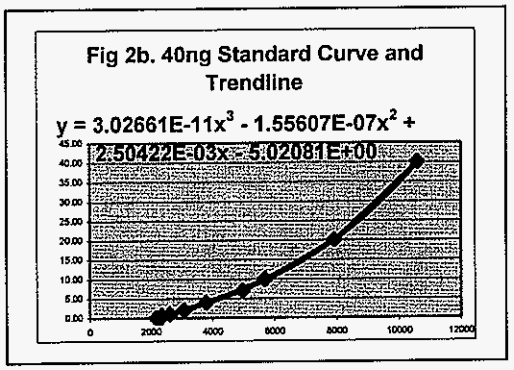
The assay was first run with standards to try and optimize the curve. This process turned out to be more complicated than anticipated, partly because we lacked some key information and the manufacturer would not provide it. However, optimizing the assay is more complicated than one would expect at the outset as well.



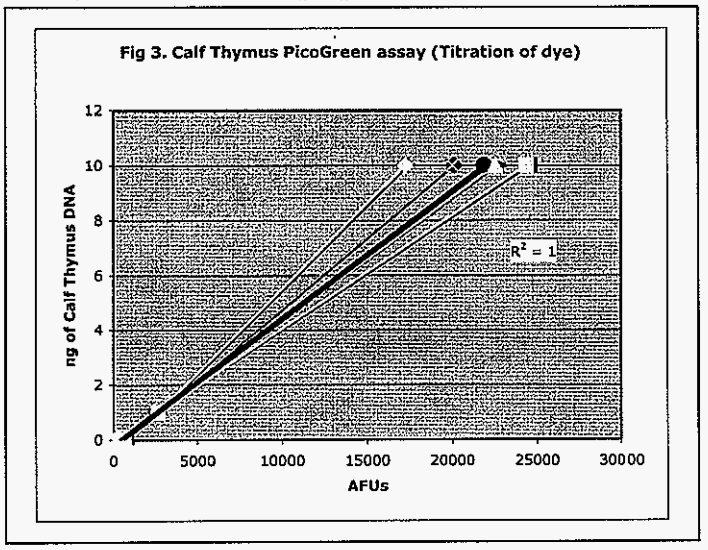

The first assays attempted produced bizarre plots (Fig. 1). Manipulation of the thermocycler used, the plate assay, reagents, and especially primer concentration, were necessary to reach an acceptable, usable plot. An example of an acceptable standard plot, taken from Bode's web site representations is shown in Figure 2. The assay has two ranges, and the 0-20 ng range is separate plotted from the 0-40 ng range.

The assay was first run using the Perkin Elmer 2400 thermal cycler, the instrument available for research at the time of the project's inception. The thermal cycler was programmed according to the BodeQuant parameters, without ramp adjustments. It was suspected that this thermal cycler was not heating evenly. When a PE 9700 thermal cycler was obtained by the lab a month later, comparison runs between the two instruments were run. Eventually the assay was completely switched over to the 9700 thermal cycler. Because the PCR cycles recommended for the BodeQuant were shorter than typical cycles of amplification, an increase in cycle lengths was tried. None of these thermal cycler modifications resulted in a usable standard curve. Modifications to the CytoFluor 4000 were attempted. The number of reads/well was increased (output AFU value is an average value of reads/well), and the gain was increased, but the output curves remained inconsistent



and unusable. To ensure that the fluorometer and dye were working correctly, an assay with calf thymus DNA (Sigma) was run following a protocol from Molecular Probes (7). Dilutions of the calf thymus DNA were prepared at concentrations from 0.001 ng/μl to 10 ng/μl. Two μL of each of the samples were added to 98 μL of $TE^{-4}$ in a 96-well Cytoplate. The PicoGreen® dye was diluted 1:200 and 100 μL added to each sample. The plate was then scanned at the same parameters as for the BodeQuant. This results from this assay showed no detectable problems with the fluorometer or dye concentration. As can readily be seen in Figure 3, neither the CytoFluor nor the dye were part of the problem. Longer incubations and dye titrations were tested, along with titration of the quantity of amplicon from 2 μL (recommended amount) up to 8 μL. None of these variations resulted in an acceptable standard curve. Freezing and re-thawing of the standard DNA was eliminated as a possible source of the problem. Fresh G152 female genomic DNA (Promega) was substituted for the K562 HMW DNA in case the original DNA had been contaminated. The amount of AmpliTaq Gold was increased, and similarly, a fresh lot of the polymerase was used for one assay to determine if the original lot had become degraded. But none of these assays resulted in an acceptable standard curve. The standard curve was finally optimized by titration of the amount of the TH01 primer set used in the PCR. All this manipulation could have been avoided had Promega simply been willing to tell us the concentration of the primers in the kit we were using. The assay with 1.0μM of the primer set resulted in a usable standard curve. A modified version of the instructions for the Bode Quant procedure, using the results from the troubleshooting experiments, is included as Appendix 1.




Validation:

Once the standard curve was optimized, preliminary validation of the assay was initiated. For validation assays, each 1 μL of sample was run with 2.5 μL of 10X GoldST*R buffer, 2.5 μL of 10 μM TH01 primer set, 0.15 μL of AmpliTaq Gold polymerase, and 18.85 μL of autoclaved ddi $H_2O$. All other PCR parameters of the assay remained the same as the original BodeQuant instructions. Samples were mixed in the plate using multiple aspirations in the multi-channel pipette once the diluted dye had been added. The plate was incubated at room temperature in darkness for approximately five minutes before scanning on the plate reader. The fluorometer settings remained as described in the BodeQuant protocol, with 25 reads/well for each scan.

Specimens used for validation – side by side comparison with QuantiBlot -- were extracted by the standard phenol-chloroform-isoamyl alcohol isolation method. Samples 9A-12A and Manipulation Blank (MB-A) were extracted on 12Dec2003. These are buccal swabs taken from the research assistant. Specimens 1B-11B and MB-B were extracted on 08Jan2004 by the research assistant. Specimens 1B-7B were from cuttings of frozen bloodstain cards from various analysts at ISP-FSCC. Specimens 8B-11B were buccals from the research assistant. Specimens A-L were training specimens extracted by an analyst in the lab.

Concentrations of each of the specimen were first estimated by running a preliminary yield (mini) gel and then employing QuantiBlot with chemiluminescence detection on x-ray film. To determine the variability in the subjective band intensity judgments required by QuantiBlot, quantities were separately determined by the research assistant, and by a DNA analyst-supervisor at the lab (referred to as "Analyst 1" and "Analyst 2"). Results are shown in Table 1.

TABLE 1. QuantiBlot vs BQ (Numbers are DNA quantities in ng/μL)

| | | | *BQ Run 1: 22Mar04* | | | | | *BQ Run 2: 29Mar04* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Specimen | Quantiblot Analyst 1 | Quantiblot Analyst 2 | Well 1, Scan 1 | Well 2, Scan 1 | Well 1, Scan 2 | Well 2, Scan 2 | **BQ 1 Average** | Well 1, Scan 1 | Well 2, Scan 1 | Well 1, Scan 2 | Well 2, Scan 2 | **BQ 2 Average** |
| **9A** | 10.00 | 10.00 | 13.07 | 11.75 | 13.10 | 11.57 | 12.37 | 11.32 | 12.64 | 11.22 | 12.62 | 11.95 |
| **10A** | 10.00 | 8.00 | 9.76 | 8.83 | 9.73 | 8.78 | 9.28 | 8.57 | no data | 8.69 | no data | 8.63 |
| **11A** | 8.00 | 10.00 | 9.10 | 7.94 | 9.02 | 7.82 | 8.47 | 10.21 | 9.97 | 10.15 | 10.02 | 10.09 |
| **12A** | 2.50 | 3.00 | 3.67 | 3.67 | 3.78 | 3.71 | 3.71 | 3.16 | 3.19 | 3.17 | 3.18 | 3.18 |
| **MB-A** | none | none | 0.34 | <0.1 | 0.33 | <0.1 | N/A | 0.12 | <0.1 | 0.18 | <0.1 | N/A |
| **1B** | 0.13 | 0.13 | 0.61 | <0.1 | 0.59 | <0.1 | --- | 0.11 | 0.12 | 0.16 | 0.19 | 0.15 |
| **2B** | 5.00 | 5.00 | 15.98 | 13.71 | 16.05 | 13.80 | 14.89 | 5.61 | 5.73 | 5.78 | 5.85 | 5.74 |
| **3B** | 1.25 | 1.25 | 2.02 | 1.14 | 2.00 | 1.13 | 1.57 | 1.76 | 1.44 | 1.89 | 1.47 | 1.64 |
| **4B** | 16.00 | 12.00 | 20.78 | 23.98 | 20.83 | 24.33 | 22.48 | 10.59 | 11.99 | 10.76 | 12.09 | 11.36 |
| **5B** | 5.00 | 5.00 | 7.21 | 7.26 | 7.13 | 7.20 | 7.20 | 6.67 | 6.95 | 6.71 | 7.11 | 6.86 |
| **6B** | 5.00 | 5.00 | 13.06 | 12.51 | 13.14 | 12.67 | 12.85 | 5.44 | 6.06 | 5.58 | 6.28 | 5.84 |
| **7B** | 12.00 | 12.00 | 16.64 | 13.76 | 16.81 | 13.89 | 15.28 | 15.03 | 14.64 | 14.92 | 14.73 | 14.83 |
| **8B** | 12.00 | 10.00 | 11.55 | 9.94 | 11.56 | 9.89 | 10.74 | 9.30 | 8.79 | 9.37 | 8.90 | 9.09 |
| **9B** | 7.00 | 8.00 | 10.78 | 9.29 | 10.86 | 9.20 | 10.03 | 8.57 | 8.92 | 8.77 | 9.06 | 8.83 |
| **10B** | 16.00 | 14.00 | 9.77 | 11.42 | 9.80 | 11.40 | 10.60 | 11.37 | 11.04 | 11.38 | 11.19 | 11.25 |
| **11B** | 11.42 | 9.14 | 11.98 | 10.31 | 12.01 | 10.37 | 11.17 | 10.10 | 10.79 | 10.07 | 10.85 | 10.45 |
| **MB-B** | none | none | 0.44 | 0.24 | 0.46 | <0.1 | N/A | <0.1 | <0.1 | <0.1 | <0.1 | N/A |

Specimens A-L show the QuantiBlot results are those of the analyst in training obtained at the time of original extraction (Table 2).

Table 2. QuantiBlot vs BQ for Specimens A-L (Numbers are DNA quantities in ng/μL)

| | | *BQ Run 2: 29Mar04* | | |
|---|---|---|---|---|
| Specimen | Quantiblot | Well 1, Scan 1 | Well 1, Scan 2 | **BQ 2 Average** |
| **A** | 0.40 | 0.51 | 0.56 | 0.54 |
| **B** | 0.75 | 0.84 | 0.89 | 0.87 |
| **C** | 0.75 | 0.81 | 0.88 | 0.85 |
| **D** | 0.38 | 0.80 | 0.84 | 0.82 |
| **E** | 0.90 | 1.34 | 1.37 | 1.36 |
| **F** | 0.65 | 1.17 | 1.22 | 1.20 |
| **G** | 1.00 | 1.75 | 1.80 | 1.78 |
| **H** | 1.00 | 1.00 | 1.03 | 1.02 |
| **I** | 1.00 | 1.17 | 1.19 | 1.18 |
| **J** | 0.46 | 0.84 | 0.87 | 0.86 |
| **K** | 0.30 | 0.46 | 0.50 | 0.48 |

The BodeQuant assay was run with the specimens listed on 22Mar2004 and 29Mar2004. The 1-12 series specimens were run both days, but specimens A-L were run only on 29Mar2004.

For both BodeQuant runs, the microplate assay was run immediately following PCR (no freezing of the amplicon). Each sample was run in two wells on each date, and the plates were scanned twice.

There are some variations in the subjective band intensity judgments of two analysts with specimens 1-12 (columns 2 and 3 of Table 1). It is fair to say that QuantiBlot results are close to the BQ2 average in all cases. Of more concern is the discrepancy between BQ1 and BQ2 averages with some specimens (2B, 4B, and 6B). The reason for these discrepancies is not immediately clear.

Only a small number of samples could be run with the functional BodeQuant standard curve assay and compared directly with QuantiBlot. Enough differences are apparent that further validation would be necessary before BQ replaced Qblot as the sole quantitation technique. The data suggest that it probably could be accomplished, however.

A limitation of BQ is that there are no set criteria for defining when the standard curve is suitable for use. Such criteria would be necessary in a final protocol. In addition, there may be problems with accuracy and reproducibility at the lower end of the curve. The manipulation blanks used in the comparison study, while estimated at 'no detectable DNA' on QuantiBlot, gave varied readings (from below 0.1 to 0.46) on the BodeQuant. More work would be required to devise ways of handling this problem.

In practice BQ is faster than Qblot in the sense that the analyst has down time during BQ to be during other things. In addition, more specimens can be run, and the range of the assay is greater. Qblot may have an advantage in that it has been used extensively for a number of years, and most of the bugs have been worked out. Still, even our limited data show that there is variability in the estimates resulting from the subjective judgment of band intensity.

Many laboratories are now beginning to use quantitation assays involving real-time PCR (9,10), as we have noted earlier. A number of different assays are possible, but one example is a TaqMan assay (11). This assay uses a probe that has a fluorescent dye bound to one end and a fluorescence quencher at the other end. This probe is added to the PCR reaction mix. The probe binds within the region of DNA that is being amplified. During the annealing step of each PCR cycle, the probe and primers bind to the DNA in the sample. During extension, the Taq exonuclease activity digests the probe, which then releases the fluorescent dye distancing it from the quencher. The real-time PCR thermal cycler can detect this fluorescence, which increases as more and more copies of the DNA are synthesized. By comparing the amount of fluorescence emitted to a known standard curve, the concentration of the DNA samples can be determined in real time. The technique uses costly equipment, which has to be purchased. Such a new assay also has to be validated in each laboratory. However, the assay is faster than either QuantiBlot or BodeQuant. And it has the potential to be automated – and issue in this climate of backlogs, and pressure to increase throughput.

## Part II. A PCR-Based Method for Determining Species of Origin

Species determination is a critical step in biological evidence analysis. For decades, it has been done using immunological methods. As DNA profiling has developed, operational laboratories have been under unrelenting pressure to increase throughput, and reduce backlogs. One strategy is to reduce the number of separate tests on a specimen to a minimum. Since several of the loci that are routinely amplified in a DNA profile are known to be human (primate) specific, obtaining an interpretable genotype at one or more of these loci can be regarded as evidence of human (primate) origin without the necessity of performing a separate test. However, if results at the relevant loci are not obtained for some reason, there are then no results indicating that the specimen was indeed human.

As DNA has taken over (some might say overwhelmed) biological evidence analysis units, there is a greater tendency to eliminate older methods based on other principles, and to try to do all the testing using DNA technologies. This makes some sense in terms of work flow and training, but in some cases, DNA-based methods are ridiculously more expensive than older, more conventional ones. The trend appears to be here to stay, however.

A PCR-based method for species determination has the prospect of fulfilling the "DNA technology" criterion, and being easy to incorporate into the work flow. Then, even in specimens that do not give genotypes at all the loci (or at the "human-specific" ones), there would still be a "test" for human species. In addition, a PCR based test is readily adaptable to a range of commonly encountered household pet and farm animal species.

Our project was based on a paper by Parodi et al. (12) describing a PCR-based method for distinguishing the species of various cell lines using the cytochrome c oxidase subunit c (cox I) locus of mitochondrial DNA (Appendix 2). We have modified the method described, to try and optimize it for current forensic DNA technology. As proof of concept, we set out to devise a method for distinguishing human, cat, dog, and horse.

### Materials and Methods

#### Extraction

The four species chosen for the project are occasionally encountered in routine forensic casework, and the original Parodi paper described primers for each of them. Moreover, we maintain dried bloodstains from these species in our laboratory. DNA obtained from extracting these bloodstains was used in the initial amplifications. In some later troubleshooting steps, K562 (a purified genomic DNA from a female cell line) from Promega was used.

DNA from most biological specimens is extracted more or less the same way using the "organic" method. Specimens are incubated in 400 µL of DNA extraction buffer (10 mM TrisHCl, 10 mM $Na_2EDTA$, pH 8, containing 100 mM NaCl and 2% SDS) and 10 µL proteinase K (10 mg proteinase K in 1 ml $dH_2O$) in a gently shaking water bath at 56°C overnight. The samples are next centrifuged through Spin-ease extraction tubes at 10,000 rpm for 5 min to remove the bloodstain substratum. This fraction is then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). After mixing and centrifugation, the aqueous layer is retained, and the chloroform layer discarded. The DNA is dialyzed using a Microcon filters, and collected in 30 µL TE buffer (10 mM TrisHCl, 0.1 mM EDTA, pH 8).

Quantitation

The quantity of DNA in an extract was determined using the QuantiBlot (Applied Biosystems) kit, and spectrophotometry (260/280) with a Spectronic Genesys 2. One reason we turned to K562 DNA for a time was doubt about the concentration of our extracts. QuantiBlot is notoriously noisy, and there is sometimes too little specimen to measure accurately using 260/280. The quantity of DNA being added to the PCR reaction was a variable we had to try and control.

PCR

The amplification reaction mixture initially contained 5 μL 10X PCR Buffer II (100 mM TrisHCl, pH 7.8, 500 mM KCl, 0.01% gelatin), 4 μL dNTPs (2.5 mM each), 3 μL 25 mM $MgCl_2$, 4 μL of 10 μM primer mix (reverse and forward), 1 μL (5 units) of Taq DNA polymerase, 23 μL of dH2O, and 10 μL DNA – final volume 50 μL. Initial amplifications were with 1 ng of target DNA. The thermal cycler was a Perkin Elmer GeneAmp PCR System 2400. Oligonucleotide primer pairs were synthesized amplify the cytochrome c oxidase subunit I (cox I) sequences in human, cat, dog, and horse. The oligonucleotides were synthesized by the University of Illinois Protein Research Laboratory/Sigma-Genosys. Original primer sequences, positions, and amplification product sizes are shown in Table 3.

Table 3. Original primer sequences in the Cox-I mt-DNA gene

| Species | Position | Dir | Sequence | Amplicon (bp) |
|---|---|---|---|---|
| Human | 5969-5988 | fwd | cggcgcatgagctggagtcc | |
| | 6173-6194 | rev | tatgcggggaaacgccatatcg | 226 |
| Horse | 5456-5475 | fwd | ccctaagcctcctaatccgt | |
| | 5674-5694 | rev | aagtaggatgatgggggaag | 239 |
| Dog | 5466-5487 | fwd | gaactaggtcagcccggtactt | |
| | 5597-5618 | rev | cggagcaccaattattaacggc | 153 |
| Cat | 7413-7434 | fwd | ttctcaggatataccttgaca | |
| | 7571-7592 | rev | gaaagagcccattgaggaaatc | 180 |

The initial amplification profile was 95°C, 10 m (hot start), then 44 cycles of 95°C 30 s, 58°C 30 s, 72°C 1 m followed by a 5 m chase at 72°C.

Over the course of efforts to establish a stable, workable set of amplification conditions, many parameters were varied. Beginning with human DNA, we spent considerable time trying to optimize PCR conditions, using both purified K562 human cell-line DNA and DNA extracted from human bloodstains and cleaned up for PCR.

Post PCR – Product Handling and Detection

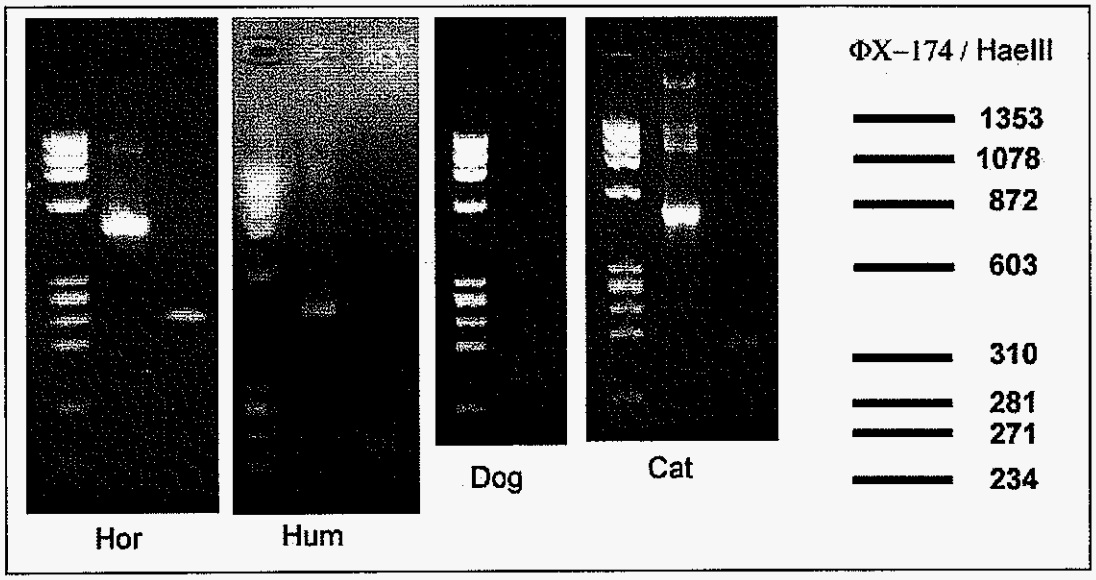
For purposes of optimizing the PCR, we ran the products on agarose gels, and visualized them with ethidium bromide (2 μL 10 mg/mL EtBr in each 20 mL 4% agarose gel, consisting of 3 parts NuSieve agarose to 1 part standard agarose, in 1X TBE or 1X TAE. Electrophoresis was typically at 110V for 1-2 hrs. A PCR "control" consisting of 1 ng linearized λ-DNA and primers that amplify a 500 bp segment, was included in most experiments. The 500 bp λ control was run on most of the gels. Because our targets are mt-DNA, and mt-DNA amplifications are often for more cycles than genomic, the λ control is usually overamplified. A ϕX-174/*Hae*III ladder appears on most gels as a sizing marker.

Although the ultimate goal was the use of fluorescent tag labeled primers, and running the PCR product on a DNA analyzer (such as the ABI 310), we relied on the State Police Forensic Science Center for time on this analyzer, and did not want to run specimens on it until we had a pretty good idea that the technique was working, based on the much cheaper and easier gel detection method.

Establishing a Consistent Method

Considerable time was spent "titrating" different parameters used in the original paper to try and establish a consistent method, and one that had some resemblance to the conditions usually used with casework evidence specimens. Turnover in graduate RAs did not aid this process either. Eventually, a set of PCR conditions was established, and will work. Some time was spent establishing which variant of *Taq* polymerase would do best – and it was *Taq* Gold. The enzyme is robust, but requires a hot start (this is not a problem). Fig. 4 shows a composite of separate amplifications for the four species.

Figure 4. Composite Photos of Post-Amplification Gels for All Four Species*




* The left-most lane is a ϕX-174-*Hae*III ladder. To the right is a diagrammatic representation of the sizing bands in this ladder. The ladder bands do not always resolve completely, but it is generally possible to verify that the size of the amplicon is approximately as expected. In the Hor, Hum and Cat gels, the middle lane is a λ-DNA PCR control product of 500 bp. Because it is genomic, the number of PCR cycles use for the mt-DNA result in overamplification of the λ control.

Another parameter we spent time on is quantity of input (template) DNA. From Part I of this project, it will be evident that DNA quantitation is not trivial (certainly not as trivial as many forensic DNA analysts appear to think, and would have us think). In this work, we noted that amplification of the mt-DNA sequences seemed to work better with larger quantities of template – sometimes 10-20 fold greater than what would normally be used in a multiplex genomic amplification. However, after going back to 260/280 to nail down the DNA concentration in the specimens, and convincing ourselves that we were on the linear part of the curve, we eventually got a pretty firm handle on this matter.

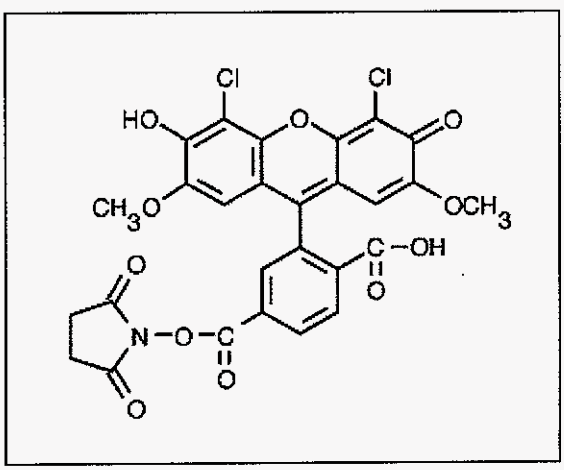
In the process of attempting to get a firm handle on the quantity of DNA in a specimen, we did a side project to see if we could devise a "quanti-blot" assay exactly analogous to the human genomic kit assay, but for mitochondrial DNA. The genomic DNA assay, as noted above, is a dot-blot assay that relies on a 40 nt probe (nt1741-nt1780 in the sequence) that is biotinylated. We designed and made a biotinylated 40 nt probe with the identical AT/GC ratio (so that the hybridization stringency would be identical) representing nt6071-nt6110 of the cox I mt-DNA sequence. The probe worked in tests with "human" DNA. We next tried to calibrate this assay by preparing mitochondria from cell lines, using a commercial isolation kit. This was only attempted one time, and it failed. We know it failed because the preparation still hybridized well with the nuclear probe (and it shouldn't have) and failed to hybridize well with the mt-DNA probe (which it should have). Although this assay would have been helpful had it been easy to develop without wasting too much time, we dropped it at this point.

In the lst few months of the project, we synthesized new primers (Table 4) – now labeled with JOE (Figure 5). It is to be noted that the relative sizes of the species changed with the new primers. The new amplicon size order is dog > cat > hum > horse. These primers work under appropriate amplification conditions. Figure 6 shows a representative gel.

Table 4. New, labeled primer sequences in the Cox-I mt-DNA gene

| Species | Position | Dir | Sequence | Amplicon (bp) |
|---|---|---|---|---|
| Human | 6957-6976 | fwd | ggcctgactggcattgtatt | |
| | 7116-7135 | rev | ttggcgtaggtttggtctag | 179 |
| Horse | 5820-5839 | fwd | tggggtgtcctcgattttag | |
| | 5966-5985 | rev | catggtaatgcctgctgcta | 166 |
| Dog | 6284-6303 | fwd | cgctattccaacgggagtaa | |
| | 6510-6529 | rev | gcaaatcctcccataatggc | 246 |
| Cat | 6727-6746 | fwd | taaaacctcctgccatgtcc | |
| | 6920-6939 | rev | ctggatggccaaagaatcag | 213 |

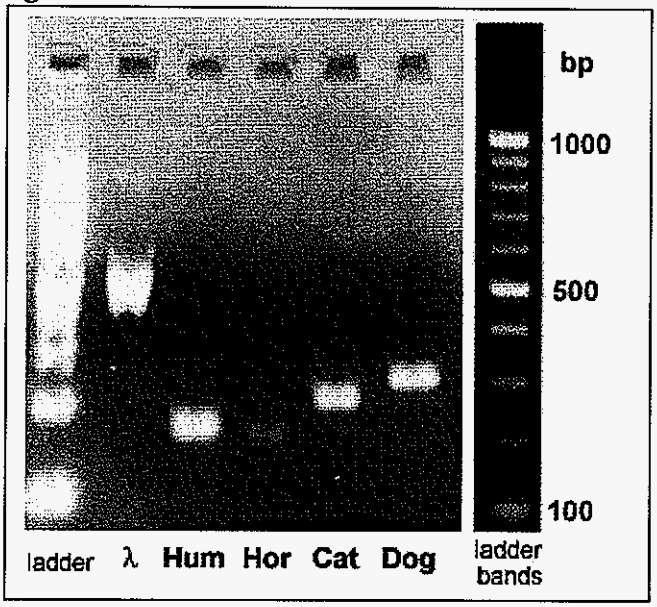
Figure 5. JOE (6-carboxy-4',5'-dichloro-2',7'- dimethoxyfluorescein, succinimidyl ester)




JOE is a fluorescein derivative, and is one of the several dyes commonly used to produce multi-colored (actually multi-wavelength fluorescence) output from a DNA analyzer. The ABI 310 to which we had access could read JOE, and we could use ROX to calibrate the molecular weights. This is common practice in everyday forensic DNA profiling done in operational laboratories. We wanted to choose one of the fluorescent dyes in routine use, and thus for which the instruments are already set up.

In Fig. 6, as above, lane 1 (leftmost) is a ladder (in this case, a 100 bp ladder different from the one in Figure 4, and not well resolved). An idealized picture of the ladder with all fragments resolved is shown to the right. And lane 2 is the 500 bp λ-DNA PCR control product. The four species DNA PCR products are as expected.

Figure 6.

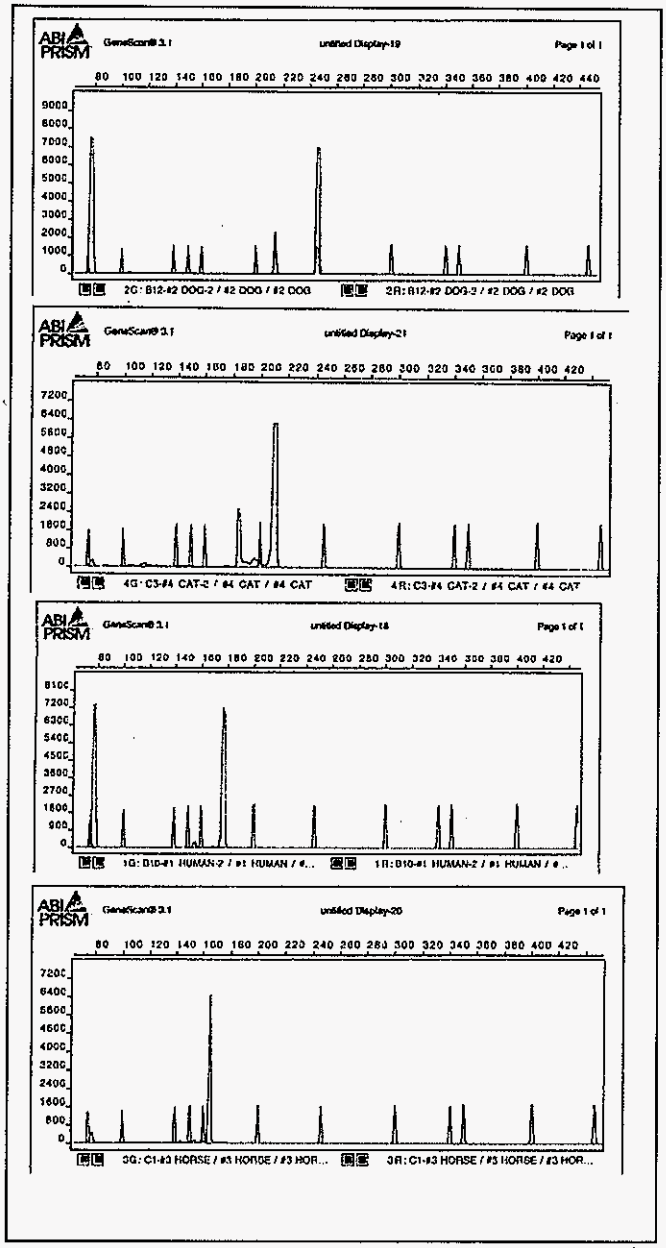



We were able to run the labeled PCR products from all the single-plexes and several multiplexes on the ABI 310, but unfortunately only one time. The instrument's availability was limited because of casework demands on it. And, although the results show that some refinements would be necessary to ready this technique for casework, the correct bands were almost always observed. There were artifact peaks present which would have to be eliminated to nicefy the assay. But in terms of proof of principle, the objective was achieved.

In Figure 7, a composite of all four individual single-plex results is seen. The expected peaks are seen in every run, except that in the top three (dog, cat and human), there are artifact peaks as well. The one at about 78 bp in dog and human is mysterious. More work would be required to sort out what this peak represents and get rid of it. It can't be primer-dimer because it is too big. And, we did not see any hint of it on gels.

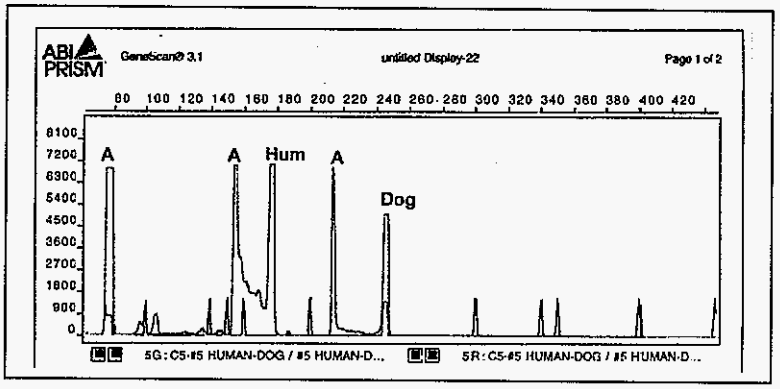
As noted, only one run on the gene analyzer was possible, so it may well be that PCR conditions could be adjusted, along with the dilution of the PCR product for instrumental analysis, to distinguish the single expected peak from background and artifacts.

Figure 7. Single-plex Results



From top to bottom: dog, cat, human, horse

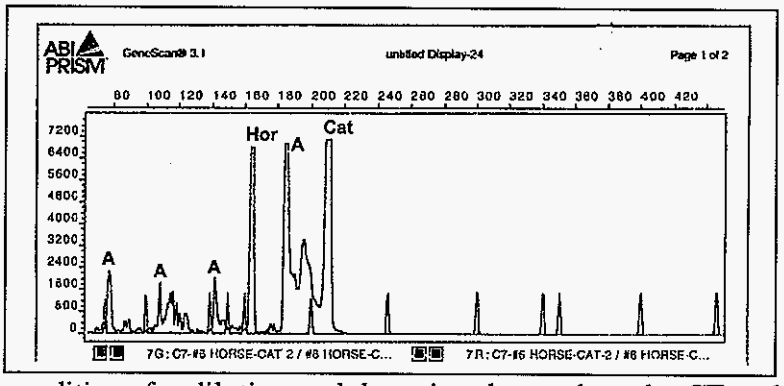
Figure 8. Human – Dog Duplex




In addition to the single plex runs, shown in Fig 7 and discussed above, we ran a few multiplexes to see how they might work. The single plex results show that the expected PCR products are obtained for each species, and that the products are detected appropriately as peaks on the analyzer.

Two duplex reactions were run. One was human-dog, and the other was horse-cat. These pairings were chosen to give maximal separation between amplicon sizes (human 179 vs dog 246; and horse 166 vs cat 213). It is clear from Figure 8 that human and dog products amplified in the duplex, and were detected as well separated peaks.

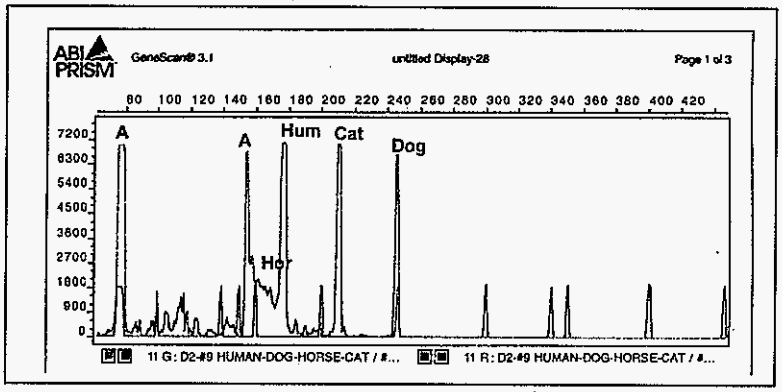
Figure 9. Horse – Cat Duplex




Something similar can be seen for the horse and cat duplex in Figure 9.

In both cases, however, there are artifact peaks that would have to be engineered out of the reactions by adjusting PCR conditions, cycle numbers, template concentrations, and the conditions for diluting and detecting the products by CE and fluorescence in the analyzer itself.

Figure 10. Quadriplex




We also amplified and ran one example of all four species in the same tube. As can be seen in Fig. 10, human, cat and dog peaks were clear – along with some artifact peaks – but horse did not amplify in the expected position.

Cleaning up the reaction would require some additional effort and adjustment of conditions.

Although the final electropherograms show that there is still a need for refinement, we are satisfied that the concept we set out to test has been successfully demonstrated – namely, that a PCR-based species test can be devised, based on the mt cox I locus. Although four species were somewhat arbitrarily chosen for this proof of concept project, the technology could easily be extended to any species for which sequence information is available for the cox I mt locus.

We may have the opportunity to do a modest bit more work on the system with the last graduate student involved in the project, but those findings will not be able to be included in this report.

The appeal of this approach, as we tried to note in the initial proposal, is that it uses amplicon detection technology identical to that routinely employed in forensic labs for the detection of DNA profiling amplicon detection. Accordingly, the specimen preparation is very similar, and the software is set up to provide peak sizings. In practice, the use of an approach like this would entail running one extra tube per specimen of interest. Assuming there was no information from the case file to suggest non-human DNA, one could use a human-animal duplex, or human-animal-animal triplex, or even human-three other animal quadriplex. The expectation would be a human DNA amplicon but no animal ones. The result would then explicitly confirm human origin.

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