# Final report of NIJ Grant # 2000 IJ-CX-K012

# Funded in three stages -

# Original grant - A Microplate Assay for the Quantitation of Human DNA (5/01/01-10/31/02),

# Supplement 1 - Simple, Rapid, and Accurate Quantitation of Human DNA (1/1/02-10/31/03) and

# Supplement 2 - Increasing the Efficiency of Forensic DNA Analysis Through Real-Time PCR (11/1/03- 4/30/06)

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**ABSTRACT:**

The thrust of the research was to develop a faster, easier and more quantitative approach to the quantitation of human DNA from forensic casework samples. Initial work focused on improving the method employed in essentially all forensic laboratories (slot blot). We worked on a human DNA specific microplate assay with reading of the plate in a fluorescence/luminescence microplate reader. The microplate assay had numerous technical difficulties and a decision was made to apply PCR technology instead. The original grant award allowed the development of an *Alu*-based assay using PCR with SYBR Green and the fluorescence based plate reader to quantitate human DNA. This grant also allowed us to begin study of a real-time PCR *Alu*-based assay. Supplement 1 allowed full development, validation, publication and implementation of this SYBR Green, *Alu*-based assay. Because of the limited dynamic range of this SYBR Green assay, we switched to a probe-based approach (Eclipse probes, TaqMan probes and LUX primers). In Supplement 2, we expanded our assay to not only quantitate total human DNA but to quantitate the male DNA as well in a duplex (Eclipse or TaqMan) assay. We validated a TaqMan-based duplex *Alu* and DYZ5 based assay. We also tried other technologies to improve the original *Alu* based assay. These included FLAP and Keyhole probes (Stratagene) and development of an assay with different sized *Alu* PCR products for mini-STRs and degraded DNA. Development and validation of a real-time based version assay of this assay is an aim of the current grant. Another aim was to develop a simple, fast screening method to differentiate the donor of specimens. We tried to use melting differences between alleles of selected STR loci; however, the results were inconsistent across genotypes. We then focused on SNPs and FRET melting technology. This technique was expanded into an aim for the current grant. Although we had suggested developing a mitochondrial DNA assay, Dr. Mark Timken

developed one and thus we stopped this line of research. The SYBR assay has been given out to over eight Forensic labs and the duplex assay to seven.

**EXECUTIVE SUMMARY:**

This final report for grant #2000 IJ-CX-K012 represents three separate monetary awards one original and two supplemental [Original grant - A Microplate Assay for the Quantitation of Human DNA (5/01/01 to 10/31/02, Supplement 1 - Simple, Rapid, and Accurate Quantitation of Human DNA (10/31/02 to 10/31/03) and Supplement 2 - Increasing the Efficiency of Forensic DNA Analysis Through Real-Time PCR (10/31/03 to 4/30/06)]. The thrust of the research was to develop a faster, easier and more quantitative approach to the quantitation of human DNA from forensic casework samples.

As the forensic community adopted new technologies to profile biological materials, quantitation methods to support these new technologies were developed. During the time when Restriction Fragment Length Polymorphism analysis was performed, simple gel electrophoresis sufficed to determine the appropriate amount of sample required for analysis. When PCR was developed, and the reverse dot blot technology of DQ alpha and Polymarker employed, the need for human quantitation of the sample was realized. To answer this need, the slot blot method for DNA quantitation was developed. The slot blot method was an appropriate technology for the time since only a crude estimate of the amount of DNA extracted was required to yield typing results. However, as the technology matured, STRs became the forensic system of choice for DNA profiling and an assay that could better predict the amount of extracted DNA became necessary in order to efficiently and judiciously profile DNA samples.

Current STR systems behave quite differently concerning the amount of template DNA required for amplification compared to the reverse dot blot techniques. The STR systems are very sensitive to and intolerant of elevated concentrations of input human DNA and have an optimum DNA range which is much narrower than the reverse dot blot technique. DNA

amounts outside this range can result in unbalanced peaks, n-1 peaks, RFU values outside the instrument range and inconsistent amplifications. For these reasons it is important to accurately estimate the concentration of extracted human DNA prior to performing an amplification. The slot blot technique used for these quantitative estimates occasionally results in the need to repeat an amplification due to an inaccurate estimate of DNA concentration. In addition to the inherent inaccuracy of the slot blot technique, the limited analytical range of the slot blot procedure necessitates the reanalysis of samples falling outside this range, adding to the time and effort required to process a sample. The slot blot technique is also very time consuming and labor intensive. The community required a new method to assay the quantity of DNA extracted in a sample that was faster, easier and better at predicting DNA amounts in order to keep up with the demand for casework analysis.

This research began during the community's transition from reverse dot blot technology to STRs. Our initial work examined the possibility of using existing technology to modify the slot blot quantitation system to yield a more accurate assessment of DNA extracts. The first proposal had the following goals: 1) to develop an accurate, non-subjective instrumental method to quantitate the amount of DNA obtained from an extracted sample 2) validate the method for forensic use and determine its effectiveness and 3) determine if an automated system can be developed to perform this quantitation.

The research was based on a human DNA specific microplate assay with subsequent reading of the plate in a fluorescence/luminescence microplate reader. The microplate employed was a 96 well plate constructed in the normal microplate format except the bottom of the plate has been replaced by a membrane. Our initial studies with the prototype microplate indicated that we could use the components of the PE Applied Biosystems slot blot kit to

perform colorimetric, fluorescent and chemiluminescent assays. The PE slot blot assay relies on the membrane binding single stranded DNA to which a biotin labeled primate specific probe can find its complement and bind under appropriate stringency conditions. We performed a number of colorimetric studies which showed the viability of the technique and then switched to a using a fluorescent reporter molecule (Amplex™ Red) to increase the sensitivity of the approach. This assay detected a greater concentration range, equal to if not exceeding the lower limit compared to when slot blot analysis utilized X-ray film development techniques. Continued work with this approach revealed some shortcomings. Unfortunately, we observed well to well differences in these microplates, and some microplates experienced "cross talk" where reagents/ fluorescent products bled through from one well to another. The assay probably could have been refined (perfected) to obtain the necessary range and reproducibility required by STR systems but at this point we took a long view of the needs of the community. The community was beginning to see a dramatic increase in the samples destined for DNA analysis, and hence no longer was it only necessary to produce better quantitation results but it was necessary to produce these faster and with less analyst time. We explored the possibility of automating the microplate assay even though the protocol has not been finalized. The washer was able to do only some of the steps in the overall process and appeared to be more of a hassle than an aid. With this in mind, we decided to examine alternative approaches to the problem, still trying to maintain the general approach of using a microplate and reader to quantitate DNA. In this approach, we maintained the microplate format and reader but we used human specific PCR primers instead of a human specific probe to determine human specificity; PCR allowed us to develop our first fast, efficient, low cost approach to human DNA quantitation. The human multicopy *Alu* sequence was chosen as the human specific sequence for PCR analysis. This first award allowed the development of

an *Alu*-based assay using PCR with SYBR Green and the fluorescence based plate reader to quantitate human DNA. This work was submitted as a manuscript at the end of the first grant. The first grant also allowed us to begin study of a real-time PCR based assay also using the *Alu* sequence. In real-time PCR, a interacting dye (SYBR Green) dye or dye-labeled primers or a dye-labeled probe is used in order to follow DNA amplification as it happens (in real-time). A special real-time PCR instrument with excitatory lasers and detectors is needed. Although this first grant did not include funds for such an instrument, NIJ, working with NSFTC, obtained a loner instrument for us to perform initial studies on real-time PCR.

Supplement 1's aims were to: 1) to determine alternative approaches to current methods for the quantitation of human DNA, 2) to evaluate each approach for ease of use, flexibility in analytical analysis and the ability to semi-automate the analysis, 3) to validate the method for use in forensic casework analysis. This grant allowed full development and validation of this SYBR Green, *Alu*-based assay. This technology was then published, implemented for casework in the VFL and also shared with other laboratories. Because of the limited dynamic range of this SYBR Green assay, we switched to a probe-based approach. We investigated molecular beacon-type Eclipse probes, TaqMan probes and LUX primers as possible superior technologies to SYBR Green. While the Eclipse and TaqMan probes gave similar results, the LUX system was discarded because of low signal. A manuscript for the Eclipse method was written up at the end of Supplement 1.

The goals of Supplement 3 were: 1) development of an assay to quantitate male and female DNA, 2) development of a simple, fast assay that detects alleles at one or two STR loci as quick sample screening method, 3) improvement of the original *Alu* PCR Real-time PCR assay

(new techniques, mini-STR assay, mitochondrial assay) and 4) dissemination of methods to the Forensic community.

Crimes involving violence against women perpetrated by men is a serious problem and one in which forensic laboratories are concerned. Often in these crimes, blood may be shed by the both the victim and attacker such that multiple stains are transferred between individuals. In these situations, it is forensically important to find the blood not "belonging to the owner" on the item of evidence (blood from the female on the male's clothing or vice versa). A method that could quickly identify those stains as male or female could allow the examiner the chance to be selective in the stains that are analyzed further by STR analysis and hence save time and money by limiting the number of stains that require further analysis. By multiplexing quantitation with sex typing, the decision about what stain should be further analyzed is made simultaneously with the quantitation of the stain and hence no further work is required than what must be done prior to STR analysis. Another important use of real-time PCR sex typing would be in the STR analysis of sexual assault cases. Often seminal fluid stain extracts contain female DNA in addition to the DNA arising from the seminal fluid. These mixtures can range from a small contribution of female DNA to a very high amount relative to the male DNA present. Under those circumstances where female DNA contributes significantly to the total DNA present in the sample, the determination of the total human DNA present may not yield definitive information to determine if a male profile could be obtained. Some of the serological testing done prior to DNA extraction to determine the presence of semen (prostatic antigen, P30) are very sensitive and can detect extremely low levels of semen; however, these tests are often not good predictors of the success of STR typing from a particular sample. A method that could determine the

amount of male DNA present in a sample could allow the forensic scientist to decide if a sample has ample male DNA for further analysis.

In Supplement 3, we expanded our assay to not only quantitate total human DNA by the *Alu* assay but to quantitate the male DNA as well. We envisioned creation of a duplex probe-based assay which would quantitate total human and male DNA simultaneously. We worked on both Eclipse and TaqMan-based approaches. Eventually the Eclipse method was discarded because of issues with the dyes that could be used in a multiplex. Issues also arose with the Y-specific sequence to be used, the initial DYZ3 sequence appeared to have ethnic variability, DYZ1 had cross-reactivity with female DNA. We finally settled on the DYZ5 sequence and we developed and validated a TaqMan-based duplex *Alu* and DYZ5 based assay. The manuscript has been submitted and accepted (with minor revisions required).

Supplement 3 had several additional goals beside development of the duplex total/male assay. One was to improve the original *Alu* PCR Real-time PCR assay. We tried other technologies to improve the original *Alu* based assay. These included FLAP and Keyhole probes which were new proprietary methods from Stratagene. Neither of these systems worked well.

Another improvement was to develop an assay to quantitate human DNA for the mini-STR assays. Many forensic samples are highly degraded and PCR of the longer length STRs found in the usual kits is not possible. John Butler at NIST and Bruce McCord at Ohio University developed a set of STRs with small PCR products ("Big Mini" STR System). Currently, their system uses primers for THO1 and FGA (6FAM), CSF1PO and D21S11 (VIC) and TPOX and D7S820 (NED). However, current quantitation methods are not designed to quantitate degraded DNA accurately. A redesigned real-time *Alu* PCR assay with a product of similar size to the Big Mini STR products would be an effective assay to quantitate such DNA.

This required development of an assay with different sized PCR products. We utilized three different sized *Alu* PCR products (82bp, 124bp and 201bp) and a gel-based readout to develop a preliminary assay. Development and validation of a real-time based assay to detect DNA degradation is an aim of the current grant.

Another aim was to develop a simple, fast assay that detects alleles at one or two STR loci as a quick screening method to differentiate biological specimens allowing the examiner to process only those stains necessary to show "contact" between two (or more) individuals. When the victim and assailant are of the same gender, then quick screening of possible stain donor identity will not be possible by the above gender tests. Clearly, identity can be determined to near certainty using the usual 13 STR loci but often such a determination is of limited value from a criminal justice point of view; for example, determining that blood on the assailant's shirt is his/her own blood. Such a complete analysis often becomes a waste of time and money. Quick analysis of one or two STR loci, while certainly not by any means a definitive test of identity, will usually allow determination of whether a blood sample did not come from an individual. This is sufficient for a quick screen of crime scene stains to answer the question – Is this stain of possible victim or assailant origin? Development of such a screen would be of benefit to the forensic community.

We tried to use melting differences between alleles of selected STR loci detected on a real-time PCR instrument with SYBR Green to accomplish this. An initial attempt with the TPOX locus gave some melting differences but they were small and inconsistent. We also tried D22 (courtesy of Mike Coble of NIST) but again the results were inconsistent across genotypes. We abandoned this line of research and decided to concentrate on SNPs instead of STRs. Some preliminary experiments were performed using a FRET melting technology. In this type assay,

PCR is performed as usual using two primers flanking the SNP. Two probes are also present: one probe with a 3' dye label which covers the SNP and a second with a 5' dye and a 3' extra phosphate to prevent elongation. The first probe is a perfect match to one allele (allele A) but obviously has one mismatch with the other allele (allele C). When the two probes both bind to the PCR product, and the 3' dye is excited, it transfers energy to the 5' dye which produces light (FRET). Determination of which allele(s) is present depends on the melting temperature where the fluorescence is lost. Obviously, when the allele with the A is present, the first probe will melt off later than if the 1 bp mismatch G is present. This technique was expanded into an aim for the current grant.

We had indicated in the grant that we would attempt to develop an assay to quantitate mitochondrial DNA. While we did make some efforts to design an appropriate set of primers and probe, it was difficult because of ethnic variability in mt DNA sequence. Also, Dr. Mark Timken developed a multiplex nuclear/mitochondrial assay and we felt that since assay worked well, that there was no need for us to develop another assay.

Our final goal was to disseminate the assays we developed to other laboratories. The SYBR assay has been given out to over eight Forensic labs and the Duplex assay to seven.

## MAIN REPORT

This final report for grant #2000 IJ-CX-K012 represents three separate monetary awards one original and two supplemental [Original grant - A Microplate Assay for the Quantitation of Human DNA (5/01/01 to 10/31/02, Supplement 1 - Simple, Rapid, and Accurate Quantitation of Human DNA (10/31/02 to 10/31/03) and Supplement 2 - Increasing the Efficiency of Forensic DNA Analysis Through Real-Time PCR (10/31/03 to 4/30/06)]. The thrust of the research was to develop a faster, easier and more quantitative approach to assess the amount of human DNA extracted from forensic casework samples.

### Original Grant - "A Microplate Assay for the Quantitation of Human DNA"

### Background:

An accurate, sensitive human DNA quantitation technique, suitable for automation became a high priority for the forensic community with the advent of STR profiling. The STR systems yield excellent results with small quantities of DNA and a statistical evaluation of the STR data results in numbers bordering on if not yielding identity. Forensic laboratories had undergone extensive validation of the STR systems and hence these loci, in addition to the separation technology behind the profiling, had become the standard for DNA profiling. Laboratories had established the profiling technology and were ready to explore methods to improve the steps leading up to the actual amplification and typing of the sample to yield STR results. This would include sample collection, preparation, extraction and DNA quatitation. The focus of our work was on the last major step prior to amplification, that of DNA quatitation.

DNA can be quantified via a number of routine analytical procedures. Gel electrophoresis using ethidium bromide or DAPI offers a comparative fluorescent gel method for

DNA quantitation. Ultraviolet and instrumental fluorescent methods also have been employed for routine DNA quantitation. However these procedures do not differentiate between human and non-human sources of DNA. These procedures simply measure the sample's total DNA content. This may be sufficient for known human standards, however when dealing with crime scene samples, alternative approaches are required to determine the amount of human DNA present in the sample. An alternative procedure that utilizes a primate specific probe to differentiate human from non-human sources has been extensively used in the forensic community. In this procedure DNA is bound to a membrane using a slot blot apparatus. The bound DNA is hybridized with a primate specific probe and through a number of steps the bound probe can be visualized by using either a colorimetric or chemiluminescence approach. The intensity of the slot blot band is dependent upon the amount of human DNA contained in the sample. The operational concentration range for this technique is approximately 10 ng to 0.16 ng for colorimetric and 10 ng to ≈ 0.04 ng for chemiluminescence analysis. Questioned samples, samples from a crime scene or reference samples, "blotted" on this membrane are compared visually by the analyst to a series of concentration standards also contained on the membrane to obtain an estimate of the amount of DNA present in the questioned sample. This visual comparison of the intensity of the "bands" is inherently inaccurate and results can vary considerably depending on the reader and operator. NIST, the National Institute for Standards and Technology, performed an STR study in which part of their study examined laboratories' ability to quantitate DNA samples. Their study, "1999 NIST Mixed-Stain Study #2: DNA Quantitation, Differential Extraction, and Identification of the Unknown Contributors," showed that a wide variation existed in the results obtained by the participating laboratories in the quantitation portion of this test. Further it appeared that at a low concentration of DNA, the

inaccuracy of the estimate increased. Most laboratories involved in this study relied upon the slot blot technique to estimate the DNA concentration of the sample.

The slot blot technique, in conjunction with the primate specific probe, was introduced at the time when the PCR techniques DQ alpha and later Polymarker were introduced. These were the first major PCR based methods used throughout the forensic community. The inaccuracy and the restricted operational range of the slot blot technique did not significantly hinder DNA analysis because these early PCR techniques are relatively insensitive to wide ranges of template DNA. Early protocols for DNA template input for DQ alpha ranged from 2 ng to 40 ng (the test presently requests a range of 2 to 10 ng). If the estimated concentration was not accurate, the test would usually still work and yield appropriate results.

A quantitative technique that yielded rough DNA concentration estimates was not a concern when introduced since both DQ alpha (now known as DQ A1) and Polymarker can work well with a wide range of DNA template amounts. Concentration estimates that were considerably off from the "true" value, would still give excellent results in these early PCR systems that were not very sensitive to template concentration variations.

Current STR systems behave quite differently concerning the amount of template DNA required for amplification compared to the reverse dot blot techniques. The STR systems are very sensitive to and intolerant of elevated concentrations of input DNA and have an optimum DNA range of approximately 0.5 ng to 2.0 ng, much narrower than the reverse dot blot technique. DNA amounts outside this range can result in unbalanced peaks due to stochastic effects when DNA amounts fall below the recommended range. Split peaks, known as minus A or n-1 peaks, high concentrations of amplification products that are outside the usable instrumental analysis range, and inconsistent amplifications are the results of high amounts of

template DNA used in amplification. For these reasons, it is important to accurately estimate the concentration of extracted DNA prior to performing an amplification. The slot blot technique used for these quantitative estimates occasionally results in the need to repeat an amplification due to an inaccurate estimate of DNA concentration. In addition to the inherent inaccuracy of the slot blot technique, the limited analytical range of the slot blot procedure necessitates the reanalysis of samples falling outside this range, adding to the time and effort required to process a sample.

As the demand for DNA analysis increased, the need for an accurate means to quantitate extracted DNA became more important. The forensic community had a definite need for an accurate, sensitive assay technique capable of evaluating a wide range of DNA concentrations. An ideal technique must be specific for human nuclear DNA, non-subjective, reliable over a wide concentration range and amenable to automation. During this time period when we wrote the first grant proposal, we felt that the method should be based on current technology to avoid extensive validation.

**Project goals:** 1) to develop an accurate, non-subjective instrumental method to quantitate the amount of DNA obtained from an extracted sample 2) validate the method for forensic use and determine its effectiveness and 3) determine if an automated system can be developed to perform this quantitation.

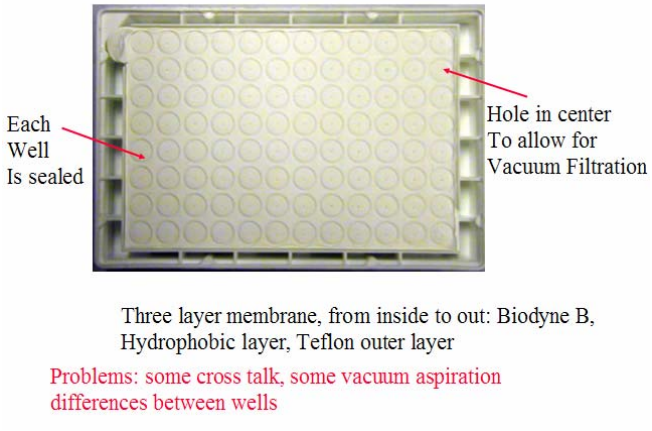
**Methods:**

**Phase I – Work on human DNA specific microplate assay with subsequent reading of the plate in a fluorescence/luminescence microplate reader**

The research was based on a human DNA specific microplate assay with subsequent reading of the plate in a fluorescence/luminescence microplate reader. The microplate is a 96

well plate constructed in the normal microplate format except the bottom of the plate has been replaced by a membrane (Figure 1).

Figure 1 – 96 well plate

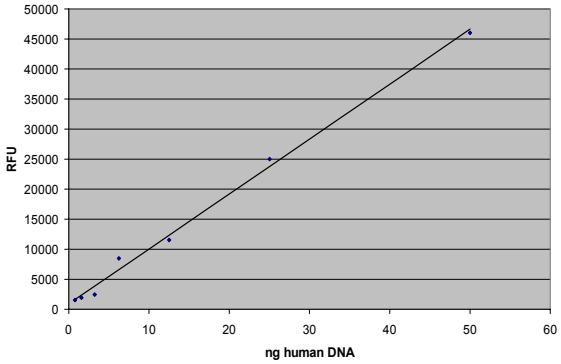



We used a number of different plates, prepared by Pall and Nunc, for the study but all contained a Biodyne® B membrane plate for use in this assay. This Biodyne® B membrane is the same as that required for the PE Applied Biosystems QuantiBlot® human quantitation kit. Our initial studies with the microplate assay indicated that we could use the components of the PE kit to perform colorimetric, fluorescent and chemiluminescent assays. The PE assay relies on the membrane binding single stranded DNA to which a biotin labeled primate specific probe (complementary to the alpha satellite DNA sequence at the D17Z1 locus) can find its complement and bind under appropriate stringency conditions. Subsequently, the enzyme horse-radish peroxidase binds via streptavidin to the biotin labeled probe. The probe is "visualized" through any number of horse-radish peroxidase substrates that can be used to detect the amount of probe bound to the membrane. We performed a number of colorimetric studies using TMB (tetramethyl-benzidine, PE Applied Biosystems, Foster City, CA) as the indicator and found that the procedure worked well enough to encouraged us to try more sensitive reporter molecules. We next tried the assay using a microplate reader (Bio-TeK FLX800; Bio-Tek® Instruments,

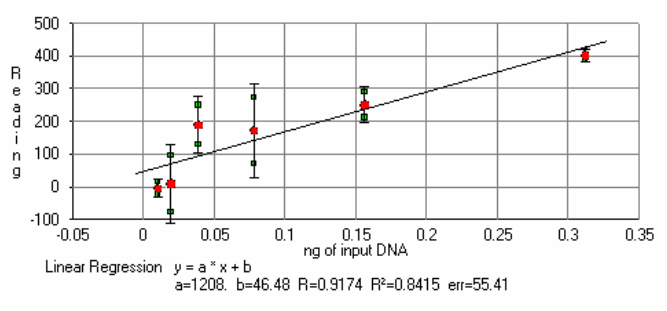
Winooski, VT) on a plate containing fluorescent (Amplex™ Red Molecular Probes, Eugene, OR) substrates. Figure 2 shows a graph of our results using a Bio-TeK FLX800 (Bio-Tek® Instruments, Winooski, VT) microplate reader, which indicates a dynamic range of 0.78ng-50ng.

Figure 2 – Amplex Red results A) Standard curve B) Lower end of standard curve showing variability C) view of a sample plate to see Amplex red coloration

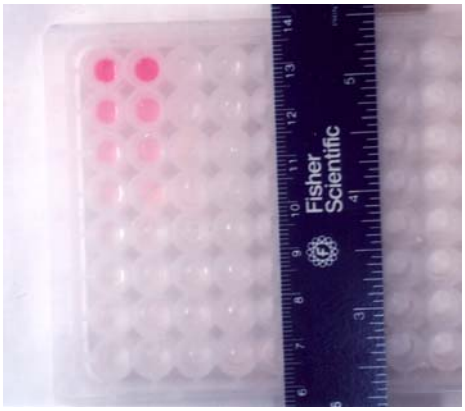
A.




B.




C.




As we continued our assay development, we relied upon the work under development by Pall, Gelman Laboratory, Ann Arbor, MI. Pall was working with us in an attempt to find the best plate for our needs. During this study, we found that there were noticeable well to well differences in the microplates tested which greatly affected reproducibility. These plates experienced "cross talk" where reagents/ fluorescent products bled through from one well to

another. Additionally, some plates appeared to have well to well variations in the application vacuum during the evacuation of the wells.

As we attempted to deal with the mechanical problems of the plate, we also examined different approaches to improve the sensitivity of the assay. We felt that the assay, to be very useful to the community, should have of a sensitivity of approximate 40 pg per well. We had never reached this level of sensitivity. In an attempt to improve sensitivity, we examined different fluorescent substrates for the assay and have modified the washing and hybridization steps. We also switched the from the primate specific probe supplied in the slot blot kit to an *Alu* probe recommended to us by Dr. Mark Batzer, another NIJ grant recipient.

*Alu* probes have a high copy number in the human genome and the thought was that this could improve the sensitivity of the assay. *Alu* sequences are found in 500,000 to 1,000,000 copies in the human genome representing 6-13% of the haploid genome (Mighell et al., 1997; Schmid, 1996). The consensus *Alu* sequence is ~280bp in length consists of two similar monomers connected by an A rich region. Because *Alu* sequences are present in many copies and are primate specific, they make an excellent target or marker for human DNA. They have been exploited by others to develop assays to detect human DNA. For example, a paper reports use of an *Alu* PCR based system (Sifis et al., 2002) to quantitate human genomic DNA from 2.5 to 100pg by determining the peak heights (RFU) obtained with an ABI PRISM™ 377 Genetic Analyzer and GeneScan software (Applied Biosystems). Also, Urban et al. (2000), used PCR of *Alu* sequences to detect template contamination. Unfortunately, our initial work with these *Alu* probes did not yielded any significantly better results than the D17Z1 probe.

Even though we had not perfected the microplate assay, we thought it best to assess the total quantitation strategy. If we perfected the assay, we understood that the forensic community

would be reluctant to adopt a quantitation method that was labor intensive. This procedure was labor intensive and so an automated washing strategy was needed to make the technique palatable to the community.

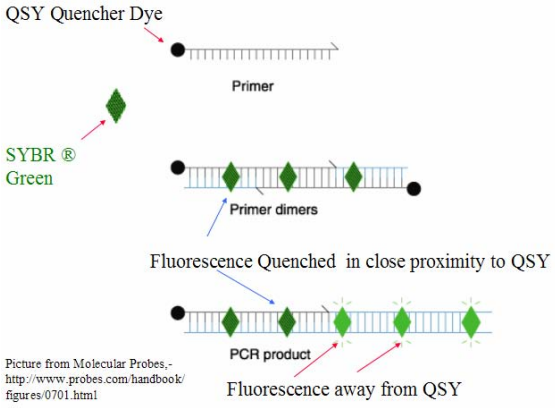
We had the opportunity to use a "loaner" plate washer from Bio-Tek® Instruments, Winooski, VT and learned some of the limitations that are inherent in low cost washers. The washers will be able to only do some of the steps in the overall process and may be more of a hassle that an aid. One of the problems we have found is with the addition of small volumes of expensive reagents. The low cost washers are not amenable for this application and hence those steps would require manual additions. Low cost washers also do not have heated chambers necessary for the hybridization step. The automation of this whole procedure, unless modified drastically from the original idea, will not be an easy endeavor using on the shelf low cost washers. This realization made us reconsider the direction of our research. The microplate technique requires considerable analyst attention. Each well of the plate must receive the appropriate addition of solution, whether it is probe, hybridization or wash solution. This takes considerable analyst time and hence the suitability of this analysis for busy forensic laboratories is diminished if it can not be coupled to simple automated washer.

At this point we changed the direction of our approach. We maintained the microplate format and reader but instead of binding the DNA to membrane and probing the DNA to obtain the selectivity of the method, we used primers instead of a probe to determine human specificity and a PCR approach to avoid the washing steps involved in the original microplate approach.

### Phase II – Development of a SYBR Green, *Alu*-based PCR Real-Time Approach to Human DNA Quantitation

In Phase II, we developed a procedure that would allow a faster, less time intensive analysis of the amount of DNA extracted in a sample. The procedure we developed was based on the PCR of an *Alu* sequence that is found in over 800,000 copies in the human genome. The primers were used to amplify a 124bp fragment of *Alu* sequence which was detected by SYBR Green I staining in a fluorescent plate reader. To reduce background in the plate reader assay, we utilized QSY 7 labeled primers (Figure 3 - method patented by Molecular Probes, Inc.) that removed background fluorescence of the primers and of primer-dimers.

Figure 3 – QSY primers

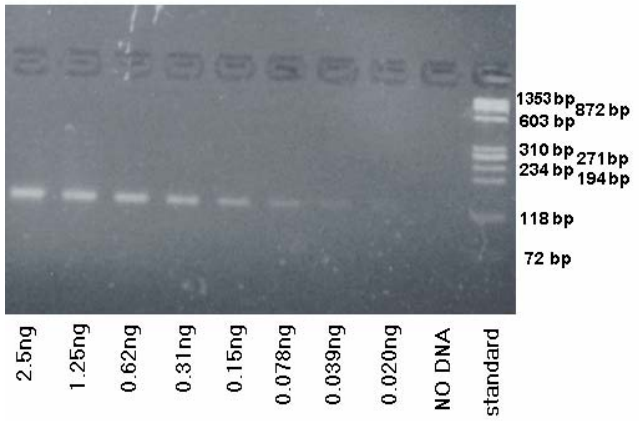



This assay quantitated both input DNA (which could contain non-human DNA) and also the amount of human DNA. The assay was optimized through the analysis of a number of variables including PCR cycles, annealing temperature, annealing time, extension time, specificity (test of animal DNAs), SYBR Green concentration, double-stranded versus single stranded input DNA and homebrew reagent mixes versus use of kits. The assay was validated using pseudo-crime samples, degraded DNA, and blood spots in comparison with the slot blot technique to demonstrate that it accurately predicts the appropriate amount of DNA required for STR analysis. We demonstrated that the assay has a dynamic range of 10ng to 10pg, is fast, quantitative and comparable in cost to the slot blot assay.

*Development of Assay*

The *Alu* PCR primers GTCAGGAGATCGAGACCATCCC (forward) and TCCTGCCTCAGCCTCCCAAG (reverse) were designed from the sequence of plasmid pPD39 (Ya5 subfamily) (Batzer et al., 1994) using the program, Oligos © 1999-2002 v.9.6 (http://wwww.biocenter.helsinki.fi/bi/bare-1_html/oligos.htm), designed by R. Kalendar of the Institute of Biotechnology, University of Helsinki. The unlabeled primers were purchased from Synthetic Genetics (now Epoch Biosciences, San Diego, CA) while those labeled with QSY 7 on the 5' end were purchased from Synthegen, LLC (Houston, TX). Initial experiments utilized *Alu* primers in a simple 20 cycle PCR with an ETBr stained gel-based readout to check for the correct size product, sensitivity, specificity and the presence of primer-dimer. The PCR gave the correct size product, and the amount of product was proportional to the amount of human input DNA although the results plateaued at high input DNA concentrations (Figure 4).

Figure 4 - *EtBr Stained gel of Alu PCR products*



Additional experiments showed that the PCR was specific for human (or other primate) DNA, that the PCR was relatively insensitive to annealing temperature (58-64ºC) and $Mg^{++}$ concentration (1mM to 5mM) (data not shown).

With these positive results, the assay was then moved to a 96 well format using the SYBR® Green JumpStart™ Taq ReadyMix™ kit (SYBR Green I is the DNA stain) and a plate reader for quantitation. PCR utilized the SYBR® Green JumpStart™ Taq ReadyMix™ kit (S4438, Sigma, St Louis, MO). The 25ul reactions contained 12.5 ul of 2X Buffer (1X final), 10pmoles of each QSY 7 labeled primer, 6.5ul of distilled $H_20$ and 5ul of TE (10mM Tris, pH 7.5, 0.1mM EDTA) containing the input DNA at various concentrations. The ReadyMix™ 2X buffer contains 20mM Tris-HCl, pH8.3, 100mM KCl, 7mM $MgCl_2$, 0.4mM each dNTP, 0.05 U/ul Taq DNA polymerase, JumpStart Taq antibody and with a proprietary amount of SYBR Green and unnamed other ingredients. Various experiments added additional components such as inhibitors (see below) or extra SYBR Green or 250ug/ml BSA. A homebrew PCR mix containing SYBR Green was also utilized for some experiments. This consisted of 10mM Tris, pH 8.3, 50mM KCl, 2.5mM $MgCl_2$, 200uM each dNTP, 8% DMSO and 1/40,000 or 1/20,000 of SYBR Green I (S7563, Molecular Probes, Eugene, OR). PCR for the QSY assay was performed in an ABI 9700 thermocycler in ABgene (Rochester, NY) 0900 PCR plates with flat caps (ABgene, AB0783). Immediately before PCR, initial readings of the loaded wells were taken in the fluorescent plate reader ($Fl_x$800, Bio-Tek, Winooski, VT). PCR consisted of 95ºC for 2 minutes (HotStart) followed by 11 to 16 cycles (depending on the experiment) of 94ºC for 15 seconds, 60ºC for 1 minute, 72ºC for 1 minute followed by 72ºC for 7 minutes and 20ºC for 10 minutes. For the last 20ºC 10min step, the hot bonnet was moved off and the PCR plate was covered by a foil cover to keep out light. This step was performed to allow the sample to cool to

the same temperature as the initial reading before the final reading was taken in the plate reader. Various experiments changed annealing times, temperatures and cycle number to optimize the assay.

Initial experiments indicated that background fluorescence from the input DNA and primers made the assay unworkable. The Molecular Probes (Eugene, OR) website suggested a patented method using QSY 7 or 9 dyes attached to the primers to quench the fluorescence of primers and primer-dimers. The background problem was essentially removed in the *Alu* assay by utilizing QSY 7 labeled forward and reverse primers (data not shown). The use of the QSY 7 labeled primers made this assay now feasible and allowed for further optimization.

Another assay improvement came with the realization that some of the leveling off seen at high concentration might be the result not of the PCR plateauing because of lack of primers or dNTPs but rather of insufficient SYBR Green in the reaction. Experiments were then performed adding extra SYBR Green to the reaction mix despite anecdotal evidence that it inhibited the PCR reaction. The plateau effect was significantly reduced by the addition of additional SYBR Green to the reaction mix. Adding an additional 1/20,000 seemed to have a slight inhibitory effect; thus, addition of 1/40,000 SYBR Green to the ReadyMix™ kit mix was chosen for future experiments.

The variables of number of PCR cycles, annealing temperature, annealing time, extension time, denaturation time and homebrew mix versus use of the ReadyMix™ kit were explored. Fourteen cycles was chosen for the final assay. Annealing temperature (56ºC to 62ºC), denaturation time (15 or 30sec) and extension time (30sec to 2min) (data not shown) changes had little effect.

The effects of changing primer concentration, dNTP and Taq concentration were also determined (Figure 5). Increasing the primer concentration slightly slowed the reaction speed. Increasing the dNTP concentration definitely decreased amplification. Both of these effects could be mediated by decreasing the available Mg++ concentration for the polymerase. Increasing the amount of Taq did increase the reaction speed and increase the plateau but did not affect the reaction sufficiently to be worth the extra cost.

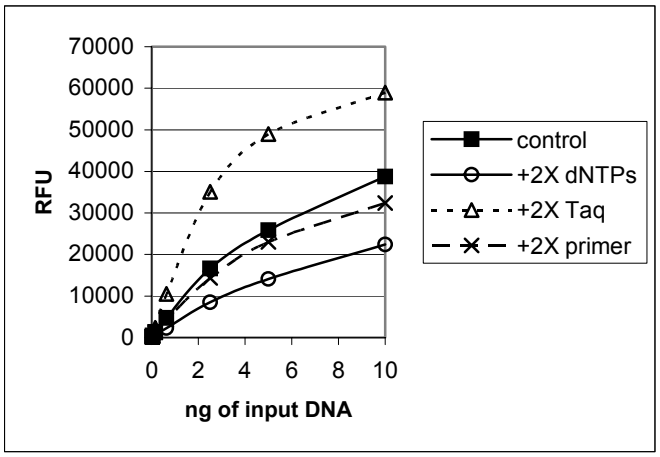



Figure 5 - *Effect of increasing primer, Taq or dNTP concentration*

Because the assay plateaus and even decreases at high input DNA, there could be concern that 5ng and 40ng could be confused because the final assay results would be identical; however, the zero time reading can discriminate between these amounts because the 40ng sample would have a much higher zero time reading. The zero time reading can, in essence, be used as a SYBR Green measurement of the amount of input DNA.

*Assay Validation*

An important part of assay validation is to determine if the assay is primate specific, i.e. that the assay gives negative results with non-primate DNA. Five primates, 13 commonly encountered animals as well as 3 bacteria, one insect and yeast were evaluated using

approximately 10 ng of each DNA. The primates gave the expected positive result but the animals, insect, bacteria and yeast were all negative (data not shown).

Another important point is to show that non-primate DNAs do not interfere with detection of human DNA. A mixing experiment of rat and human DNA (Figure 6) was performed where each well contained the same total amount of DNA (2.5ng) but with different ratios of human and rat DNA. This figure shows that as the percent human DNA increases, RFUs increase. The rat DNA neither contributed to the final reading nor inhibited the PCR reaction.

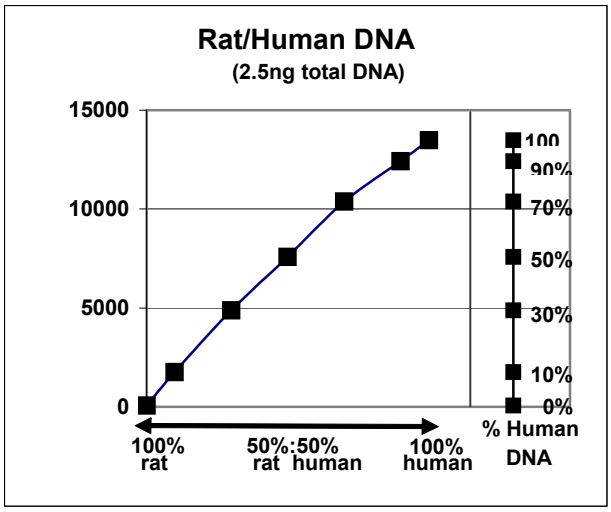
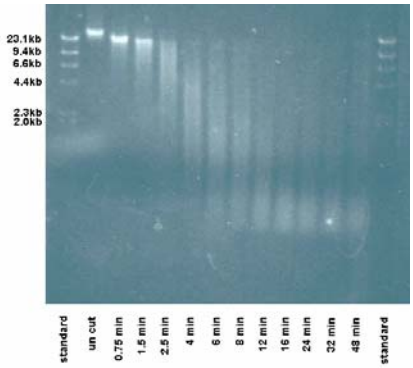



Figure 6- *Human/Rat Mixing Experiment*

Degraded human genomic DNA was created by treatment with DNAseI for varying lengths of time (0.75min to 48min) (Figure 7).

Figure 7 – Agarose gel of DNaseI treated DNA



These degraded samples were quantitated by slot blot and the QSY assay (Table 1). The samples were then diluted to 0.1ng/ul based on the results of the QSY assay, amplified using the COfiler™ STR kit (Applied Biosystems) and analyzed on an ABI 310. All of the results for the samples diluted based on the QSY results were within acceptable ranges for peak heights (150-5500 for each heterozygous peak) (data not shown). This indicates that the QSY assay successfully quantitated the amount of "PCRable" DNA within the degraded sample so that a correct prediction of the dilution of sample necessary for STR analysis could be made.

TABLE 1 - *Results on DNAse I Treated Samples*

| Sample Treatment | Slot Blot (ng/5ul) | QSY Assay (ng/5ul) | COfiler™ result - RFU Amelogenin X | COfiler™ result - RFU D7S820 allele 10 |
|---|---|---|---|---|
| None | 2.25 | 2.77 | 2777 | 842 |
| 0.75 min | 2.25 | 2.51 | 3648 | 886 |
| 1.5 min | 1.25 | 2.48 | 3614 | 678 |
| 2.5 min | 0.62 | 2.97 | 2090 | 505 |
| 4 min | 0.62 | 2.29 | 2216 | 268 |
| 6 min | 0.62 | 2.04 | 2043 | 245 |
| 8 min | 0.31 | 1.06 | 2911 | 363 |
| 12 min | 0.15 | 0.72 | 2665 | 262 |
| 16 min | 0.15 | 0.71 | 2284 | 164 |
| 24 min | 0.15 | 0.66 | 1932 | 165 |
| 32 min | 0.15 | 0.87 | 1559 | 211 |
| 48 min | 0.15 | 0.92 | 2094 | 398 |

Results from the slot blot assay were also compared to the results from the new QSY *Alu* PCR assay on blood and semen samples. A set of 13 blood spots, 6 mock crime samples, 24 samples of DNA placed on different surfaces with different treatments (RT, 37°C, sunlight) for different periods of time and 4 samples of DNA from colored cloths (denim) were studied. In general, the determined concentrations for the blood spots based on the slot blot or QSY assay results were close but the results for the degraded, environmental samples were much lower for the QSY assay. This is to be expected if the DNA is so degraded that PCR cannot occur. The

values for the denim cloth samples also deviated from expected clearly due to the known inhibitory effects of agents within denim.

All of the above samples were analyzed with the COfiler™ STR kit. Using 1ng of DNA based on the readings from the QSY experiment, all of the samples gave results on the ABI 310 within the acceptable range (150-5500 RFU for each heterozygous peak) except for one cloth sample that was high (cloth #1), and one sample from concrete (concrete -1 day).

To address the fact that the QSY assay appeared to give low concentration values that resulted in too much DNA being added to the COfiler™ amplifications for the denim samples, a final concentration of 250ug/ml BSA was added to the QSY assay. The addition of BSA did not change the values for the standard curve (data not shown) nor for samples such as blood spots; however, it did raise the concentration values for difficult samples such as denim, now making COfiler™ amplifications based on the QSY+BSA assay result in STR profiles within acceptable limits (data not shown).

Two inhibitors of PCR, EDTA and hematin, were evaluated. EDTA chelates the Mg++ needed by the polymerase. As the EDTA concentration increased in the assay, the resulting RFU values fell accordingly resulting in 50% inhibition at 0.75mM (data not shown). The zero time point was unaffected by EDTA; thus, inhibition could be detected as an incongruity between the zero time and final readings. Hematin is a derivative of heme that is known to inhibit PCR reactions. Fifty percent inhibition was observed at 0.5uM hematin without BSA in the *Alu* assay; however, it required increasing the hematin concentration to 25uM to give 50% inhibition when BSA was added to the assay as above.

While the ReadyMix™ kit worked well in this assay, it is expensive and for optimal assay performance requires the analyst to supplement the kit with additional SYBR Green. We

compared results obtained with the ReadyMix™ kit to that of a homebrew mix that costs approximately 60% of the ReadyMix™ kit/well. Although the absolute RFU values with the homebrew were slightly lower than that obtained with the ReadyMix™ kit, the assays are comparable and the homebrew mix may even give a more linear response. Depending on the laboratory's needs, the homebrew mix would be a viable alternative with only slight in-house modifications required, e.g. perhaps the addition of more polymerase, SYBR Green or Mg++.

The QSY +BSA *Alu* assay has many advantages over the current slot blot assay. It is reproducible; Table 2 shows the results of a standard curve performed in triplicate and with values and standard deviations for each datapoint. The assay is cost effective as use of a homebrew mastermix results in a test actually costing less than the ABI Quantiblot® kit in our hands. The QSY assay is also fast requiring approximately 1/2 hour of setup time, an hour of PCR amplification (during which the analyst can be performing other tasks) and then the quantitation results are immediately calculated by the plate reader software and printed out. Lastly, the dynamic range is larger than the slot blot assay using either colorimetric or luminescence readout.

TABLE 2 - *Results of triplicate QSY Alu assay experiment*

| input DNA | Mean Result (triplicates) | Standard Deviation |
|---|---|---|
| 20ng | 36958 | 1597 |
| 10ng | 36100 | 1384 |
| 5ng | 28899 | 273 |
| 2.5ng | 18562 | 300 |
| 1.25ng | 10419 | 169 |
| 0.62ng | 5234 | 253 |
| 0.31ng | 2692 | 28 |
| 0.15ng | 1266 | 38 |
| 0.078ng | 628 | 2 |
| 0.039ng | 337 | 50 |
| 0.02ng | 238 | 57 |
| 0.01ng | 131 | 43 |

That fact that *Alu*s are primate specific has been exploited by others to develop assays to detect human DNA. A recent paper reports use of an *Alu* PCR based system (Sifis et al., 1995) to quantitate human genomic DNA from 2.5 to 100pg. This system quantitated the samples using an ABI PRISM™ 377 Genetic Analyzer and GeneScan software using peak height (RFU) as the readout. The QSY assay developed here has several advantages over these systems in simplicity, analyst time and sensitivity.

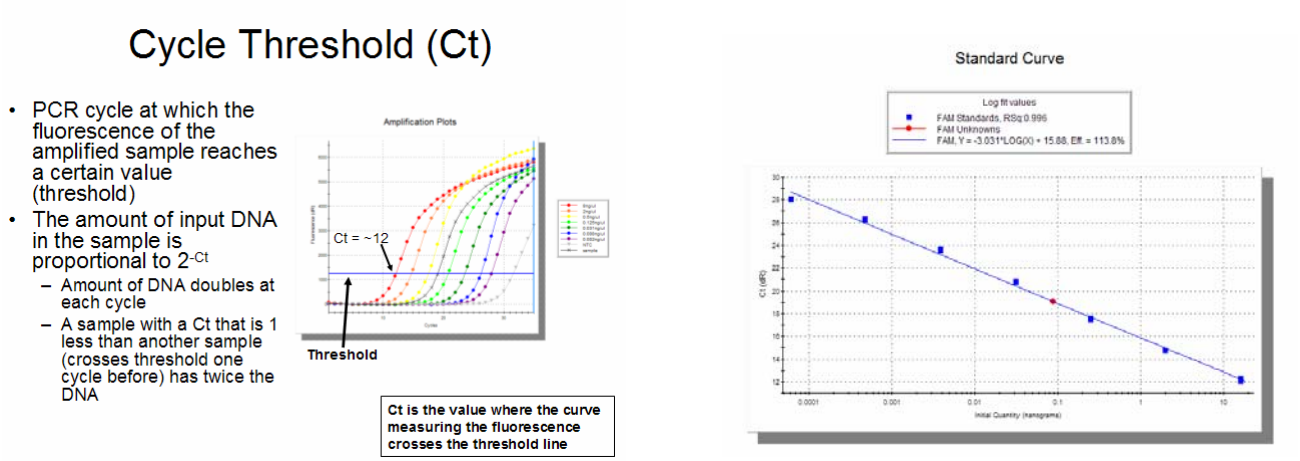
The QSY *Alu* assay has shown it can correctly determine the amount of human DNA in a wide range of samples and predict the proper dilution for STR analysis. Because both the QSY *Alu* assay and STR analysis are PCR based, the QSY *Alu* assay should have better predictive value for STR success than the slot blot assay. The QSY assay also gives additional information that the slot blot assay does not. Study of the initial zero time reading and comparison with the final result allows for determination of the presence of too much human DNA or the presence of animal DNA or inhibitors. The reference for the published paper detailing the results of the QSY assay is: Nicklas JA, Buel, E. Use of QSY *Alu* PCR for Quantitation of Human DNA in Forensic Samples. J Forensic Sci 2003; 48:282-91.

**Phase III – Real-time PCR assay**

As discussed above, the QSY assay had a number of limitations. First, it required quite a bit of hands-on manipulation. The plate had to be moved in and out of the PCR instrument and the plate reader several times and it had to be performed at reasonably precise times so that the technician was essentially tied to the instruments. Second, the dynamic range of the assay was not large, and in fact, not linear. The QSY primers were also expensive. We, thus, sought a faster, less hands-on, simpler and less expensive assay.

In real-time PCR, fluorescence measurements are taken at each cycle by an instrument designed as a thermocycler which can read the accumulated fluorescence from each PCR tube. The PCR cycle at which the fluorescence of the amplified sample reaches a certain value (threshold) is called the cycle threshold (Ct). The amount of input DNA in the sample is proportional to $2^{-Ct}$. In other words, a plot of the log input concentration versus the Ct value gives a straight line for a set of dilution standards with Ct decreasing as DNA concentration increases. The concentration of the unknown sample can be read off the standard curve from its Ct value.

Figure 8 - Real-time PCR assay results A) definition of cycle threshold in amplification plot showing Ct line and B) standard curve




The generation of fluorescence during PCR for use with real-time instruments can be divided into essentially two distinct methodologies. One relies on a dye intercalating with the accumulated PCR product and the other is a probe based technology. The dye typically used for intercalation is SYBR® Green I which is heat stable and is included in the PCR. As the PCR product is generated, SYBR® Green I binds to the DNA and an increase in fluorescence is observed. The specificity of the assay is based upon the selection of the primers and care in

design to avoid primer dimers which would also bind dye and yield unwanted fluorescence. The other approach uses dual labeled probes which will be discussed in more detail under grant #2.

We undertook to develop a real-time *Alu*-based Human DNA quantitation assay using the same PCR primer sequences used in the QSY assay and the SYBR Green intercalating dye. We used the Corbett Research MX2000 instrument that had been loaned to us by the NSFTC. This work was started under grant #1. The full development of the assay is described under grant #2.

**Supplement 1 - Simple, Rapid, and Accurate Quantitation of Human DNA**

**Background:**

As discussed above, in an attempt to find a quantitation method suitable for forensic casework analysis, we wrote and were awarded grant #1 to examine the possibility of improving the slot blot procedure and to determine if the procedure could be automated. Our approach was to migrate the slot blot procedure to that of a 96 well microplate with the results of the analysis read in a microplate reader. We utilized a plate with the normal microplate format except the bottom of the plate has been replaced by a Biodyne® B membrane. This Biodyne® B membrane is the same used in the Applied Biosystems QuantiBlot® human quantitation kit. In essence, DNA is bound to the membrane, a hybridization is undertaken with a human specific probe labeled with biotin/ horseradish peroxidase and a reporter molecule added to observe the extent of probe binding. A number of reporter molecules were examined and a fluorescent molecule (Amplex™ Red Molecular Probes, Eugene, OR) designed for use with horseradish peroxidase labeled probes was used to for most of our analyses.

We also tried a number of different probes for detecting DNA on the membrane. In addition to the D17Z1, which is the probe supplied with the QuantiBlot® kit, we have tried

probes based on *Alu* sequences and also a LINE element probe (see Table 3 for sequences). Briefly, the results we obtained from these experiments did not yield the results we desired. Reproducibility was a problem and the technique was not as sensitive as we had hoped (*Alu* AS and EBHRP1probes). Also, difficulties arose with cross-reaction with DNAs from non-primates such as dog, cat and rat (LINE probe). The MGB-Eclipse *Alu* probe gave a very high blue background on a slot blot. In addition, the overall process in using the microplates was very labor intensive and not amenable to automation using inexpensive microplate washers. Although we attempted other approaches to solve some of the problems we have encountered, we believed there was a better approach to solve the quantitation problem.

**TABLE 3 - Probes**

| **Probe** | **Sequence** |
|---|---|
| D17Z1 | B-TAG AAG CAT TCT CAG AAA CTA CTT TGT GAT GAT TGC ATT C |
| *Alu* - EBHRP1 | HRP-GCC ATT CTC CTG CCT CAG CCT CCC AAG TAG C |
| *Alu* – AS | HRP-GAT CAC GAG GTC AGG AGT TC |
| LINE | B-TAT AAA TCA TGC TAT AAA GAC ACA TGC ACA CGT ATG |

Key: HRP - horseradish peroxidase, B - Biotin

Another human quantitation method has been introduced by the Promega company. Their system, known as the AluQuant™ Human DNA Quantitation System, relies on a coupled enzymatic reaction linked to a probe that is specific to repetitive human sequences. Two enzymes are required to generate ATP from a probe bound to the human DNA contained in the sample. A luminometer is then used to read light generated from the luciferase-ATP reaction in the second incubation step of the assay. Promega states that the optimum quantitation range for this method is from 0.1 ng to 50 ng of DNA. The AluQuant™ procedure has the advantage over the slot blot procedure in that blotting or binding of the DNA to a fix surface is not required for this assay. However, the assay requires two separate samplings of each standard and crime scene sample, requiring more operator time and supplies and also requiring the use of additional

forensic sample. In addition, the coupling of three different enzymes in an assay makes it inherently prone to problems. All separate enzymatic reaction must work properly to obtain a result. We have tried this AluQuant™ procedure but had mixed results possibly due to the failure of one of the reagents. We did not perform any further examination of this kit.

We had preliminary data that suggested that a PCR approach to human DNA quantitation will allow for a quick, reproducible method that requires only limited hands on attention. We believe that there may be a number of ways that this technology could be implemented into a laboratory depending upon the resources available. Our preliminary results below show that such a method is an accurate, simple and rapid means to quantitate extracted DNA over a large dynamic range [40ng to 5pg (one cell)]. These PCR assays are specific for human nuclear DNA, non-subjective and reliable over a wide concentration range. The method should also be amenable to automation and should require only limited analyst attention. Such an accurate, specific and sensitive technique capable of evaluating a wide range of DNA concentrations would be of great assistance to the forensic community.

**Project goals:** 1) to determine alternative approaches to current methods for the quantitation of human DNA, 2) to evaluate each approach for ease of use, flexibility in analytical analysis and the ability to semi-automate the analysis, 3) to validate the method for use in forensic casework analysis.

**Methods:**

*Development of Assay*

Previous studies with the QSY assay demonstrated the specificity of the *Alu* primers and their use in a fluorescence plate reader assay. With these positive results, the assay was then moved to a real-time format and a real-time PCR instrument for quantitation. PCR utilized

the SYBR® Green JumpStart™ Taq ReadyMix™ kit (S4438, Sigma, St Louis, MO). Some experiments used the Brilliant™ SYBR® Green QPCR Master Mix (600548, Stratagene, La Jolla, CA). The 10ul reactions contained 5ul of 2X Sigma or Stratagene Master Mix (1X final), 0.2ul of 20 pmoles/ul each primer, 0.025ul of 1:100 (in DMSO, D-8779 Sigma, St. Louis, MO) SYBR® Green I (S7563, Molecular Probes, Eugene, OR), 0.4ul of 250ug/ml BSA (A-9647, Sigma, St. Louis, MO), 2.175ul distilled $H_20$ and 2ul of TE (10mM Tris, pH 7.5, 0.1mM EDTA) containing the input DNA at various concentrations. Some initial optimization experiments varied the concentrations of components such as SYBR® Green I or primers. The SYBR® Green I stock is a 10,000X concentrate. The 10,000X concentrate was diluted 1/100 in DMSO and either 0.025ul or 0.050ul was added to the 10ul reaction (0.25X final or 0.50X final concentration, respectively). Real-time PCR for the *Alu* assay was performed in a Corbett Research Rotorgene (Phenix Research, Hayward, CA) using the small 0.1ml tubes (MPCR-72, Phenix Research, Hayward, CA). PCR consisted of 95ºC for 2 minutes ("hotstart") followed by 35 cycles of 95ºC for 15 seconds, 68ºC for 30 seconds, 72ºC for 30 seconds. Various initial experiments changed annealing and/or extension times and temperatures to optimize the assay. A melt curve was also performed to check for specificity of the reaction. This consisted of 20 seconds at 72ºC followed by a ramp up of 1 degree step with 5 second hold at each step.

Previous studies with the plate reader assay has also indicated that it would be probably be necessary to add additional SYBR® Green I to the master mix because the large number of *Alu* PCR copies generated quickly bound all the available SYBR® Green I. The Sigma SYBR® Green JumpStart™ Taq ReadyMix™ contains a proprietary amount of SYBR® Green I. Additional SYBR® Green (diluted 1:100 in DMSO) was added to the reactions prior to PCR amplification to increase the amount of. SYBR® Green in the final assay above the proprietary

amount in the Sigma mix. Experiments using the SYBR® Green JumpStart™ Taq ReadyMix™ kit with 3 concentrations of SYBR® Green I were performed (no additional SYBR® Green I added to the Master Mix, additional 0.25X final concentration SYBR® Green I added and additional 0.50X final concentration SYBR® Green I added) (data not shown). Use of no additional SYBR® Green I caused loss of linearity at the higher DNA concentrations and also curve shape was significantly altered. Also, the fluorescence intensity was very low when no SYBR® Green I was added. The addition of SYBR® Green I did slow the PCR reaction (increased the cycle thresholds (Cts)) with the 0.50X having the greatest effect as expected. Thus, as with the plate assay, added 0.25X was chosen for the final assay. Our previous studies had also indicated that addition of BSA (10ug/ul) to the PCR overcame the effects of the inhibitors examined and made the assay more reflective of results with the STR assays, thus, 10ug/ul BSA was routinely added to the PCR mix. It did not change values for the real-time assay (data not shown).

The next studies focused on changing the annealing temperature. Two annealing temperatures, 60°C and 68°C were compared. As expected 68°C increases the Cts slightly; however, there was a dramatic effect on the melt curve with the higher temperature resulting in a much sharper peak. Since *Alu*s are a family with many related but not identical members, a lower annealing temperature allows amplification of many diverse sequences while the higher temperature selects a more uniform product. The 68°C anneal was thus chosen for the assay.

A denaturation time of 15 seconds, an annealing time of 1 minute and an extension time of 1 minute (1min/1min) were initially used for the assay. However, theoretically with a small PCR product like the 124bp *Alu* PCR in these experiments, shorter times should be sufficient. A comparison was made between 30 second anneal and extension with 60 second anneal and

extension. The assays were very similar except that the longer anneal experiments had slightly lower Cts. Experiments using 30 second anneal with 60 second extension as well as 60 second anneal with 30 second extension were also performed with results intermediate to the 30 second anneal and 30 second extension and the 60 second anneal and 60 second extension (data not shown). Since the results were similar for all cases, and the 30 second anneal and 30 second extension time saved 35 minutes per assay over use of 60 seconds for each, the shorter times were chosen for the assay.

The effect of changing primer concentrations was also investigated. The forward and reverse primer concentrations were varied independently (0.2uM to 0.8uM). While increasing the primer concentrations 4 fold did decrease the Ct slightly (~0.4 Ct) with a slightly greater decrease for the forward primer, the effects were not significant.

*Assay Validation*

An important part of assay validation is to determine if the assay is primate specific, i.e. that the assay gives negative results with non-primate DNA. Three primates, 12 commonly encountered animals as well as 3 bacteria, one insect and yeast were evaluated using approximately 10ng of each DNA. The primates gave the expected positive result but the animals, insect, bacteria and yeast were all essentially negative (i.e. gave Cts approximately that of the no template control (27.44) and or at least close to the Ct of 1pg of human DNA (21.5)).

Another important point is to show that non-primate DNAs do not interfere with detection of human DNA. A mixing experiment of rat and human DNA was performed where each well contained the same total amount of DNA (2ng) but with different ratios of human and rat DNA. The theoretical values for the amount of added human DNA are shown as grey triangles. As the percentage of human DNA increases, Cts decrease accordingly with very good

agreement between the expected, theoretical and the observed values. The rat DNA neither contributed to the final reading nor inhibited the PCR reaction.

Degraded human genomic DNA was created by treatment with DNAseI for varying lengths of time (0.5 min to 256 min). These degraded samples were quantitated by slot blot and the real-time *Alu* assay. The *Alu* assay gave higher values for DNA concentration especially at longer digestion times; this is probably the result of small fragments not binding to the slot blot membrane (data not shown). The samples were then diluted to 0.1ng/ul based on the results of the *Alu* assay, 1.0ng of template DNA was amplified using the COfiler™ STR kit and the product analyzed on an ABI 310. All of the results for the samples diluted based on the *Alu* results were within laboratory acceptable ranges for peak heights (150-5500 RFU for each heterozygous peak) for the THO1 locus; however, the results for the highly degraded DNAs for the D7S820 locus were <150 RFU (data not shown). This is to be expected as the 124bp *Alu* product will successfully predict amplification of similarly sized STR products such as THO1 (~ 175bp) but not for the larger D7S820 product (~275bp) in a highly degraded sample. These results indicate that the *Alu* assay successfully quantitated the amount of "PCRable" DNA within a moderately degraded sample so that successful dilution of the sample for STR analysis could be made.

Results from the slot blot assay were also compared to the results from the new real-time *Alu* assay from a wide variety of samples types. Table 4 lists the *Alu* assay versus slot blot results for these different sample types. Figure 9 plots the real-time assay results against the slot blot results for the 50 samples in Table 4. The slope of the least squares regression line is 1.0076 indicating that on average the assays give the same result although there is a bit of scatter [correlation coefficient (R=0.698), coefficient of determination ($R^2$=0.4874)].

TABLE 4 – *Results for various samples*

| Sample | Slot blot result (ng/ul) | *Alu* assay result (ng/ul) | THO1 (RFU) (smaller allele) | D7S820 (RFU) (smaller allele) |
|---|---|---|---|---|
| Databank #1 | 0.44 | 0.322 | 2110 | 715 |
| Databank #2 | 0.40 | 0.941 | (1012)[a] | (399)[a] |
| Databank #3 | 0.04 | 0.193 | 1706 | 449 |
| Databank #4 | 0.12 | 0.283 | 1482 | 738 |
| Databank #5 | 0.24 | 1.096 | 485 | 192 |
| Databank #6 | 0.24 | 0.461 | 1113 | 413 |
| Databank #7 | 0.03 | 0.057 | IS[b] | IS[b] |
| Proficiency test 01-02 | 0.7 | 0.258 | 2380 | 1714 |
| Proficiency test 01-03 | 0.4 | 0.339 | 1824 | 751 |
| Proficiency test 01-04 | 1.0 | 0.522 | 1951 | 712 |
| Female fraction – F [c] | 0.08 | 0.093 | 1193 | 708 |
| Female fraction - G | 0.4 | 0.664 | 1679 | 768 |
| Female fraction - H | 0.5 | 0.670 | (1008)[a] | 893 |
| Female fraction - I (dilution 1) | 0.06 | 0.064 | IS[b] | IS[b] |
| Female fraction – I (dilution 2) | 0.5 | 0.548 | (1116)[a] | 884 |
| Female fraction – J | 0.6 | 0.612 | 1435 | 809 |
| Male fraction – E | 0.48 | 0.425 | 1352 | 761 |
| Male fraction – G | 0.24 | 0.428 | 2194 | 675 |
| Male fraction – H | 0.01 | 0.012 | IS[b] | IS[b] |
| Male fraction – J | 0.4 | 0.207 | 2386 | 728 |
| Standard - I | 0.1 | 0.121 | (2941)[a] | 760 |
| Standard – J | 0.02 | 0.02 | IS[b] | IS[b] |
| Computer keys swab #1 [c] | 0.1 | 0.079 | 536 | 105 |
| Computer keys swab #2 | 0.2 | 0.208 | 1510 | 560 |
| Inside glove swab #3 | <0.03 | 0.001 | IS[b] | IS[b] |
| Phone swab #1 [c] | 0.03 | 0.016 | 227 | 87 |
| Phone swab #2 | 0.2 | 0.109 | 1186 | 575 |
| Phone swab #3 [c] | <0.03 | 0.007 | No peaks | No peaks |
| Envelope seal #1 | 0.2 | 0.262 | 1238 | 391 |
| Envelope seal #2 | 0.16 | 0.195 | 1014 | 403 |
| Envelope seal #3 [c] | 0.06 | 0.046 | 772 | 279 |
| Swab of fingerprint #1 | <0.03 | 0.000 | IS[b] | IS[b] |
| Swab of fingerprint #2 [c] | 0.03 | 0.037 | 371 | (193)[a] |
| Swab of fingerprint #3 | <0.03 | 0.001 | IS[b] | IS[b] |
| Blood spot #1 – 3 mo in dark | 0.6 | 0.888 | 1388 | (653)[a] |
| Blood spot #2 – 3 mo in dark | 0.5 | 0.901 | 1602 | 830 |
| Blood spot #1 – 3 mo in sunlight | 1.0 | 1.784 | 1034 | (290)[a] |
| Blood spot #2 – 3 mo in sunlight | 0.2 | 0.377 | 1189 | 418 |
| Blood spot #1 – 3 mo at 37°C | 0.4 | 0.689 | 853 | (236)[a] |
| Blood spot #2 – 3 mo at 37°C | 0.7 | 1.671 | 958 | 280 |
| Swab of blood on stick | 0.5 | 0.325 | 1883 | 748 |
| Swab of blood on metal | 0.5 | 0.401 | 1893 | 883 |
| Swab of blood on concrete | 0.5 | 0.402 | 1860 | 945 |
| Swab of blood on leaves [c] | 0.2 | 0.084 | 1540 | 887 |
| Swab of blood on cardboard | 0.24 | 0.266 | 1450 | 715 |
| Swab of blood on soapy cloth [c] | 0.05 | 0.039 | 577 | 216 |
| Blood on denim #1 | 1.0 | 0.245 | 1237 | 1060 |
| Blood on denim #2 | 1.0 | 1.696 | 1847 | 874 |
| Blood on denim, 6 weeks in dark | 1.0 | 0.606 | 1274 | 980 |
| Blood on denim #3 | 0.36 | 0.657 | 1314 | 676 |

[a] numbers in parenthesis are ½ peak height of single peak (homozygote) [b]IS = insufficient sample to perform STRanalysis [c]Used neat because original concentration was below 0.1ng/ul

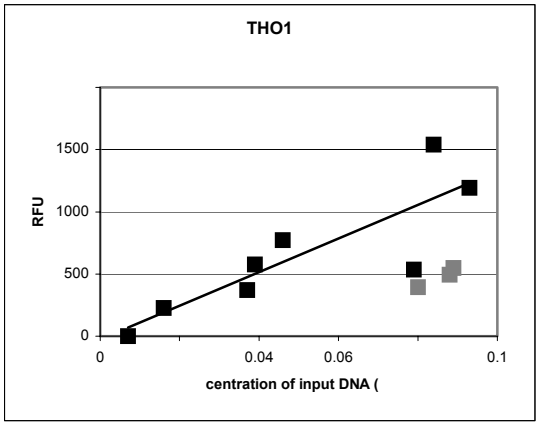
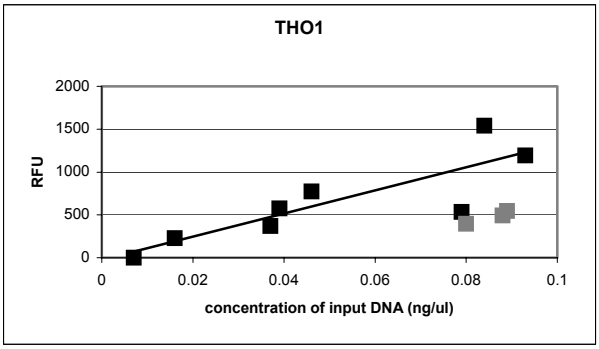



Figure 9 – Plot of Quantiblot® versus *Alu* PCR results

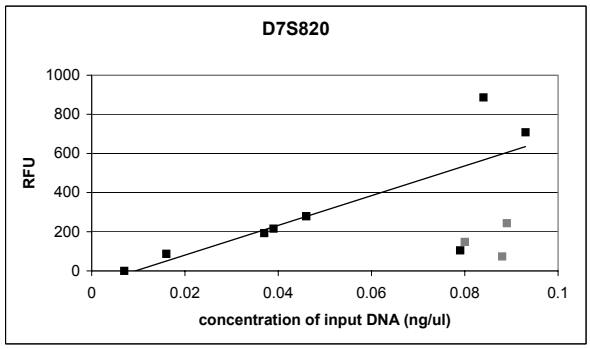
Most of the above samples (those with sufficient DNA) were analyzed with the COfiler™ STR kit. For samples where the sample concentration was above the usually used 0.1ng/ul, samples were diluted to 0.1ng/ul and 10ul (1.0 ng total) was used for COfiler™ STR analysis. All of these samples gave results on the ABI 310 within the laboratory's acceptable range (150-5500 RFU for each heterozygous peak) and Table 4 shows the results for the THO1 and D7S820 loci. For those 13 samples below the 0.1ng/ul level, the samples were amplified using 10ul of the neat sample. The THO1 and D7S820 results for these samples are shown in Figure 10A and 10B, respectively. The DNAse I treated samples are shown in grey and were not used to generate the shown trendline. A linear relationship between initial concentration and RFU was found. From these results, an *Alu* assay measurement of approximately 0.04ng/ul (i.e. 0.4ng input DNA) will be required to guarantee a RFU >150 (laboratory cutoff) for the D7S820 locus. These results indicate that the *Alu* assay is able to correctly quantitate DNA even at low concentrations. One sample (computer keyboard #1, concentration 0.079ng/ul) did give unexpectedly low RFUs; this sample gave results similar to the DNAseI treated DNAs; thus, this sample was apparently highly degraded.

Figure 10 –STR results for samples with concentrations less than 0.1ng/ul. A.THO1, B. D7S820

A.




B.




The reproducibility of the assay was also investigated in several ways. Quadruplicates of the standard curve performed on the same day had percent standard deviations for the Ct values of less than 1.4% (except for the NTC which was 3.8%). Seven samples repeated three times over 3 days had percent standard deviations for the Ct values less than 2% (data not shown) while the percent standard deviation for the concentration values ranged from 7 to 19% (Table 5). In terms of expected variation and reproducibility, standard deviations for Ct values are quoted 0.05 in the Corbett Research brochure and values of 0.12-0.28 have been reported in other sources (Bio-Rad Laboratories, Livak and Schmittgen, 2001). For concentration values, Bustin reported variability of between 0 and 5% between different runs and %CV differences of 20-30% between different kits or probe lots on concentration values (Bustin, 2000, 2002). As shown in Table 5, the duplex assays compares favorably with these values. Lastly, an experiment testing three lots of the Sigma ReadyMix™ showed comparable results with the different lots (data not shown).

TABLE 5 - *Repeats of 7 samples on 3 different days*

| Sample | Mean Concentration | stdev | %stdev |
|---|---|---|---|
| Male fraction – J | 0.43 | 0.05 | 11.36 |
| Female fraction – E | 2.35 | 0.17 | 7.25 |
| Blood on denim | 0.47 | 0.06 | 12.33 |
| Swab of blood on leather | 0.18 | 0.02 | 10.25 |
| Databank#5 | 0.96 | 0.15 | 15.80 |
| Proficiency test# 01-02 | 0.75 | 0.14 | 19.29 |
| Envelope seal #2 | 0.37 | 0.03 | 8.87 |

Assay performance with addition of hematin an inhibitor of PCR was evaluated. Hematin is a derivative of heme that is known to inhibit PCR reactions. Fifty percent inhibition was observed at ~15uM hematin (data not shown).

The Stratagene Brilliant™ SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA) and the Sigma SYBR® Green JumpStart™ Taq ReadyMix™ kit were comparison tested on a large number of samples. For reasons that are not understood, the concentration values with the Brilliant master mix were much lower than those obtained with the Sigma master mix and much lower than those obtained with the slot blot technique (on average 0.29) (data not shown); thus, further use of the Brilliant kit was discontinued.

The real-time *Alu* assay has many advantages over the current slot blot assay and also advantages over the *Alu* plate reader assay. The assay is cost effective, as use of 10ul reactions results in a test actually costing less than the ABI Quantiblot® kit in our hands. The real-time *Alu* assay is also fast, requiring approximately 1/2 hour of setup time, 72 minutes of PCR amplification (during which the analyst can be performing other tasks) and then the quantitation results are immediately calculated by the real-time instrument software and can be printed out. Also, the dynamic range is larger than the slot blot assay using a colorimetric readout. Lastly, variation of assay conditions such as annealing temperature, primer concentrations, or extension

time do not have any major effect on the assay, suggesting it may be robust over the variations seen between instruments or laboratories. Advantages over the plate reader assay include: no need for QSY labeled primers, no need to move plates back and forth between instruments, use of less reagents and a greater dynamic range. Because both the A*lu* assay and STR analyses are PCR based, the *Alu* assay should have better predictive value for STR success than the slot blot assay. The published manuscript for the real-time SYBR, *Alu* assay is: Nicklas JA, Buel E. Development of an *Alu*-based, real-time PCR method for quantitation of human DNA in forensic samples. J Forensic Sci 2003; 48:936-44.

We have incorporated this assay into casework analysis in the VFL since July, 2003. It has proved very valuable, saving a great deal of analyst time and providing more accurate results than Quantiblot.

Real-time PCR with probes can offer an increase in specificity and greater dynamic quantitative range. The dual labeled probes rely on a process known as FRET, fluorescence resonance energy transfer. When a fluorescent molecule is excited with by a light source, the molecule can emit light at a longer wavelength or the energy can be transferred to another molecule. This transfer of energy between one molecule, the fluor, and another molecule, the quencher, occurs within a defined distance. If the intermolecular distance is too great, no transfer of energy occurs and the energy absorbed by the fluor is transmitted as fluorescence. When the fluor and the quencher are in close proximity, the energy is transferred to the quencher, that can then release the energy in the form of light at a longer wavelength or it can be released as heat. Creating fluorescence through and “increase” in fluor, quencher distance during a PCR to indicate the amount of product can be achieved through a number of different mechanisms. Two popular approaches involve a digestion of the probe to release the fluor from the probe after

binding to a target sequence (TaqMan®) (Figure 11) and the other is a molecular beacon approach where the probe and quencher in solution are in close proximity but upon the probe binding to the DNA complement sequence the distance is increased to allow observable fluorescence.

We investigated both the TaqMan and the Eclipse (Molecular Beacon-like, Figure 12) methods.

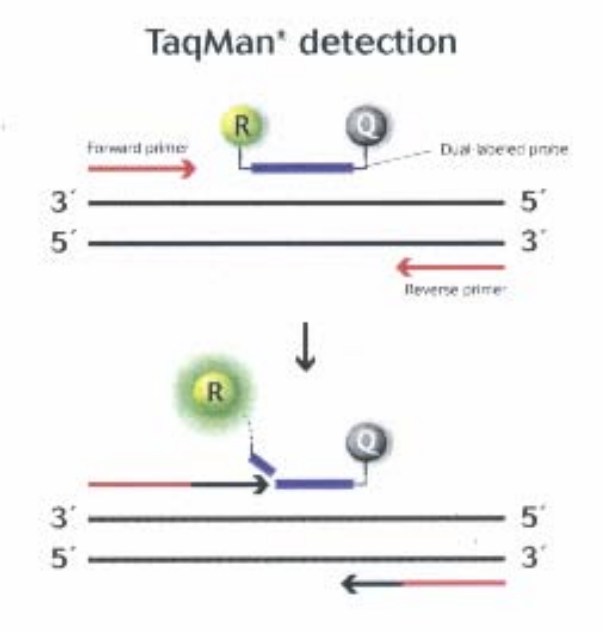



Figure 8 – Real-time TaqMan method (Applied Biosystems)

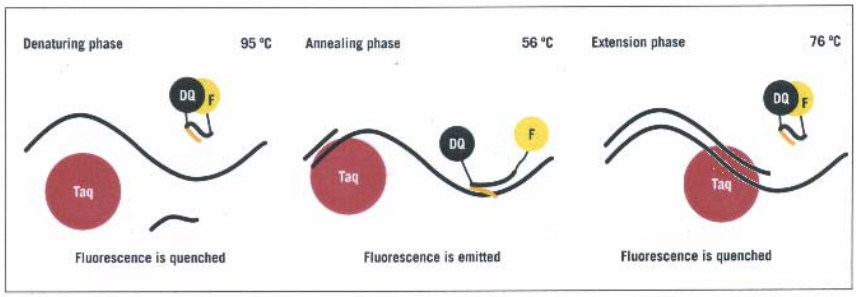



**Fig 2.** The MGB Eclipse Probe System mechanism of action involves three stages for each PCR cycle. During the initial phase, MGB Eclipse Probes assume a random coil at denaturing temperatures due to the hydrophobicity of the MGB. Fluorescence from the reporter dye is quenched by the proximity of Eclipse Dark Quencher. During the annealing phase, the MGB folds back into the minor groove of the resulting DNA duplex formed by the probe and target to stabilize hybridization. Additionally, Eclipse Dark Quencher is spatially separated from the reporter, allowing fluorescence. Following annealing, MGB Eclipse Probe is melted from its target during the primer extension phase, which allows more efficient primer extension.

Figure 9 - Real-time Eclipse

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*Development of Assay*

The *Alu* PCR primers 5'-GAG ATC GAG ACC ATC CCG GCT AAA-3' (forward) and 5'-CTC AGC CTC CCA AGT AGC TG-3' (reverse) and the Eclipse™ probe 5'-GGG CGT AGT GGC GGG-3' (FAM fluor - Eclipse™ quencher) were designed by and purchased from Synthetic Genetics (now Epoch Biosciences, Bothell,WA) from the sequence of plasmid p*PD39* (Ya5 subfamily) (Batzer et al., 1994). These PCR primers generate a 113 bp product that was confirmed on an agarose gel (data not shown). PCR utilized JumpStart™ Taq DNA polymerase (Sigma) and the Eclipse™ 10X buffer (Epoch Biosciences). The 10 µl reactions contained 1 µl of Eclipse™ 10X buffer (1X final), 0.5 µl of primer mix (0.1 µM forward, 1 µM reverse), 0.5 µl probe, 0.4 µl of 6.25mg/ml BSA (A-9647, Sigma), 1.6 µl of 2.5mM each dNTP (Applied Biosystems), 0.08 µl JumpStart™ Taq DNA polymerase (D4184, Sigma, 2.5 U/µl), 3.92 µl distilled $H_20$ and 2 µl of TE (10 mM Tris, pH 7.5, 0.1 mM EDTA) containing the input DNA at various concentrations. Some initial optimization experiments varied the concentrations of components such as Taq polymerase, primer or probe. Some experiments tested pre-made mastermixes [Jumpstart™ Taq ReadyMix™ polymerase (P2893, Sigma), Platinum® PCR SuperMix (11306-016, Invitrogen, Carlsbad, CA), HotMasterMix (954 14 018-1, Eppendorf, Hamburg, Germany)]. Real-time PCR for the *Alu* assay was performed in a Corbett Research Rotorgene using the small 0.1 ml tubes. PCR consisted of 95ºC for 2 min ("hotstart") followed by 40 cycles of 95ºC for 15 sec, 56ºC for 20 sec, 72ºC for 30 sec. Various initial experiments changed annealing and/or extension times and temperatures to optimize the assay. A melt curve was also performed after the assay to check for specificity of the reaction. This consisted of one cycle of 95°C for 15 sec, 56°C for 20 sec followed by a ramp up of 1 degree/step with 5 sec hold at each step.

Mentioned in the paragraph above) Real-time PCR using the Eclipse™ probe resulted in consistent Ct values for duplicates and large dynamic range (128 ng/µl to 0.5 pg/µl) on standard curves (Figure 13*A* and 13*B*). As with the SYBR® Green assay (Nicklas and Buel, 2003), the no template control (NTC) had a Ct of ~31, presumably because of a very low amount of ambient human DNA in the air and water.

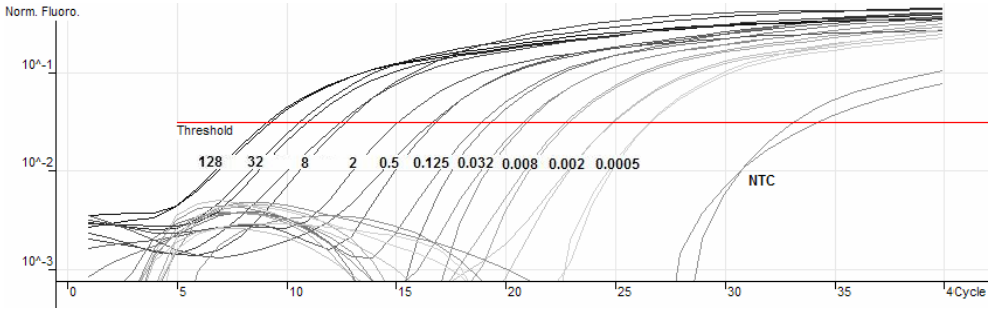



Figure 13A - Eclipse™ *Alu* real-time PCR assay results for standard curve dilution series of human DNA. Normalized RFU are plotted versus cycle number for 11 serial 1:4 dilutions of human DNA (128ng/µl, 32ng/µl, 8 ng/µl, 2 ng/µl, 0.5 ng/µl, 0.125 ng/µl, 0.032 ng/µl, 0.008 ng/µl, 0.002 ng/µl, 0.0005 ng/µl) plus a no template control (NTC).

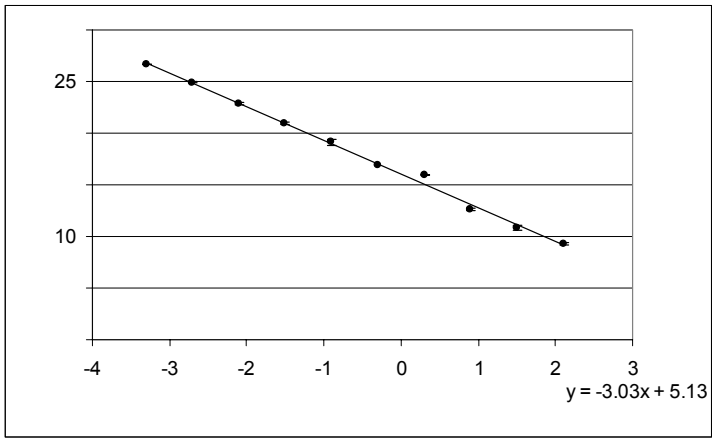



Figure 13*B* - Using the Ct values at a threshold of 0.02 generates a standard curve ($R^2$ = 0.999). The slope of the standard curve (m=-3.31) indicates the reaction has an efficiency of 100%.

The next studies attempted to improve the assay (save on reagents, shorten experiment running, increase specificity) by varying the DNA range in the standard curve, annealing time and temperature, extension time, polymerase concentration and primer concentration. The first studies focused on changing the annealing temperature. Annealing temperatures, of 52°C, 54°C,

56°C, 60°C, 64°C and 68°C were compared. In the SYBR® Green assay increased annealing temperature caused a narrowing of the melting peak, probably due to the fact that *Alu*s are a family of related sequences; lower annealing temperatures allow amplification of slightly more distantly related species that, thus, will generate a broader melting curve. In contrast, using just the Eclipse™ primers in a SYBR® Green based assay (no probe added) gave essentially equivalent amplification and melting curve profiles (87°C peak) with all of the annealing temperatures tested (data not shown). This is possibly due to increased specificity of the Eclipse™ primers. However, when the Eclipse™ probe was used in a full Eclipse™ assay, each increase in the annealing temperature decreased the RFU until the reaction essentially failed at 68°C. Increasing the annealing temperature had no effect on the melting curve of the Eclipse™ product (data not shown). Differences in the mechanism of the two assays perhaps account for these findings; there is melting of the entire PCR product in the SYBR® Green assay whereas in the Eclipse™ assay, only melting of the probe with the PCR product is measured. Furthermore, the minor groove binder (MGB) may also affect the melting. The manufacturer's recommended 56°C annealing temperature was chosen for the final assay because increasing the temperature did not enhance the specificity (SYBR® Green-based primer study) but did cause RFUs to decline. However, the annealing temperature probably could be increased to 60°C without any detriment to the assay and a possible saving in a few minutes in assay length.

A denaturation time of 15 sec, an annealing time of 20 sec and an extension time of 30 sec were initially used for the assay as per vendor recommendations. Experiments were performed comparing annealing times of 10, 20 and 40 sec. Little difference in Ct was observed between the annealing times (data not shown); therefore, the recommended 20 sec was chosen for the final assay. Experiments comparing extension times of 15, 30 and 60 sec (data not

shown) revealed a slight decrease of Ct and plateau RFU with decreased extension time; again the 30 second recommended time was chosen for the final assay.

Absolute amounts of primer and probe were varied to determine optimal concentrations. Probe concentrations of 50 nM, 100 nM, and 200 nM and primer concentrations (forward:reverse) of 25 nM:250 nM, 50 nM:500 nM and 100 nM:1000 nM were independently varied for a single fixed DNA concentration (2 ng/μl). For a fixed probe concentration, as the primer concentration decreased, the Ct increased about 0.8 cycles from the highest to lowest primer concentration. For a fixed primer concentration, as the probe concentration increased, the Ct also increased about 0.4 cycles. The first is understandable as decreased primer could slow the reaction and thus increase the Ct. Why the decrease in probe causes a decrease in Ct is not so clear. The probe does come in a buffer; it is possible that this buffer may have an effect on the amplification independent of the effect of probe. This was a reproducible effect as it was observed in an additional experiment that tested the probe at three concentrations (100 nM, 200 nM, 400 nM) over a standard curve DNA dilution series. The Ct values for the 400 nM probe were on average 0.80 more than with the 200 nM probe which was on average 0.40 greater than the average for the 100 nM probe. Since the Ct increased substantially with the 400 nM probe, the recommended 200 nM probe was chosen for the final assay.

When the original primer mix was obtained from Synthetic Genetics (now Epoch Biosciences), it came as a 20X 1:10 mix (2 μM forward primer: 20 μM reverse primer). However, separately packaged primers were also obtained and tested in ratios of 0.05uM:1uM, 0.1 uM:1 uM, 0.2 uM:1 uM, 0.5 uM:1 uM and 1 uM:1 uM final concentration. Figure 14 shows that the RFU decrease with increased forward primer concentration. This is to be expected as extension off the forward primer will displace the Eclipse™ probe from the PCR

product, reducing signal or possibly the increased primer sequesters Mg++, reducing polymerase efficiency. However, the Ct values remained fairly stable across the different ratios (data not shown). The recommended 1:10 ratio was therefore chosen for the assay. Of note, currently Epoch Biosciences now ships primer mixes as a 20X 1:1 mix. An experiment comparing the 1:1 ratio versus the 1:10 ratio on eight samples was therefore performed. While the raw data had slightly different characteristics and the Ct values were perhaps slightly lower with 1:1 primer ratio, the $R^2$ values for the standard curves and the PCR efficiencies were identical (data not shown). The differences in calculated concentrations at 1:10 and 1:1 for the 8 samples were within replicate and day to day variation (data not shown).

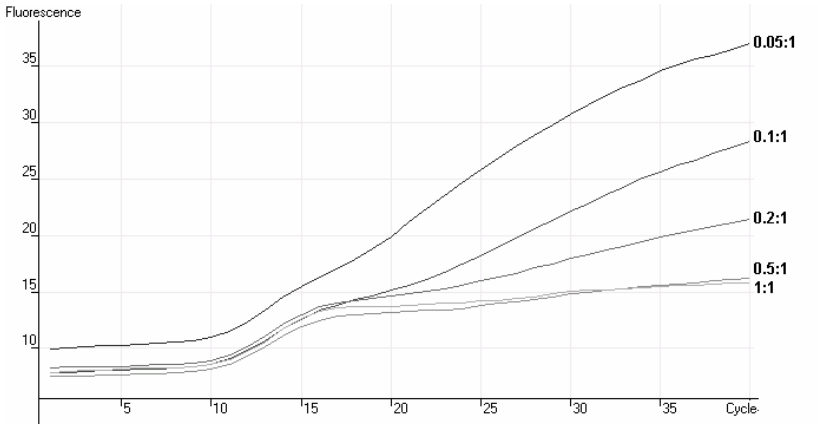



Figure 14 – Raw RFU data for 2 ng/µl input human DNA plotted versus cycle number for Eclipse™ real-time PCR assays performed at different ratios of forward to reverse primer (0.05 µM:1 µM, 0.1 µM:1 µM, 0.2 µM:1 µM, 0.5 µM:1 µM, 1 µM:1 µM). RFU values decrease as the amount of forward primer is increased.

In general, the above experiments showed that Epoch Biosciences suggested conditions were essentially optimal. While the experiments indicated that less probe or primer or shorter cycle times could be utilized in our laboratory with our thermal cycler, since the intent was to develop this assay for a wide audience, we chose to be conservative and pick temperatures and concentrations far from any values where the assay deteriorated, i.e., values that would be robust in any laboratory.

One important deviation from Epoch Bioscience's suggested protocol that was determined from experimental results was the use of a lower Taq concentration. The original methods received from Synthetic Genetics suggested using 0.4 U per 10 µl reaction; however, more recent directions from Epoch Biosciences suggested using 0.8 U per 10 µl reaction. Initial experiments performed here used 0.4 U/10 µl reaction; however, experiments were performed to titrate down the amount of enzyme for cost considerations. Initial experiments showed paradoxically that Ct decreased about 0.6 cycles as Taq polymerase concentration was lowered from 0.4 U/10 µl reaction to 0.05 U/10 µl reaction (data not shown). An experiment was performed to determine if Taq polymerase concentration affected the results with difficult samples (blood on denim or degraded DNA). Table 6 shows results of parallel experiments using 14 samples at 0.4 U/10 µl reaction or 0.2 U/10 µl reaction. The variations seen in Table 6 between the two concentrations were within replicate and day to day variation of the assay [see below]. The higher Taq polymerase concentration did not result in higher determined concentrations for the difficult samples. Thus, 0.2 U/10 µl reaction was chosen as the assay concentration because Taq is the major cost consideration for the assay and less Taq actually seemed to decrease Ct.

*Assay Validation*

An important part of assay validation is to determine if the assay is primate specific, i.e. that the assay gives negative results with non-primate DNA. Three primates, twelve commonly encountered animals as well as three bacteria, one insect and one yeast were evaluated using approximately 5ng/ul DNA of each DNA. The primates gave the expected positive result while the others all yielded Ct values ranging between that obtained for the NTC to that observed for the 0.5 pg/µl human DNA sample.

TABLE 6 – Differences in Eclipse™ assay results (ng/µl) for 11 samples using varying amounts of Taq polymerase

| Sample | 0.4 U Taq/ 10 µl reaction | 0.2 U Taq/ 10 µl reaction |
|---|---|---|
| Denim #1 | 2.07 | 2.96 |
| Denim #2 | 0.33 | 0.36 |
| Denim #3 | 11.8 | 7.8 |
| Cloth #1 | 0.67 | 0.50 |
| Cloth #2 | 0.79 | 0.88 |
| Blood on soapy cloth | 0.07 | 0.08 |
| Blood on stick | 0.77 | 0.71 |
| Blood on leather | 0.10 | 0.15 |
| Blood on metal | 0.78 | 0.89 |
| Blood on cardboard | 0.39 | 0.57 |
| female fraction J | 0.68 | 0.73 |
| Blood, 3 mo dark | 0.76 | 1.33 |
| databank #1 | 0.45 | 0.89 |
| databank #2 | 0.96 | 0.75 |

Another important point is to show that non-primate DNA does not interfere with detection of human DNA. A mixing experiment of rat and human DNA (Figure 15) was performed (triplicates) where each well contained the same concentration of human DNA (1.0 ng/µl) but with different concentrations of rat DNA from 10ng/ul to 0.0 ng/ul (8 fourfold dilutions from 10ng/ul to 0.025ng/ul plus no added rat DNA). This figure shows that the measured concentration of human DNA is essentially constant as the amount of rat DNA increases. Thus, the rat DNA neither contributed to the final reading nor inhibited the PCR reaction.

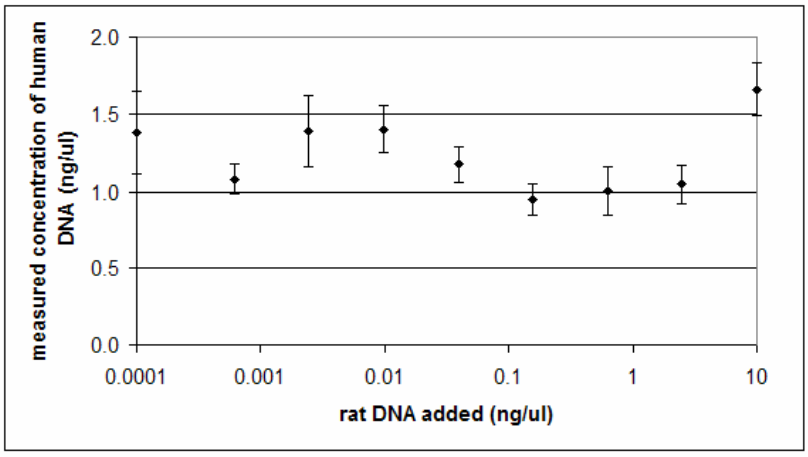



Figure 15– Rat and human DNA mixture Eclipse™ real-time PCR assay. Plot of measured amount of human DNA versus the input rat DNA. All assays contained the same 1.0ng/µl of human DNA but with different concentrations of added rat DNA (0.0ng/ul, 0.025ng/ul, 0.01ng/ul, 0.04ng/ul, 0.15ng/ul, 0.63ng/ul, 2.5ng/ul, 10ng/ul). Error bars for triplicates are shown.

The new real-time Eclipse™ assay was compared to the SYBR® Green assay on a wide variety of samples types (Table 7 and Figure 16). Results were quite similar for both assays except for two datapoints (blood on stick and denim#2). Repeats of the blood on stick samples brought the two values much closer, 0.51 ng/µl and 0.63 ng/µl, respectively, suggesting that the original values were both just statistical extremes in opposite directions. For the denim#2 sample, repeats increased both concentration determinations (2.18 ng/µl and 6.33 ng/µl, respectively). Examination of the raw data curves showed that the curve shapes were flat, a symptom of the presence of an inhibitor which explains the inconsistent results and differences between the assays for this sample.

TABLE 7 – Comparison of SYBR® Green assay and Eclipse™ assay concentration results for samples and results of STR analysis with the COfiler™ kit. DNA dilutions for the STR analysis based on Eclipse™ assay concentration results

| Sample | SYBR® Green *Alu* assay result (ng/µl) | Eclipse™ *Alu* assay result (ng/µl) | COfiler™ *THO1* peak height | COfiler™ D7S180 peak height |
|---|---|---|---|---|
| Databank #1 | 0.57 | 0.48 | 1379 | 703 |
| Databank #2 | 0.89 | 0.91 | (1341)[a] | 462 |
| Databank #3 | 0.87 | 1.17 | (2441)[a] | 1091 |
| Databank #4 | 2.09 | 1.62 | 2390 | 901 |
| Databank #5 | 2.31 | 2.43 | 1317 | 771 |
| Proficiency #1 | 0.18 | 0.15 | 1635 | (957)[a] |
| Proficiency #2 | 0.96 | 1.19 | 1523 | (770)[a] |
| Female fraction F | 0.22 | 0.32 | 1352 | 763 |
| Female fraction J | 0.69 | 0.65 | 1543 | 845 |
| Male fraction D | 0.053 | 0.055 | 724 | 427 |
| Male fraction 01-6-1 | 0.046 | 0.039 | not done[b] | not done[b] |
| Blood, 3 mo dark | 0.73 | 0.79 | 1175 | (462)[a] |
| Blood on stick | 0.31, 0.51 | 0.78, 0.63 | 757 | 339 |
| Blood on metal | not done[b] | 0.65 | 1074 | 502 |
| Denim #2 | 1.46, 2.18 | 4.21, 6.33 | 2576 | 1958 |
| Cloth #1 | 0.47 | 0.55 | 928 | 681 |

[a]value is half of homozygous peak
[b]insufficient sample

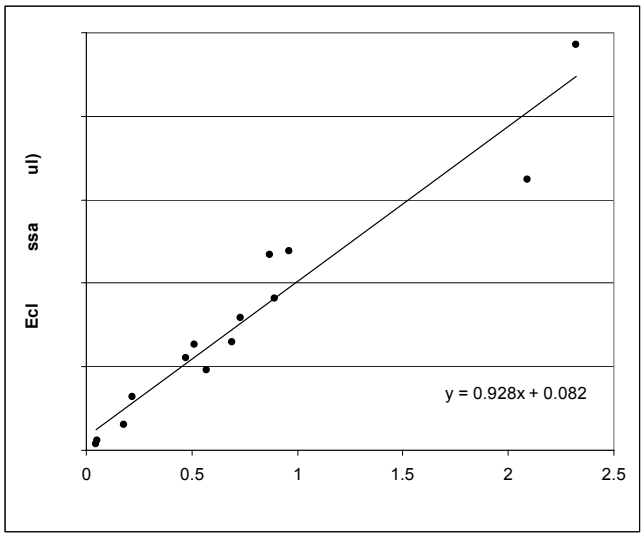



Figure 16 – Graph of the comparison of Eclipse™ *Alu* and SYBR® Green *Alu* assays from Table 7. The Eclipse™ *Alu* assay results for 15 samples (denim #2 is not shown) plotted against the results for the SYBR® Green assay. The slope of the trend line is approximately 1 and the $R^2$ value is 0.93.

Fifteen of the 16 above samples (the male fraction 01-6-1 sample had insufficient DNA) were analyzed with the COfiler™ STR kit. Fourteen samples were diluted to 0.1ng/µl and 10µl (1.0 ng total) was used for COfiler™ STR analysis. One sample, male fraction D, was used neat because the concentration was below 0.1 ng/µl. All of these samples gave results on the ABI 310 within the laboratory's acceptable range (150-5500 RFU for each heterozygous peak) and Table 7 shows the results for two selected loci representing small and large amplicons (*THO1* and D7S820, respectively).

The question arises as to the lowest DNA concentration (as determined by the Eclipse™ assay) that will allow generation of a complete STR profile. Establishing this cutoff concentration would allow a laboratory to cease testing on those samples containing insufficient DNA to reasonably expect generation of a complete or near complete STR profile with peak heights within the acceptable RFU range (i.e. suitable for inclusion into CODIS). In other cases, where a complete profile may not be necessary, such as suspect exclusion, knowing this level would help with an expectation of results, decisions as to whether to switch to mtDNA testing or perhaps triage of multiple samples from one crime scene. To determine this cutoff concentration, six samples of approximately 0.1 ng/µl were serially diluted (1:1) three times. The Eclipse™

assay was then performed on all the samples and the serial 1:1 dilutions to determine the concentration of each. STR analysis (COfiler) was also performed on each sample and dilution (all neat). The RFU results for *THO1* and D7S820 were graphed versus the input DNA concentration to determine what the lowest input concentration was (as measured by the Eclipse™ assay) that would allow successful STR analysis (Figure 17). As can be seen, RFUs decrease linearly as input DNA decreases below 0.1ng/µl (the recommended input DNA concentration) with RFUs lower as expected for the larger D7S820 product (~255-295 bp) than the smaller THO1 product (~165-190 bp).

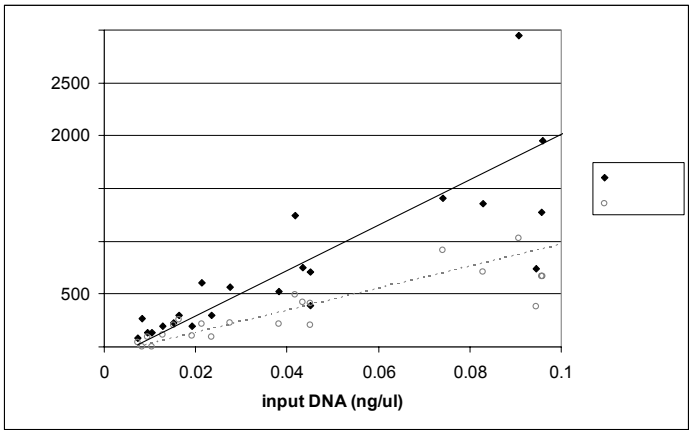



Figure 17 – Graph of COfiler™ STR RFU versus input DNA as determined by the Eclipse™ *Alu* assay. Six DNA samples were serially diluted 1:1 and the Eclipse *Alu* assay performed on all dilutions to determine DNA concentration. COfiler™ analysis was performed on neat samples. The DNA samples were isolated from three convicted offender FTA blood spots, one commercial mixture, a buccal swab and one aged blood sample.

An experiment was also performed to check on the amplification of a number of reagent blanks from casework. These should amplify in the range of the NTC (Ct≈32) or at least below 0.5 pg/µl (Ct≈25). Ten reagent blanks were tested; they had Ct values of 25.21 to 37.11 which gave concentrations of 0.0000 ng/µl to 0.0005 ng/µl.

The reproducibility of the assay was also investigated in several ways. Triplicates of the standard curve performed on the same day had average percent standard deviations for the Ct values of less than 2.5% (data not shown). Triplicates of concentration values for eight samples performed on the same day are shown in Table 8 (percent standard deviations of 3.5 to 24%).

Eight samples repeated five times over different days had percent standard deviations for the concentration values from 9% to 24% (Table 9). These same 8 samples were repeated 3 times with the SYBR® Green assay (Table 9) and the percent deviations ranged from 4% to 21%. Thus, the two assays are generally similar in terms of reproducibility.

TABLE 8 – Triplicate Eclipse™ assay sample concentration (ng/µl) results

| Sample | Replicate 1 | Replicate 2 | Replicate 3 | mean | stdev | %stdev |
|---|---|---|---|---|---|---|
| databank #1 | 0.62 | 0.47 | 0.42 | 0.50 | 0.10 | 20.12 |
| databank #4 | 2.25 | 2.46 | 3.52 | 2.74 | 0.68 | 24.77 |
| databank #5 | 3.21 | 2.08 | 2.51 | 2.60 | 0.57 | 21.90 |
| female fraction J | 0.54 | 0.55 | 0.51 | 0.53 | 0.02 | 3.57 |
| Quantiblot™ StdA | 2.22 | 2.16 | 1.97 | 2.11 | 0.13 | 6.12 |
| Quantiblot™ Cal2 | 0.09 | 0.08 | 0.08 | 0.08 | 0.01 | 8.94 |
| blood, 3 mo dark | 0.99 | 0.99 | 1.03 | 1.00 | 0.02 | 2.44 |
| ABI 9947A | 0.11 | 0.12 | 0.08 | 0.10 | 0.02 | 19.16 |

TABLE 9 – Eclipse™ assay concentration (ng/µl) results on eight samples over five days compared to SYBR® Green assay results over three days

| Sample | Eclipse™ assay | | | SYBR® Green assay | | |
|---|---|---|---|---|---|---|
| | mean | stdev | %stdev | mean | stdev | %stdev |
| databank #1 | 0.44 | 0.05 | 11.3 | 0.60 | 0.09 | 15.1 |
| databank #4 | 1.76 | 0.43 | 24.2 | 2.52 | 0.45 | 17.6 |
| databank #5 | 2.63 | 0.30 | 11.2 | 2.74 | 0.56 | 20.2 |
| female fraction J | 0.54 | 0.08 | 14.4 | 0.66 | 0.04 | 5.8 |
| Quantiblot™ StdA | 1.87 | 0.25 | 13.5 | 2.78 | 0.58 | 20.9 |
| Quantiblot™ Cal2 | 0.08 | 0.01 | 9.2 | 0.09 | 0.02 | 21.1 |
| Blood, 3 mo dark | 0.73 | 0.17 | 22.6 | 1.06 | 0.05 | 4.1 |
| cloth#1 | 0.48 | 0.06 | 13.1 | 0.49 | 0.04 | 8.6 |

*Test of Commercial Mastermixes*

Use of a pre-made mastermix would simplify assay set up; therefore, the performance of several pre-made mastermixes were directly compared on a standard curve dilution series. The "homemade" mix resulted in an RFU of ~40 (for 32 ng/µl input DNA), a reaction efficiency of 1.00 and an R value of 0.998. The Invitrogen Platinum® PCR SuperMix gave a low RFU of 15, a reaction efficiency of 0.69 and a low R value of 0.784. The Sigma Jumpstart™ ReadyMix™

Taq polymerase gave an RFU of 30, a reaction efficiency of 0.98 and an R value of 0.998. Lastly, the Eppendorf® HotMasterMix gave an RFU of 20, a reaction efficiency of 0.98 and an R value of 0.998. The latter two mixes would probably be acceptable although the "homemade" mix appears to perform slightly better based on the RFU.

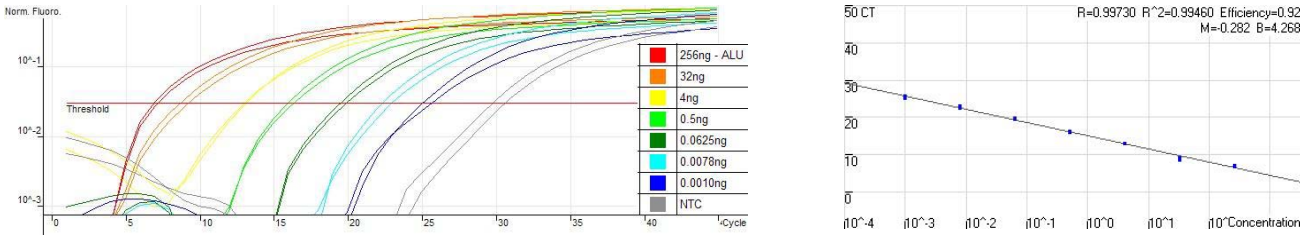
The main advantage of the MGB Eclipse™ real-time *Alu* assay over the SYBR® Green *Alu* assay is its much greater dynamic range. The assay is linear from 256 ng to 1 pg input DNA (128 ng/µl to 0.5 pg/µl sample concentration). The Eclipse™ assay does have a slightly longer run time (87 min versus 72 min) and the Eclipse™ probe costs more than SYBR® Green. However, when purchased in bulk, the probe becomes a negligible cost/reaction (~$0.04). The major cost is the price of Taq polymerase and/or the mastermix kit (~$0.30). Both assays actually cost less than the Applied Biosystems Quantiblot® kit in our hands. As with the SYBR® Green assay, variation of Eclipse™ assay conditions such as annealing temperature, primer concentrations, or extension time do not have any major effect on the assay, suggesting it may be robust over the variations seen between instruments or laboratories. Comparisons between the mastermix made in the laboratory and the three different commercial brands of mastermix that were tested showed that the mastermix made in the laboratory gave higher RFU values than the commercial mixes. The Invitrogen Platinum® PCR SuperMix gave unacceptably low RFU values and variable results.

The published reference for this work is: Nicklas JA, Buel E. An A*lu*-based, MGB Eclipse™ real-time PCR method for quantitation of human DNA in forensic samples. J Forensic Sci 2005; 50(5):1081-90.

We started similar experiments with TaqMan probes and primers (For primer - GACCATCCCGGCTAAAACG, Rev primer - CGGGTTCACGCCATTCTC, probe -

FAMCCCCGTCTCTACTAAAAMGBNFQ) about halfway through grant #2. The Eclipse primers/probe generally seemed to perform slightly better so we did complete validation and manuscript on the Eclipse method although we continued to test the TaqMan method. Figure 18 shows an amplification (A) and a standard curve (B) using the TaqMan method which is quite comparable to Eclipse results.

Figure 18 - A) Standard curve of *Alu* TaqMan results in duplicate and B) standard curve




**Supplement 2 - Increasing the Efficiency of Forensic DNA Analysis Through Real-Time PCR**

**Background:**

The initial focus of our work thus has been to develop a technique to quantitate human DNA in crime scene samples that is not labor intensive, uses minimal sample, and yields quick, accurate results that allow the analyst to perform amplifications of template DNA yielding usable STR analytical results. We based our analysis on PCR of an *Alu* sequence. In our previous assays, the DNA dye SYBR® Green I (Molecular Probes, Eugene, OR) was included in the amplification mix which allowed an estimate of the PCR product when assayed in a fluorescent plate reader or in real-time PCR. The amount of input DNA was shown proportional to the quantity of PCR product produced. Our first assay used PCR amplification and then detection in a fluorescence plate reader. However, another approach to DNA quantitation employs the use of

a real-time PCR instrument (Ong and Irvine, 2002; Ginzinger, 2002; Jung et al., 2000). This instrument monitors the accumulation of PCR product with each cycle and allows assessment of each sample individually during the exponential growth phase. The final readout for each sample is the cycle threshold (Ct) that is defined as the point where the amplification curve crosses a set fluorescence value. Ct is thus inversely proportional to DNA concentration. In other words, as DNA concentration increases, amplification and therefore fluorescence increase accordingly and, thus, the fluorescence threshold is crossed at a lower number of cycles (lower Ct). Real-time assays have the advantages of a greater dynamic quantitative range and require only limited analyst attention. We, thus, developed a real-time version of the *Alu* PCR assay using SYBR® Green I staining. This assay was validated by study of animal samples, blood spots, mock casework and degraded DNA and is shown to quickly, reliably and inexpensively quantitate human DNA over a range of 16ng to 1pg.

Crimes involving violence against women perpetrated by men is a serious problem and one in which forensic laboratories are concerned. Often in these crimes, blood may be shed by the both the victim and attacker such that multiple stains are transferred between individuals. In these situations, it is forensically important to find the blood not "belonging to the owner" on the item of evidence (blood from the female on the male's clothing or vice versa). A method that could quickly identify those stains as male or female could allow the examiner the chance to be selective in the stains that are analyzed further by STR analysis and hence save time and money by limiting the number of stains that require further analysis. By multiplexing quantitation with sex typing, the decision about what stain should be further analyzed is made simultaneously with the quantitation of the stain and hence no further work is required than what must be done prior to STR analysis.

Another important use of real-time PCR sex typing would be in the STR analysis of sexual assault cases. Often seminal fluid stain extracts contain female DNA in addition to the DNA arising from the seminal fluid. These mixtures can range from a small contribution of female DNA to a very high amount relative to the male DNA present. Under those circumstances where female DNA contributes significantly to the total DNA present in the sample, the determination of the total human DNA present may not yield definitive information to determine if a male profile could be obtained.

Some of the serological testing done prior to DNA extraction to determine the presence of semen (prostatic antigen, P30) are very sensitive and can detect extremely low levels of semen; however, these tests are often not good predictors of the success of STR typing from a particular sample. A method that could determine the amount of male DNA present in a sample could allow the forensic scientist to decide if a sample has ample male DNA for further analysis. The STR analysis of semen stains takes considerable time and effort and could be streamlined by eliminating stains early in the DNA analysis process not meriting further attention.

We intended to develop a real-time PCR sex-typing assay that is predictive of STR results. Through an appropriate evaluation of the assay, we believe we can predict whether a male pattern can be obtained from a given sample. The ability to selectively amplify those samples that will yield results will speed the analysis of sexual assault cases by eliminating analysis of those samples that will not yield results and allow the forensic scientist to focus on those samples that will yield usable data. Our plan is to develop a multiplex system of gender determination in conjunction with human DNA quantitation so that no additional work is required than that needed prior to STR analysis. The research will attempt to define criteria which will aid the examiner in deciding what samples merit STR analysis. A wide variety of

samples will be assessed, from mock case work to adjudicated case work material, to determine this criteria and the likelihood of appropriately determining sample outcome.

A number of groups have utilized PCR of different genes or sequences to detect gender of a DNA sample. A classic method, that is incorporated into most STR kits, involves a 6bp size difference in the X-linked and Y-linked copies of the human amelogenin gene (Sullivan et al., 1993; Mannucci et al., 1994). The size difference can be detected on an ABI 310 instrument or on a simple gel with followed by ethidium bromide staining (Sullivan et al., 1993; La Fountain et al., 1998). The Y chromosome copy is 6bp longer in intron 4 in a region slightly 3' of exon 3. However, there are rare reported cases of males with no amplification of the Y-linked amelogenin gene with the generally utilized primers (Roffey et al., 2000; Steinlechner et al., 2002: Santos et al., 1998; J. Butler, personal communication) probably due to a deletion. Other groups have exploited other differences between the X- and Y-linked amelogenin genes (Eng et al., 1994; Faerman et al., 1995). Other groups have used sequences in the homologous ZFX and ZFY genes for gender determination (Reynolds and Varlaro, 1996; Roy and Steffens, 1997; Stacks and Witte, 1996; Wilson and Erlandsson, 1998). In the Reynolds and Valaro method, PCR gives a 209bp fragment with an X and Y common HaeIII site and a unique Y site; thus, restriction with HaeIII allows determination of the gender of the sample. The method used by Roy and Steffens used a common forward primer and specific X and Y reverse primers while that of Wilson and Erlandsson uses an *Alu* insertion found only in the ZFX gene.

Amplification of the alpha satellites from the X (DZX1) and Y (DZY1) chromosomes (Neeser and Liechti-Gallati, 1995; Gaensslen et al., 1992; Fattorini et al., 1993; Witt and Erickson, 1989; Hashiba et al., 2000) has also been used for gender determination. Primer pairs were developed for each satellite that gave slightly different sized fragments, these were detected

on a gel either by staining or use of a labeled primer. Some examples of female individuals giving a product with the male primers and or males producing little amplification with the male primers have been found (Hanaoka and Minaguchi, 1996). Still other groups have used other genes or combinations such as the SRY gene and the X-ALD gene (Gold et al., 2001) or DYZ1 with the repeat from DXS424 (Pfitzinger et al., 1993), or SRY and DXZ4 (Palmirotta et al., 1998; Naito et al., 1994). Use of the alpha satellites for gender determination have the advantage of multiple copy number rather than the single copy amelogenin or ZFX/ZFY genes. This would allow quantification on a smaller or less concentrated sample or multiplexing with the *Alu* assay.

When the victim and assailant are of the same gender, then quick screening of possible stain donor identity will not be possible by the above gender tests. A different test to quickly determine possible stain origin will be required. Clearly, identity can be determined to near certainty using the usual 13 STR loci but often such a determination is of limited value from a criminal justice point of view; for example, determining that blood on the assailant's shirt is his/her own blood. Such a complete analysis often becomes a waste of time and money. Quick analysis of one or two STR loci, while certainly not by any means a definitive test of identity, will usually allow determination of whether a blood sample did not come from an individual. This is sufficient for a quick screen of crime scene stains to answer the question – Is this stain of possible victim or assailant origin? Development of such a screen would be of benefit to the forensic community.

Many forensic samples are highly degraded and PCR of the longer length STRs found in the usual kits is not possible. John Butler at NIST and Bruce McCord at Ohio University are developing a set of STRs with small PCR products ("Big Mini" STR System) (Butler et al., 2003; Wiegand and Kleiber, 2001). Currently, their system uses primers for THO1 and FGA

(6FAM), CSF1PO and D21S11 (VIC) and TPOX and D7S820 (NED). However, current quantitation methods are not designed to quantitate degraded DNA accurately. A redesigned real-time *Alu* PCR assay with a product of similar size to the Big Mini STR products would be an effective assay to quantitate such DNA.

Often forensic samples are too degraded for the usual STR analysis that requires nuclear DNA to be in fragments greater than 400bp for the larger alleles. For such samples, many laboratories have turned to using mitochondrial typing because many more copies are present per cell (perhaps 10,000/cell) and the probability of some copies being sufficiently intact is increased (Bender et al., 2000; Butler et al., 1998; Graw et al., 2000; Melton et al., 2001; Piercy et al., 1993; Primorac and Schanfield, 2000; Reynolds et al., Seo et al., 2002; Szibor et al., 2000; Wilson et al., 1995). Usually the hypervariable I (HV-1) or II (HV-2) region of the control region is sequenced. Guidelines for mitochondrial DNA typing have been published by the DNA Commission of the International Society for Forensic Genetics (Bar et al., 2000). The disadvantage of mitochondrial typing is that mitochondria are inherited only from the mother; thus, sibs and other close family members through the female line can have (modulo possible mutation and segregation differences) identical typings. However, sequencing of the HV regions can give reasonably high probabilities when carefully performed.

Quantitation of mitochondrial DNA for these assays will be different than for nuclear DNA. Several methods have been developed using real-time PCR (either TaqMan® or molecular beacons) (Lim et al., 2001; Gahan et al., 2001; Reynier et al., 2001; Rodriguez-Santiago et al., 2001; Szuhai et al., 2001; Steuerwald et al., 2000; Heid et al., 1996); however, these methods were mostly developed to study human disease and not to quantitate for forensic analysis. Meissner et al (2000) used real-time PCR to quantitate mtDNA in human

blood. Andréasson et al. (2002) recently report the use of real-time PCR to detect human nuclear and mitochondrial DNA; however, while they tested their assay on a variety of samples, testing of non-human DNA by this assay was not detailed. The mtDNA quantitation assay was also tested for its ability to allow determination of copy number for mtDNA sequencing (HVII region).

Increases in mitochondrial DNA mutations, especially deletions, have been associated with exposure to mutagens and with aging. A common deletion (4977bp) resulting from recombination between 13bp direct repeats (at 8470-8482 and 13,447-13459) is known to increase in tissues of aged individuals. Heteroplasmic inheritance of this deletion causes Pearson syndrome or Kearns-Sayre syndrome (Holt et al., 1988, 1989; Rotig et al., (1991); Moraes et al., 1989; Schon et al., 1989). Two groups have developed real-time PCR methods to detect this deletion. Koch et al. (2001) used a non-deleted region probe and a probe specific for the deletion for its quantitation in human keratinocytes, while Meissner et al. (2000) used similar probes to detect the deletion in human blood.

**Project goals:** 1) development of an assay to quantitate male and female DNA, 2) development of a simple, fast assay that detects alleles at one or two STR loci as quick sample screening method, 3) improvement of the original *Alu* PCR Real-time PCR assay, and 4) dissemination of methods to the Forensic community.

**Methods**:

*Assay Development*

We designed a duplex Eclipse assay *Alu* for total human DNA and the DYZ3 repeated Y chromosome sequence for male DNA. We spent a great deal of time working on this assay but ran into problems getting two different dyes that would work well in our real-time instruments.

We initially obtained the Eclipse probes from Synthetic Genetics which developed this technology. We obtained the *Alu* probe labeled with FAM and the DYZ3 probe labeled with Cy5. This assay seemed to work pretty well. Synthetic Genetics was then taken over by Epoch BioSciences and apparently the division that made had the Cy5 was sold to Eurogentec and Cy5 was no longer available. We were sent the DYZ3 probe with TET as the dye but this is a very suboptimum dye for any real-time instrument except ABI real-time instruments; thus, it did not work well in our hands. They offered a new dye, Yakima Yellow, but this was abysmal. We tried switching the dyes on the two probes but this was worse. We switched to the TaqMan because we could now get FAM and VIC dyes on the TaqMan MGB probes and these dyes both work well on the Corbett RG3000 and the Stratagene MX3000 (a loaner instrument we acquired from Stratagene). We did keep complaining to Epoch but to no avail until they were bought out by Nanogen. Another new dye (PY) was then forthcoming which we tried but we were still not satisfied; thus, we developed our duplex, total/male assay with the TaqMan technology.

We also tried using a different set of *Alu* primers and probe based on the Yd6 subfamily rather than the Ya5 family that we had been using. This was because there were fewer members of the Yd6 family and we thought this would balance the fewer members of the DYZ3 sequence.

We worked for a number of months on the TaqMan *Alu*/DYZ3 based assay optimizing it and bringing it near to validation. However, when we tested a large number of men, we found that to our surprise and dismay that there was a great deal of variation in the ratio of male:total among men (0.2 to 1.2) especially within Caucasians where the ratio was often very low. This suggested that the number of copies of DYZ3/male varied. It was clear that this DYZ3 based-assay would not be viable. This sent us back to the drawing board to find a new repeated Y chromosomal sequence. After a week or two of literature research, we decided that DYZ1 and

DYZ5 were the next best options and we designed primers and probes for these sequences. We quickly determined that the DYZ1 was not sufficiently male specific, i.e. there was a low but significant reaction with female DNA. DYZ5, however, passed this test and a preliminary screen with males showed little variation. We then began optimization of this new *Alu*/DYZ5 assay. We initially tried the Yd6 *Alu* primer/probe set but the NTC did not always have a Ct <40 so we switched back to the Ya5 set. This new assay was then fully optimized and validated.

*Development of Assay*

The assay was developed as a real-time TaqMan duplex to detect both total human DNA (*Alu* sequence, VIC labeled MGB probe) and male DNA (DYZ5 sequence, FAM labeled MGB probe). For a human specific sequence, the human *Alu* sequence (Ya5 subfamily) was chosen (Batzer et al., 1994; Otieno et al., 2004). Currently there are 2473 identified Ya5 sequences in the human genome, of which 75 to 80% are fixed (present in all individuals) and 20 to 25% are polymorphic (Otieno et al., 2004; Dale J. Hedges and Jerilyn A. Walker, personal communication). There are estimates of another ~557 polymorphic *Alu* sequences that are not yet discovered (Dale J. Hedges and Jerilyn A. Walker, personal communication). DYZ5 is a Y-specific sequence (Yp11.2) with a repeat of approximately 20,300 bp (Tyler-Smith et al., 1988). The testis-specific protein, Y encoded, genes are part of the DYZ5 repeat unit (Manz et al., 1993). There is one array of ~540-800 kb and another minor block of 60 kb on the Y chromosome (Tyler-Smith et al., 1988). The DYZ5 repeat is conserved in the great apes (Guttenbach et al., 1992) but is not present in other mammals. Based on the 20.3 kb repeat size and the array size of 300-800 kb, there will be ~15-50 copies of DYZ5 per Y chromosome. The total human (*Alu*) and Y specific (DYZ5) primers and TaqMan MGB probes were designed using Applied Biosystems' (Foster City, CA) Primer Express program. BLAST searches

(NCBI) were performed on the primer and probe sequences to check for complementarity of the DYZ5 sequences to the X or autosomes, as well as to check the *Alu* sequence against non-primates. The unlabeled primers (*Alu,* Forward gaccatcccggctaaaacg and Reverse cgggttcacgccattctc; DYZ5, Forward gctattgagttgttgggagttcctt and Reverse aagtgcttcaccacaccatagaaa) and *Alu* (VIC-ccccgtctctactaaaMGBNFQ) and Y probes, (6FAM-ctgtgactattccccMGBNFQ) were ordered from Applied Biosystems (Foster City, CA).

The final duplex human/Y DNA quantitation assay utilized the ABsolute QPCR Mix (ABgene, Rochester, NY). The 20 µl reactions contained 1X mix, 100 µM *Alu* forward primer, 200 µM *Alu* reverse primer, 200 µM DYZ5 forward primer, 100 µM DYZ5 reverse primer, 200 µM each probe and 160 ng/µl BSA (A-9647, Sigma, St. Louis, MO). Two µl of sample or standard suspended in TE (10 mM Tris, pH 7.5, 0.1 mM EDTA) were added to each well. Initial optimization experiments varied the concentrations of primers or probes and some experiments compared other pre-made mastermixes [QuantiTect Multiplex PCR kit (Qiagen, Valencia, CA), Brilliant Multiplex QPCR Master mix (Stratagene, La Jolla, CA), QPCR Mastermix Plus (Eurogentec, San Diego, CA), Universal Mastermix (Applied Biosystems, Foster City, CA), FastStart TaqMan Probe Master, Roche Applied Science, Indianapolis, IN)].

Real-time PCR for the assay was performed primarily in a Stratagene MX 3000P (La Jolla, CA), but also occasionally in a Corbett Rotorgene 3000 (San Francisco, CA) using either 96-well plates or the 0.1 ml Corbett tubes, respectively. The DYZ5 FAM-labeled probe fluorescence was read in the FAM channel while the VIC-labeled *Alu* probe fluorescence was read in the HEX channel. PCR consisted of 95ºC for 15 min ("hotstart") followed by 45 cycles of 95ºC for 30 s, 60ºC for 1 min. While developmental experiments used 45 cycles of PCR, 40 cycles of PCR are sufficient for a routine assay. The instrument software (Stratagene MX3000P

ver. 2 or Corbett Research RG3000 ver. 5) were used to analyze the data. Reaction efficiency is defined as $E = 100\% \times (10^{-s} - 1)$ where s is the slope of standard curve.

Figure 19 shows a 3% agarose gel of the PCR products generated from male and female DNA. The *Alu* primers yield the expected 127 bp PCR product with either male or female DNA as a template, while the DYZ5 primers yield the expected 137 bp product only with male DNA as a template (the 137bp male product is light with 0.0046ng male DNA but clearly visible on the original gel).

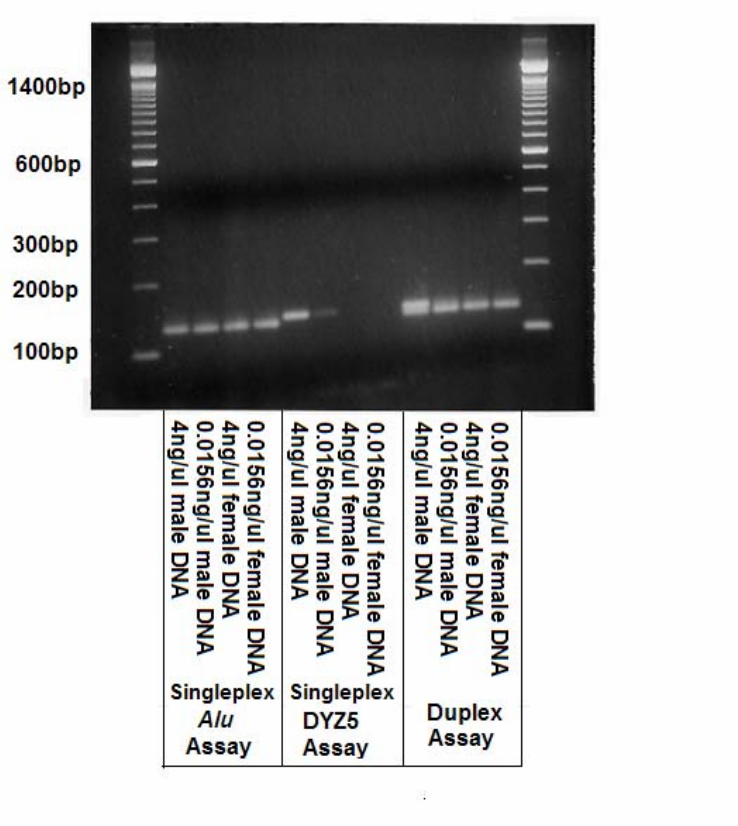


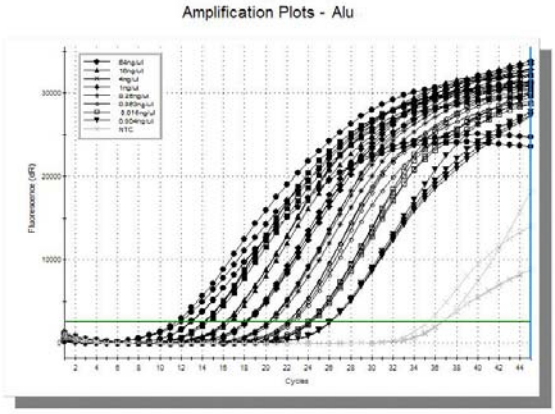

Figure 19–Gel electrophoresis of assay products. PCR was performed as described in Materials and Methods except that only 32 cycles were performed, no probes were used and three reactions were performed: only DYZ5 primers, only *Alu* primers and both pairs of primers. Male DNA (two concentrations of Promega human genomic DNA: male) and female DNA (two concentrations of Promega human genomic DNA: female) were used as samples. The PCR products were run on a 3.5% agarose gel with ΦXHindIII as a size standard.

Figure 20 shows Ct graphs for quadruplicate samples using the *Alu* probe (VIC channel) (top) and DYZ5 probe (FAM channel) (middle), as well as their standard curves (bottom) using a

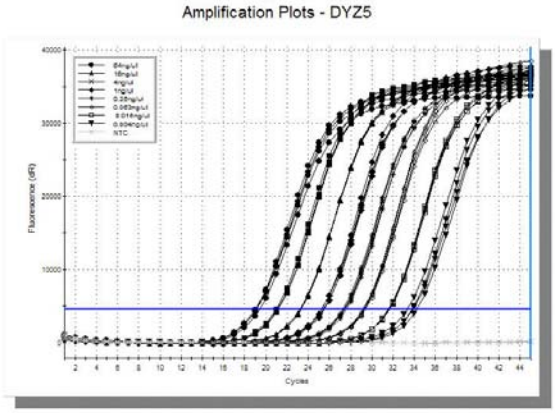
dilution series of male DNA. The assay has a large dynamic range of 64 ng/µl to less than 4 pg/µl (2 µl of sample is added per reaction) with a tight grouping of Ct values for each sample.

FIG. 20–Duplex assay results run in quadruplicate. PCR was performed as described in Materials and Methods. The standard curve dilution series samples were run in quadruplicate on the same plate. a. *Alu* probe results. b. DYZ5 probe results. c. Standard curve results. The DYZ5 FAM-labeled probe fluorescence was read in the FAM channel while the VIC-labeled *Alu* probe fluorescence was read in the HEX channel.

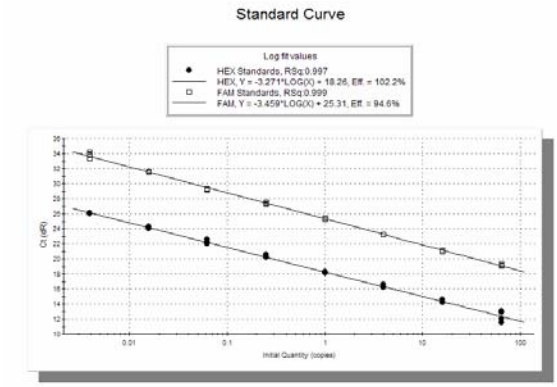
A.




B.




C.

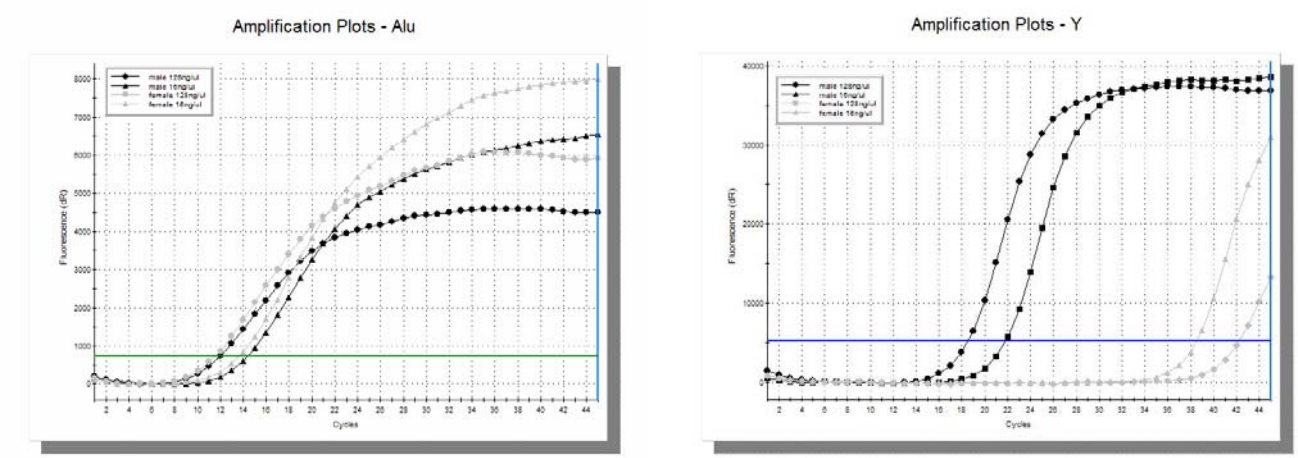



To show that the Y-assay was specific for male DNA, the assay was tested with both male and female DNA at 16 ng/µl and 128 ng/µl (Figure 21). In Fig. 18a, the *Alu* probe shows that the male and female 16 ng/µl DNAs are at equal concentrations as well as the male and female 128 ng/µl DNAs. In Fig. 18b, the Y probe shows that the 128 ng/µl samples have a Ct

difference of 20 (38.6-18.6). This is a selectivity difference of ~1,000,000 fold ($2^{20}$) between male and female DNA.

Figure 21–Duplex assay results on male versus female DNA. PCR was performed as described in Materials and Methods. Male DNA (two concentrations of Promega human genomic DNA: male) and female DNA (two concentrations of Promega human genomic DNA: female) were used as samples. a. *Alu* probe results. b. DYZ5 probe results.

A. B.



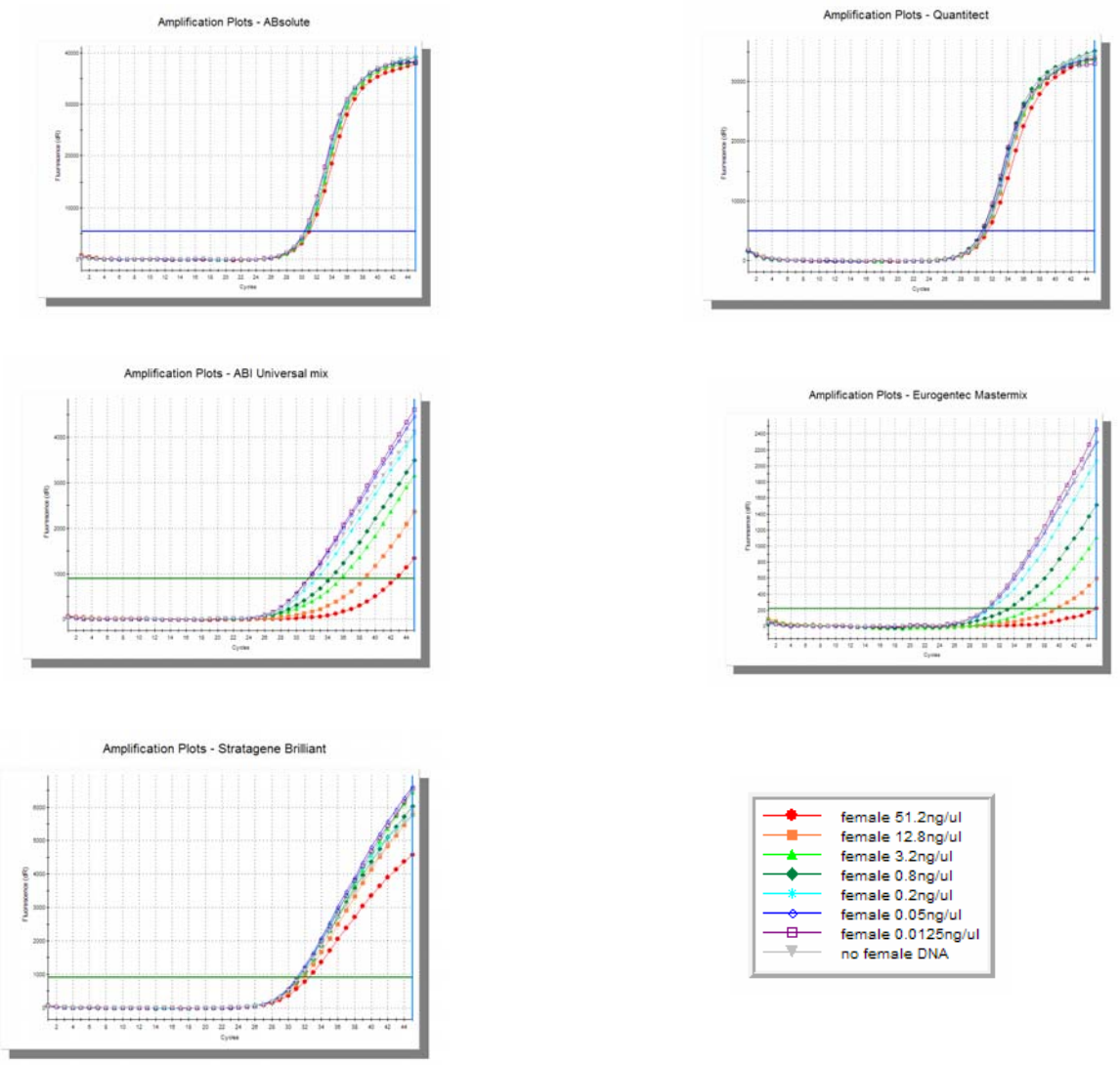

The assay was optimized by changing several parameters. Primer/probe titrations were performed essentially as recommended by Applied Biosystems (Foster City, CA) (User Bulletin #5). A range of primer concentrations (50, 100, 200, 400 and 800 μM) and probe concentrations (50, 100, 150, 200, 250 and 300 μM) were tested. Forward and reverse primer concentrations for *Alu* were maintained at 200 μM while the forward and reverse primers were varied independently for the DYZ5 and vice versa. Once the optimal primer concentrations were chosen based on Ct plateau effects, the probe optimization experiments were performed for each probe separately. The optimal primer/probe concentrations were chosen to be: 100 μM *Alu* forward primer, 200 μM *Alu* reverse primer, 200 μM DYZ5 forward primer, 100 μM DYZ5 reverse primer, and 200 μM each probe (data not shown).

BSA was added to the mastermix at the concentration (250 µg/µl) used in our previously reported SYBR Green *Alu* assay (Nicklas and Buel, 2005). However, this concentration was inhibitory to the PCR (data not shown). The BSA concentration was reduced to 160 ng/µl, which is the concentration used in the Applied Biosystems Profiler Plus and COfiler kits. This concentration was not inhibitory to the reactions and gave similar results with or without BSA added with a test set of samples (data not shown).

We performed an experiment where we added increasing amounts of female DNA to samples with a fixed amount of male DNA to determine if the female DNA interfered with detection of the male DNA. This test consisted of a standard curve and eight samples. The eight samples contained 0.05 ng/µl male DNA and various amounts of female DNA (0 ng/µl to 51.2 ng/µl in 1:4 dilutions). The expected result was an incremental increase in amount of DNA (0.5 ng/µl to 51.25 ng/µl) for the *Alu* probe but always a constant 0.5 ng/µl (the constant amount of male DNA in each sample) from the DYZ5 probe. In our initial experiments, we found that there was interference in that increasing amounts of female DNA lowered the final plateau and changed the Ct. To address this problem, we tried a variety of commercial mastermixes [QuantiTect Multiplex PCR kit (Qiagen, Valencia, CA), Brilliant Multiplex QPCR Master mix (Stratagene, La Jolla, CA), QPCR Mastermix Plus (Eurogentec, San Diego, CA), Universal Mastermix (Applied Biosystems, Foster City, CA,), Absolute QPCR mix (ABgene, Rochester, CA), FastStart TaqMan Probe Master (Roche Applied Science, Indianapolis, IN)] and found that the results varied a great deal (Figure 22). The ABgene, Qiagen and Roche mixes both yielded the expected constant 0.5 ng/µl results for DYZ5 with good (>95%) reaction efficiency and the Stratagene mastermix was nearly as consistent (91-94%). The efficiency of the PCR reactions seemed to be slightly less (85-90%) for the Roche product. The other two mastermixes showed

corresponding inhibition of the DYZ5 results with increasing female DNA, however. A difference of 10 Ct was observed between the control sample (no female DNA) and the sample with 51.2 ng/µl female DNA. Clearly, the mastermixes drastically affected the observed results. The reasons for these differences are unknown since the ingredients are proprietary but could include buffer composition (salts and $Mg^{++}$), concentration of dNTPs, concentration of enzyme or ingredients such as DMSO. The ABgene mastermix was chosen for future experiments based on results and cost. The experiments using the ABgene mastermix showed that female DNA does not interfere with the detection of male DNA over a 1024-fold difference in concentration.

Figure 22 – Comparison of different PCR mastermixes



*Validation of Assay*

Experiments to validate the assay included reproducibility, human specificity, evaluation of potential ethnic variability, artificial mixtures, adjudicated casework, and the effect of inhibitors and state of DNA degradation.

Table 10 shows the consistency in Ct for standard curve quadruplicate values on the same 96-well plate. As with our *Alu* SYBR® Green assay (Nicklas and Buel, 2003), the no template control (NTC) for the *Alu* part of the duplex assay had a Ct of ~37. With the SYBR assay, this product had a melting point spread identical to the *Alu* product from a tube with added DNA. The amplification of the NTC has also been seen by others using an *Alu*-based assay (Urban et al., 2000; Walker et al., 2003); thus, it is presumably due to ambient human DNA in the air and water. That the NTC for the DYZ5 assay did not rise above the threshold most likely reflects the relatively lower copy number of this sequence compared to the *Alu* sequence. The percent standard deviations for the Ct values varied from 0.13 to 5.73% but most were less than 1%.

TABLE 10a–*Ct values for standard curve quadruplicates – Alu.*

| Standard Point | Repeat 1 | Repeat 2 | Repeat 3 | Repeat 4 | Mean | Stdev | %Stdev |
|---|---|---|---|---|---|---|---|
| 64 ng/µl | 19.09 | 19.03 | 19.12 | 19.38 | 19.16 | 0.15 | 0.81 |
| 16 ng/µl | 21.11 | 20.99 | 21.13 | 21.16 | 21.10 | 0.07 | 0.35 |
| 4 ng/µl | 23.27 | 23.27 | 23.21 | 23.27 | 23.26 | 0.03 | 0.13 |
| 1 ng/µl | 25.47 | 25.24 | 25.26 | 25.40 | 25.34 | 0.11 | 0.44 |
| 0.25 ng/µl | 27.51 | 27.34 | 27.21 | 27.38 | 27.36 | 0.12 | 0.45 |
| 0.0625 ng/µl | 29.23 | 29.16 | 29.35 | 29.26 | 29.25 | 0.08 | 0.27 |
| 0.0156 ng/µl | 31.53 | 31.6 | 31.64 | 31.54 | 31.58 | 0.05 | 0.16 |
| 0.0039 ng/µl | 34.23 | 33.87 | 33.78 | 33.30 | 33.80 | 0.38 | 1.13 |
| NTC | No Ct | No Ct | No Ct | No Ct | | | |

TABLE 10b–*Ct values for standard curve quadruplicates – DYZ5.*

| Standard Point | Repeat 1 | Repeat 2 | Repeat 3 | Repeat 4 | Mean | Stdev | %Stdev |
|---|---|---|---|---|---|---|---|
| 64 ng/µl | 13.01 | 11.56 | 12.90 | 11.97 | 12.36 | 0.71 | 5.73 |
| 16 ng/µl | 14.25 | 14.16 | 14.23 | 14.54 | 14.30 | 0.17 | 1.17 |
| 4 ng/µl | 16.28 | 16.27 | 16.16 | 16.56 | 16.32 | 0.17 | 1.05 |
| 1 ng/µl | 18.07 | 18.32 | 18.10 | 18.17 | 18.17 | 0.11 | 0.61 |
| 0.25 ng/µl | 20.27 | 20.19 | 20.21 | 20.57 | 20.31 | 0.18 | 0.87 |
| 0.0625 ng/µl | 22.14 | 22.01 | 22.31 | 22.54 | 22.25 | 0.23 | 1.03 |
| 0.0156 ng/µl | 24.11 | 24.00 | 24.20 | 24.33 | 24.16 | 0.14 | 0.58 |
| 0.0039 ng/µl | 26.12 | 26.03 | 26.11 | 26.14 | 26.10 | 0.05 | 0.19 |
| NTC | 36.61 | 36.66 | 35.29 | 36.73 | 36.32 | 0.69 | 1.90 |

Table 11 shows the replicates (different days) of determined concentrations (ng/ul) for a set of 11 samples [five normal males, two males believed to be XYY based on Amelogenin ratios, a Promega stock mixture (Madison, WI) of male and female DNA diluted to ~4 ng/µl, the 9947 DNA from an Applied Biosystems STR kit (Foster City, CA) (0.1 ng/µl), a mixture of DNA from one male and one female from a proficiency test and DNA from a normal female]. The *Alu* and DYZ5 results in Tables 11a and 11b, respectively, are consistent (except for the fourth repeat for normal male #2 which is abnormally high, especially with the *Alu* probe). The percent standard deviations are between ~8-71%; removing that one abnormal point changes the range to ~8-35%. The dilution of Promega DNA (Madison, WI) (4 ng/µl) and the neat solution of Applied Biosystems 9947 (Foster City, CA) yielded results consistent with the stated concentrations. Table 11c gives the ratio of male DNA to total DNA which is approximately 1.0 for the five males samples, closer to 2.0 for the XYY samples (suggesting these individuals do have two Y chromosomes), 0.64 and 0.29 for the two mixtures, respectively, and 0.0 for the two female samples (9947, normal female). The percent standard deviations range from 5- 30%.

TABLE 11a–*Replicates of samples – Alu. Determined concentrations (ng/ul) for 11 samples.*

| Standard Point | Repeat 1 | Repeat 2 | Repeat 3 | Repeat 4 | Mean | Stdev | %Stdev |
|---|---|---|---|---|---|---|---|
| normal male #1 | 4.44 | 7.36 | 6.34 | 10.35 | 7.12 | 2.47 | 34.66 |
| normal male #2 | 39.64 | 28.21 | 32.00 | 104.90 | 51.19 | 36.12 | 70.57 |
| normal male #3 | 0.51 | 0.74 | 0.56 | 0.79 | 0.65 | 0.14 | 21.10 |
| normal male #4 | 1.08 | 1.29 | 1.22 | 1.04 | 1.16 | 0.12 | 10.13 |
| normal male #5 | ND | 0.62 | 0.56 | 0.54 | 0.57 | 0.04 | 7.59 |
| XYY #1 | 0.12 | 0.17 | 0.12 | 0.15 | 0.14 | 0.02 | 17.20 |
| XYY #2 | 0.63 | 0.85 | 0.64 | 0.60 | 0.68 | 0.11 | 16.48 |
| Promega mix | ND | 4.62 | 4.16 | 5.74 | 4.84 | 0.81 | 16.79 |
| Mixture | ND | 0.77 | 0.53 | 0.65 | 0.65 | 0.12 | 18.65 |
| 9947 | 0.08 | 0.09 | 0.09 | 0.13 | 0.10 | 0.02 | 23.03 |
| normal female | 0.75 | 1.05 | 0.82 | 0.93 | 0.89 | 0.13 | 14.81 |

TABLE 11b–*Replicates of samples – DYZ5. Determined concentrations (ng/ul) for 11 samples.*

| Standard Point | Repeat 1 | Repeat 2 | Repeat 3 | Repeat 4 | Mean | Stdev | %Stdev |
|---|---|---|---|---|---|---|---|
| normal male #1 | 6.00 | 5.33 | 6.16 | 7.66 | 6.29 | 0.98 | 15.64 |
| normal male #2 | 25.58 | 19.89 | 25.28 | 40.89 | 27.91 | 9.04 | 32.39 |
| normal male #3 | 0.42 | 0.68 | 0.65 | 0.84 | 0.65 | 0.17 | 26.82 |
| normal male #4 | 0.88 | 0.97 | 0.98 | 1.01 | 0.96 | 0.06 | 6.07 |
| normal male #5 | ND | 0.44 | 0.39 | 0.44 | 0.42 | 0.03 | 7.22 |
| XYY #1 | 0.20 | 0.27 | 0.24 | 0.31 | 0.25 | 0.05 | 18.40 |
| XYY #2 | 0.92 | 1.17 | 1.00 | 1.08 | 1.04 | 0.11 | 10.13 |
| Promega mixture | ND | 2.84 | 2.82 | 3.57 | 3.08 | 0.43 | 13.89 |
| Mixture | ND | 0.19 | 0.19 | 0.18 | 0.18 | 0.01 | 3.60 |
| 9947 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | |
| normal female | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | |

TABLE 11c–*Replicates of samples – DYZ5:Alu Ratio**

| Standard Point | Repeat 1 | Repeat 2 | Repeat 3 | Repeat 4 | Mean | Stdev | %Stdev |
|---|---|---|---|---|---|---|---|
| normal male #1 | 1.35 | 0.72 | 0.97 | 0.74 | 0.95 | 0.29 | 30.89 |
| normal male #2 | 0.65 | 0.71 | 0.79 | 0.39 | 0.63 | 0.17 | 27.25 |
| normal male #3 | 0.83 | 0.92 | 1.18 | 1.07 | 1.00 | 0.15 | 15.51 |
| normal male #4 | 0.81 | 0.75 | 0.80 | 0.97 | 0.83 | 0.10 | 11.41 |
| normal male #5 | ND | 0.71 | 0.70 | 0.81 | 0.74 | 0.06 | 8.33 |
| XYY #1 | 1.63 | 1.60 | 1.98 | 2.03 | 1.81 | 0.23 | 12.55 |
| XYY #2 | 1.46 | 1.38 | 1.55 | 1.80 | 1.55 | 0.18 | 11.56 |
| Promega mixture | ND | 0.61 | 0.68 | 0.62 | 0.64 | 0.03 | 5.42 |
| Mixture | ND | 0.25 | 0.36 | 0.27 | 0.29 | 0.06 | 20.05 |
| 9947 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | |
| normal female | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | |

* DYZ5 determined concentration÷*Alu* determined concentration

DNA from nine male animals (ferret, bobcat, bear, dog, cat, horse, donkey, herring, goat) and two bacteria (*E. coli* and *Clostidium*) at concentrations of 5-10 ng/µl were tested using the assay. Reactions with animal and bacterial DNA gave apparent concentrations between 0.0 (no

Ct) and 0.00081ng/ul or are at least 13,000-fold less sensitive than human DNA for both the *Alu* and DYZ5 probes (data not shown).

The duplex assay was tested for consistency with DNA isolated from a number of males and females representing the major ethnic groups. The expected results are that males should have a DYZ5:*Alu* concentration ratio of approximately 1.0 while females should have a ratio of approximately 0.0. Figure 23 graphs the results of these experiments for 54 males (nineteen Caucasians, five Native Americans, fourteen African Americans, eight Asians, seven Hispanics and one unknown). The DYZ5 or male DNA concentration (ng/ul) is graphed against the *Alu* or total DNA concentration (ng/ul). The males yielded DYZ5:*Alu* ratios between 0.54 and 1.23 with a mean of 0.83±0.14. The values hold tightly to the trendline ($R^2$=0.994) although its slope is less than 1.0 (0.722). Experiments were also performed on 18 females (fourteen Caucasians, three African Americans, one Native American) (data not shown). The females had ratios of 0.00 to 0.01 with a mean of 0.00 (17 had ratios of 0.00 and one had a ratio of 0.01).

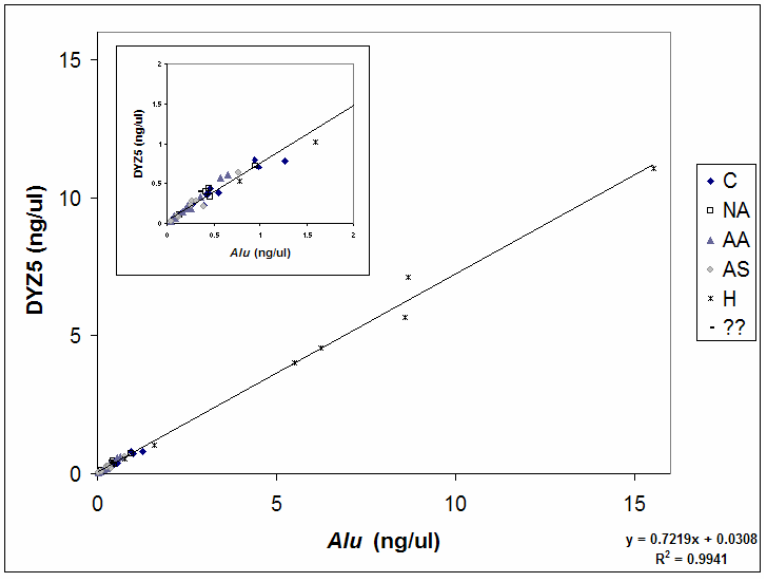



Figure 23–Plot of *Alu* and DYZ5 results for DNA samples from 54 male individuals of different ethnic groups. The inset is a blowup of the results for concentrations less than 2ng/ul to show detail. A trendline is also graphed and the $R^2$ value (0.9941) and equation (y=0.7219x + 0.0308) given. The key for the ethnic groups is C=Caucasian, AA=African American, NA=Native American, H=Hispanic, AS=Asian, ??=unknown.

The assay was tested on twenty artificial mixtures. Table 12 shows the input male and female DNA and expected male DNA:total DNA ratio for each mixture. The fifth column gives the observed ratio using the duplex assay. The correlation between the observed and expected ratio is $R^2 = 0.89$. The last two columns give STR results on selected samples which were diluted to 0.1 ng/µl based on the *Alu* results from the duplex assay (10ul used for a 25ul amplification). A male:total DNA ratio was calculated based on the AMEL X and Y peaks as well as using an autosomal STR where the male and female peaks could be clearly distinguished.

TABLE 12–*Results on a series of artificial male DNA/female DNA mixture.s*

| Mixture | Input Female DNA (ng/µl) | Input Male DNA (ng/µl) | Male:Total Input Ratio* | Male:Total Observed Ratio† | AMEL RFU (Y:X) for 0.1 ng/µl Dilution‡ (Male:Total ratio§) | STR Male:Total Ratio (Locus)‖ |
|---|---|---|---|---|---|---|
| 1 | 0.025 | 0.025 | 0.50 | 0.42 | | |
| 2 | 0.05 | 0.2 | 0.80 | 0.53 | | |
| 3 | 0.15 | 0.35 | 0.70 | 0.68 | 1469:2914 (0.67) | 0.78 (THO1) |
| 4 | 0.6 | 0.4 | 0.40 | 0.21 | 694:2433 (0.44) | 0.34 (D16) |
| 5 | 1.5 | 0.5 | 0.25 | 0.19 | 310:1947 (0.27) | 0.28 (D16) |
| 6 | 1.0 | 3.0 | 0.75 | 0.49 | 371:653 (0.72) | 0.76 (D16) |
| 7 | 10.0 | 0 | 0.00 | 0.00 | | |
| 8 | 2.5 | 0 | 0.00 | 0.00 | | |
| 9 | 0.3 | 0 | 0.00 | 0.00 | | |
| 10 | 0 | 1.25 | 1.00 | 0.68 | | |
| 11 | 0 | 1.0 | 1.00 | 0.66 | | |
| 12 | 0.1 | 0 | 0.00 | 0.00 | | |
| 13 | 20.0 | 0 | 0.00 | 0.00 | | |
| 14 | 0 | 20.0 | 1.00 | 0.85 | | |
| 15 | 2.5 | 47.5 | 0.95 | 0.81 | | |
| 16 | 1.0 | 44.0 | 0.98 | 1.21 | | |
| 17 | 1.25 | 18.75 | 0.94 | 0.90 | | |
| 18 | 34.0 | 1.0 | 0.03 | 0.01 | | |
| 19 | 29.0 | 1.0 | 0.03 | 0.01 | | |
| 20 | 1.0 | 0.5 | 0.33 | 0.24 | | |

*input male DNA÷(input male DNA+input female DNA)
†DYZ5 determined concentration÷*Alu* determined concentration
‡ RFU of the AMEL peaks (Y:X). DNA was diluted to 0.1 ng/µl based on the results of the duplex assay and COfiler analysis performed
§AMEL Y peak RFU÷(AMEL Y peak RFU+ AMEL X peak RFU)
‖RFU of known male peaks÷(RFU of male+female peaks) for the given locus. The male and female peaks were determined on a mixture by mixture basis by reference to the known profiles of the individual DNA donors.

Table 13 depicts the results with five mixtures that were serially diluted down to 1:64. As expected, the DYZ5:*Alu* ratio stays somewhat constant upon dilution.

TABLE 13–Male:total (*DYZ5/Alu) ratio* on serial dilution of selected male DNA/female DNA mixtures from Table 12.*

| | Dilution | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Mixture | neat | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | mean | stdev | %stdev |
| 1 | 0.36 | 0.33 | 0.34 | 0.48 | 0.15 | 0.53 | 0.46 | 0.38 | 0.12 | 33 |
| 5 | 0.22 | 0.2 | 0.15 | 0.18 | 0.18 | 0.14 | 0.13 | 0.17 | 0.03 | 19 |
| 6 | 0.56 | 0.54 | 0.49 | 0.45 | 0.56 | 0.42 | 0.35 | 0.48 | 0.08 | 16 |
| 15 | 0.76 | 1.01 | 1.02 | 0.96 | 1.42 | 0.71 | 0.92 | 0.97 | 0.23 | 24 |
| 18 | 0.0027 | 0.0054 | 0.0062 | 0.0049 | 0.0055 | 0.0038 | 0.0034 | 0.0045 | 0.0013 | 28 |

*DYZ5 determined concentration÷*Alu* determined concentration

Table 14 shows the results with 32 casework samples. These ranged from standards to differentials to reagent blanks. Duplex assay results were compared to the original STR results in terms of male:total DNA. For selected samples, STR results were re-run based on the concentration determined in the duplex assay. This was to determine if the duplex assay correctly determined both male:total DNA and DNA amount. In general, the duplex assay results are similar to the results from the original STR analysis. The fact that the RFUs for the TPOX RFUs (Table 14, last column) were between 518 and 2639 show that the duplex assay correctly determined the DNA concentrations.

TABLE 14–*Results with casework samples.*

| Sample Type | AMEL Male:Total Ratio* | STR Male:Total Ratio (Locus)† | Duplex Assay Male:Total ratio‡ | AMEL Y:X RFU§ (Male:Total ratio*) 0.1 ng/µl Dilution | TPOX RFU for 0.1 ng/µl Dilution‖ |
|---|---|---|---|---|---|
| cutting | 0.41 | 0.58 (D18) | 0.33 | | |
| cutting | 0.00 | 0.0 (THO1) | 0.00 | | |
| cutting | 0.00 | 0.0 (THO1) | 0.00 | | |
| cutting | 0.95 | 1.0 (D3) | 0.86 | 799:744 (1.04) | 621, 518 |
| swab | 0.00 | 0.0 (THO1) | 0.00 | | |
| swab | 0.97 | 1.0 (D3) | 1.11 | | |
| tape lift | 0.94 | 1.0 (D3) | 0.86 | | |
| fingernail scraping | 0.89 | 1.0 (D16) | 0.60 | | |
| male fraction cutting | 0.99 | 1.0 (D7) | 1.37 | | |
| male fraction cutting | 0.04 | 0.12 (D5) | 0.02 | | |
| male fraction cutting | 0.00 | 0.0 (THO1) | 0.00 | | |
| male fraction cutting | 0.00 | 0.0 (D18) | no amp | | |
| male fraction cutting | 1.04 | 1.0 (D18) | 1.92 | | |
| male fraction cutting | 0.94 | 1.0 (D3) | 1.67 | | |
| male fraction swab | 0.72 | 0.81 (D7) | 1.00 | | |
| male fraction swab | 0.81 | 0.82 (D3) | 0.91 | | |
| female fraction cutting | 0.03 | 0.074 (FGA) | 0.01 | | |
| female fraction cutting | 0.39 | 0.42 (D16) | 0.33 | | |
| female fraction cutting | 0.00 | 0.0 (D18) | 0.00 | 0:2551(0.0) | 887, 775 |
| female fraction cutting | 0.68 | 0.74 (D3) | 0.71 | 944:1877 (0.66) | 848, 730 |
| female fraction cutting | 0.18 | 0.17 (D3) | 0.14 | 206:2344(0.16) | 714, 817 |
| female fraction cutting | 0.31 | 0.12 (CSF) | 0.16 | | |
| female fraction swab | 0.00 | 0.0 (CSF) | 0.00 | 0:2142 (0.0) | 1575 |
| female fraction swab | 0.00 | 0.0 (CSF) | 0.00 | 0:2261 (0.0) | 1313 |
| male standard | 1.34 | 1.0 (D16) | 0.79 | 1017:861 (1.08) | 572, 622 |
| male standard | 0.95 | 1.0 (TPOX) | 0.50 | 1152:1718 (0.80) | 1140 |
| male standard | 1.09 | 1.0 (D8) | 1.36 | 1661:1285 (1.13) | 2639 |
| male standard | 0.93 | 1.0 (D3) | 1.02 | | |
| female standard | 0.00 | 0.0 (D16) | 0.00 | 0:2609 (0.0) | 2051 |
| female standard | 0.00 | 0.0 (D8) | 0.00 | | |
| reagent blank | | | no amp | | |
| reagent blank | | | no amp | | |

*AMEL Y peak RFU÷(AMEL Y peak RFU+ AMEL X peak RFU), †(RFU of known male peaks)÷(RFU of male+female peaks) for the given locus. The male and female peaks were determined on a case-by-case basis by reference to the known standards. ‡ DYZ5 determined concentration÷*Alu* determined concentration, § RFU of the AMEL peaks (Y:X). DNA was diluted to 0.1 ng/µl based on the results of the duplex assay and COfiler analysis performed. ‖RFU of the TPOX peak(s). DNA was diluted to 0.1 ng/µl based on the results of the duplex assay.

The effects of four PCR inhibitors (hematin, indigo carmine, bark extract and soil extract) on the duplex assay were determined by adding a dilution series of each chemical or extract. Figure 24 shows the effect of increasing amounts of hematin on the assay. A subset of these

inhibitor concentrations were added to COfiler amplifications to determine if the duplex assay was more, less or equivalently as sensitive to inhibition as the COfiler STR assay.

Figure 24–Duplex assay results on hematin containing samples. PCR was performed as described in Materials and Methods for the inhibitor experiments. a. DYZ5 probe results. b. *Alu* probe results.

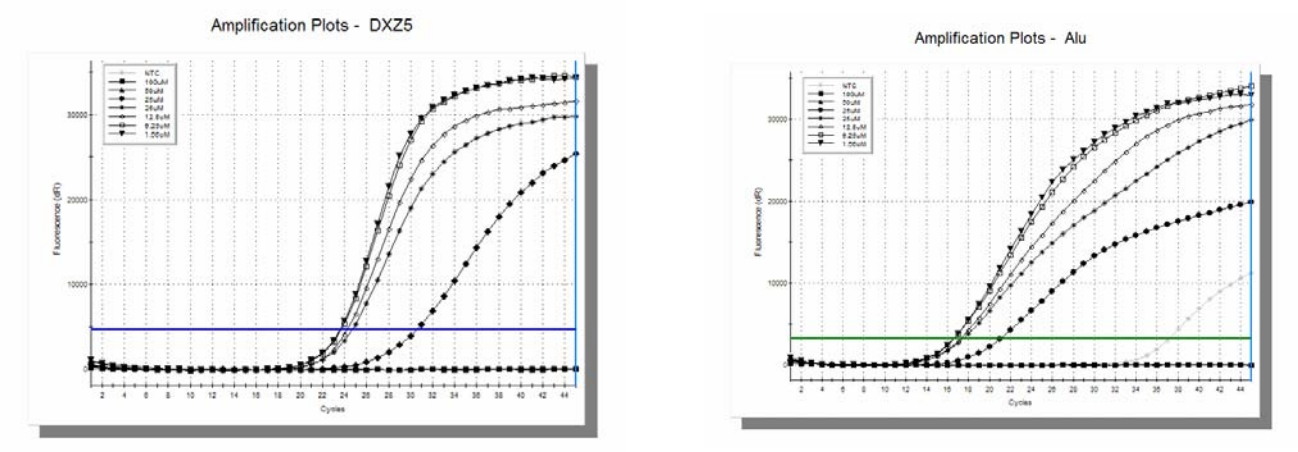



Table 15 gives the change in Cts ($\Delta Ct = Ct_{inhibitor} - Ct_{no\ inhibitor}$) for the different concentrations of each inhibitor and the COfiler TPOX peak heights. For the bark extract, the duplex assay is slightly more sensitive since there is STR amplification (although it is greatly reduced) at the 1/125 concentration while there is no duplex assay amplification. For the indigo carmine, neither the duplex assay nor the STR amplification demonstrates significant inhibition. Both assays have similar results with the soil extract. The STR assay is slightly more sensitive to hematin than the duplex assay as it fails at 25 μM while the duplex assay amplifies, but at a reduced efficiency. The DYZ5 and *Alu* components of the duplex assay have similar results although the DYZ5 component is more sensitive to the bark and soil extracts.

TABLE 15–*Results with inhibitors.*

| Inhibitor | DYZ5 ΔCt* | *Alu* ΔCt* | STR result (TPOX RFU)† |
|---|---|---|---|
| bark 1/125 final | no Ct w/inhibitor | no Ct w/inhibitor | 929, 844 |
| bark 1/250 final | -11.46 | -3.53 | 1772, 1697 |
| bark 1/500 final | -1.85 | -0.81 | 1188, 1192 |
| bark 1/1000 final | 0 | 0 | 1102, 1100 |
| indigo 400 µM final | -4.87 | -5.33 | 1661, 1609 |
| indigo 200 µM final | -1.58 | -2.38 | 1649, 1531 |
| indigo 100 µM final | -0.68 | -0.73 | 1284, 1142 |
| indigo 50 µM final | 0 | 0 | 1787, 1997 |
| soil 1/125 final | no Ct w/inhibitor | no Ct w/inhibitor | 0 |
| soil 1/250 final | -12.01 | -3.30 | 1357, 1231 |
| soil 1/500 final | -2.75 | -0.58 | 733, 761 |
| soil 1/1000 final | 0 | 0 | 1657, 1534 |
| hematin 50 µM | no Ct w/inhibitor | no Ct w/inhibitor | 0 |
| hematin 25 µM | -6.92 | -4.49 | 0 |
| hematin 12.5 µM | -0.99 | -0.95 | 294 |
| hematin 6.25 µM | -0.65 | -0.65 | 299 |
| hematin 3.12 µM | 0 | 0 | 197 |

*ΔCt =Ct with no inhibitor – Ct with inhibitor, †RFU of the TPOX peak(s). DNA was diluted to 0.1 ng/µl based on the results of the duplex assay and COfiler analysis performed.

Lastly, the assay was used with DNAseI treated DNA (single source male DNA) to simulate DNA degradation (Table 16). As expected, the Cts go up (DNA concentration goes down) as DNAseI digestion time increases. A number of these samples were diluted to 0.1 ng/µl based on the *Alu* assay results and used as a template for STR analysis. The results are shown in Table 16. The *Alu* portion of the duplex assay generally correctly predicted input DNA for STR analysis although some RFU values are too low.

TABLE 16–*Results for DNAseI treated DNA.*

| Degradation Time | DYZ5 result for 1:20 dilution (ng/µl) | *Alu* result for 1:20 dilution (ng/µl) | THO1 RFU for 0.1 ng/µl Dilution (based on *Alu* results) |
|---|---|---|---|
| 0 sec | 2.43 | 2.98 | 974, 1110 |
| 15 sec | 1.86 | 3.52 | 160, 153 |
| 30 sec | 1.22 | 2.44 | 602, 443 |
| 1 min | 0.75 | 1.97 | 199, 177 |
| 2 min | 0.21 | 0.75 | 94, 82 |
| 4 min | 0.036 | 0.124 | 452, 324 |
| 8 min | 0.013 | 0.017 | Not done |
| 16 min | 0.0004 | 0.0001 | Not done* |
| 32 min | 0.0039 | 0.0025 | Not done |
| 64 min | 0.00002 | 0.00001 | Not done |
| 128 min | 0.000000 | 0.000002 | Not done |

*STR results not performed as DNA less than 0.1ng/ul.

A duplex assay for total human and male-specific DNA determination using the multicopy *Alu* sequence and a multicopy Y (DYZ5) chromosome sequence has been developed. The assay was shown to be human specific, reproducible and to correctly determine the ratio of male to total DNA for a large number of male and female individuals, as well as artificial mixtures and casework samples.

Although the ratios for normal males are expected to be 1.00, the measurements of total (*Alu)* and male (DYZ5) concentrations, as stated above, can vary by ~20% due to day-to-day variability, therefore, the ratio will vary by considerably more. For example, for a male DNA sample in the extreme case where the *Alu* determined (total) concentration is 20% too high and the DYZ5 (male) concentration is 20% too low, the ratio will be $0.8x \div 1.2x = 0.67$. Alternatively, if the *Alu* determined (total) concentration is 20% too low and the DYZ5 (male) concentration is 20% too high, the ratio will be $1.2x \div 0.8x = 1.5$. These results taken together imply that a normal male will vary between 67% male and 150% male. The variability in male samples in our studies is somewhat higher than this with four samples having ratios lower than 0.67 and the mean being $0.83 \pm 0.14$ rather than 1.0. The ratio does generally run below 1.0 but varies from day-to-day (data not shown). There is variability in the number of DYZ5 sequences in different males since the size of the major block appears to vary (Tyler-Smith et al., 1998). This variability does not appear to be significant for this DYZ5-based assay based on our results. This was a major problem in a previous assay we attempted to develop using the centromeric DYZ3 repeat; with this assay, male ratios varied from 0.2 to 1.4, and were influenced by ethnic background (data not shown).

The duplex assay also correctly determined input DNA for STR analysis in all but extreme circumstances of degradation or presence of inhibitors. The assay has a broad dynamic

range from 64 ng/µl to less than 4 pg/µl making it useful for any sample input. No endogenous control is needed as the background *Alu* amplification provides proof that PCR has occurred. Furthermore, the assay has a built in detector for PCR failure or inhibitors. If the *Alu* assay has not crossed the threshold by ~38 cycles which is the Ct for the NTC (no added DNA) then some component is missing or there was inhibition. The assay, thus, has its own equivalent to an endogenous control.

This duplex assay can be compared to several other assays that are available to determine male DNA in forensic samples. Combined use of the Applied Biosystems Quantifiler™ Human and Quantifiler™ Y kits (Green et al., 2005) will give a male to total DNA ratio similar to that determined here. The Quantifiler™ TaqMan-based assays both use single copy genes and have small amplicons, 62 bp and 61 bp/64 bp, respectively. Each assay also contains an endogenous control. The use of single copy genes reduces sensitivity down to 23 pg (as compared to 0.5 pg for the *Alu* part of the duplex assay). The small amplicon size will make the assay less predictive of amplifiable DNA concentrations at higher levels of degradation than the duplex assay, which has amplicon sizes of 127 bp and 137 bp that overlap that of some of the CODIS autosomal STRs. The combined use of the two Quantifiler™ assays requires twice as much sample and wells (and associated tubes, tips etc.), and is also considerably more expensive than the old Applied Biosystems Quantiblot™ even for only the human quantitation assay. For comparison purposes, the Quantifiler™ User's Manual (Applied Biosystems, Foster City, CA) reports results on six DNAs (with 3 concentrations for each) analyzed in three different runs. The percent standard deviation for the Quantifiler™ Human kit ranged from 0.43 to 29.8% while for the Quantifiler™ Y kit, it ranged from 3.27 to 27.7%. These are also within the ranges reported by Bustin (Bustin, 200, 2002). For the Quantifiler™ assays, the User's Manual data give Y:Total

ratios from 0.79 to 2.62 with a mean of 1.18±0.24 (percent standard deviation ranged from 3.92 to 57.63. Thus, both assays have similar variation (the duplex assay does have a smaller standard deviation); the Quantifiler™ male estimates appear to be on the high side (>1.0) while the duplex assay estimates are on the low side (<1.0).

A triplex assay based on determination of human nuclear, mitochondrial and male DNA has been developed in addition to a duplex X chromosome/Y chromosome assay (Walker et al., 2005). The X and Y assays are based on a 90 bp deletion on the X chromosome relative to the Y chromosome. The triplex assay uses a Yb8 family *Alu* for the nuclear sequence, a conserved mitochondrial region and the Y sequence from the X/Y assay. The triplex assay has a range of 100 ng to 100 pg. This assay has the advantages of being a combined assay, thus saving on reagents, and using the multicopy *Alu* (which should afford the equivalent of an endogenous control); however, the Y sequence is single copy and reduces the assay sensitivity to 100 pg. The amplicon sizes are small (71 bp, 79 bp, 77 bp and 69 bp for the *Alu*, mitochondrial, X and Y components, respectively) and although they are larger than the Quantifiler™ PCR products, they are still smaller than any of the STR PCR products. Dr. Sudhir K. Sinha of ReliaGene Technologies has also presented (Sinha, 2005) an assay based on insertion of a single *Alu* sequence into the Y chromosome which was discovered by Dr. Mark Batzer's group (Louisiana State University). This assay is single copy but is reported to have sensitivity down to 0.5 pg. The amplicon size is ~270 bp. This is a singleplex assay run on a capillary electrophoresis unit. No associated total human assay was reported although a ~200 bp avian endogenous control is utilized. Lastly, the Promega Corporation has announced that a duplex male/human assay will be forthcoming based on their new real-time Plexor technology; however, no details were available at the time this manuscript was prepared.

The duplex assay presented in this study should allow examiners to decide how to best proceed with samples early in DNA analysis. Because of day-to-day variability and slight variations in copy number for both *Alu* and DYZ5, the duplex assay will never give an exact percentage of male DNA for mixtures (i.e. it won't discriminate between 20% and 40% male); however, the estimate will be sufficient to determine STR input DNA and to decide on autosomal vs Y STRs. Samples with very low amounts of total DNA would indicate a low probability of success with STR analysis and would allow a better utilization of resources, or indicate that mitochondrial DNA analysis would be a better choice for such a sample. Those samples with a high female to male ratio could indicate that Y-STR analysis would be a more appropriate analysis, or that it may be appropriate to re-extract the crime scene sample with greater emphasis on female fraction clean-up. Those samples without any male DNA component could either be dropped from analysis if a male profile is expected or have autosomal STR performed for a female profile. There is also a possibility that this assay could be used very early in the screening process to quickly identify stains containing a male DNA component. Such a test could be employed through simplified extraction protocols to select stains for further in depth analysis. The cost of the assay ingredients is ~$0.70 per sample which is comparable to the old Quantiblot™ assay; however, the duplex assay gives faster, less-hands-on results for human quantitation, as well as for male DNA quantitation, which makes the assay very cost effective. The reference for this work is: Nicklas JA and Buel E. (2006) Simultaneous Determination of Total Human and Male DNA Using a Duplex Real-Time PCR Assay, J Forensic Sci (in press).

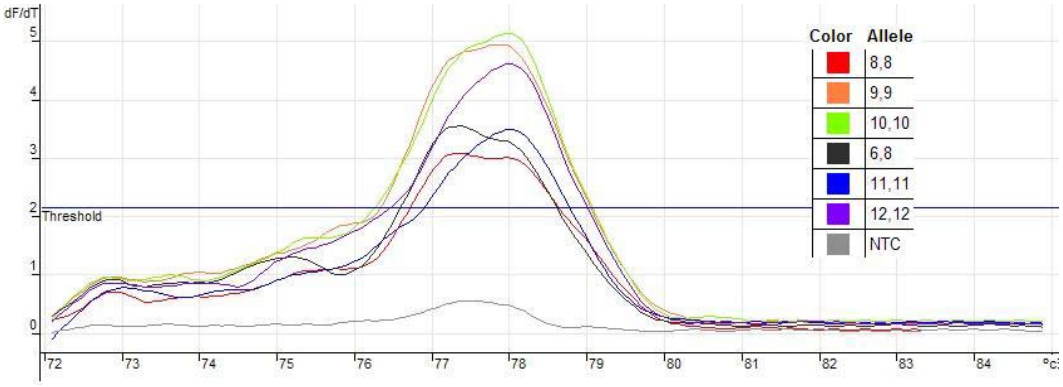
Goal 2 of this grant was to develop a simple, fast assay that detects alleles at one or two STR loci as quick sample screening method. While STR profiling is completely definitive for

sample identification, it is expensive and time-consuming and, for these reasons, cannot be performed on every single sample that may be found at a crime scene. A faster, cheaper method is needed to quickly screen crime scene samples to determine which samples require complete profiling. This new method need not be perfectly definitive nor give a complete profile; it simply must be able to differentiate between most victim and suspect and, perhaps resident, samples.

We started with the hope that because different alleles of the STR loci had different lengths, that they would melt at different temperatures. Perhaps not every allele would melt distinctly differently (i.e. enough to give conclusive typing like STRs) but most alleles would give detectable differences. Intuitively, one would think that the longer alleles would melt at higher temperatures. However, it turned out that this notion was way too simplistic. In fact, longer alleles could melt at lower temperatures if the tetrameric repeat was AT rich. We attempted initially to look at the TPOX locus because the amplicon was small and we hoped that melting differences would be apparent. While DNAs from different individuals did give different melting profiles (Figure 25), it was not consistent between individuals of the same genotype nor was there any predictable pattern for allele 8 vs allele 9 vs allele 10, etc. We thus gave up on TPOX as a locus. We asked the folks at NIST if they had any suggestions for loci and Mike Coble generously sent us samples of primers for D22 as well as DNA from genotyped individuals. Again, the results were similar, the melting pattern was not consistent between individuals of the same genotype nor was there any predictable pattern for the alleles. We then surveyed the sequences of all the forensic STRs looking at the sequence of the repeat (all are 3 A:T and one G:C or 2 A:T and 1 G:C) . It was hoped to find some 3 G:C and 1 A:T as then melting temp should increase with length but none were found. Checks of melting temperatures

of the various alleles for over ten most likely candidate loci, all were disappointing as the melting differences were small and often not linear with length. This line of STR-based melting methodology was, thus, abandoned.

Figure 25 – Melting patterns of TPOX alleles




While full profiling for CODIS requires 13 STRs, SNPs are commonly used for identification purposes, for example, paternity testing. SNPs have the advantage that many molecular methods have been developed for their rapid and inexpensive detection. Using an assay with four SNPs, each with p=q=0.5, the chance of two random individuals having the same result (same genotype) is only 2% while for 8 SNPs it is only 1/2500 (see Insert 1).

**Insert #1 - SNP identity percentage calculation**

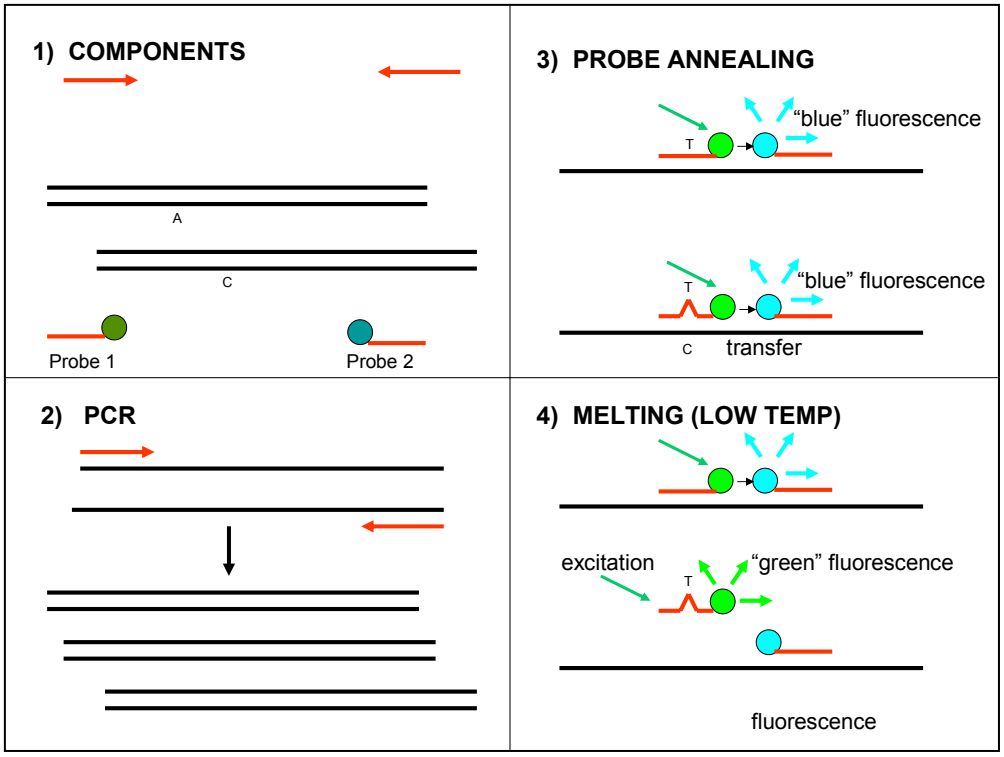
Determine freq of two people being identical: AA+AA (freq =0.25x0.25=0.0625) plus AB+AB

For n independent SNPs = $(0.375)^n$; in particular for four SNPs = $(0.375)^4$ = 0.01978 or ~2% or for 8 or 0.04% or ~1 in 2500.

Such a screening test would be sufficient for most crime scene samples. Many forensic labs are now acquiring real-time PCR instruments for human DNA quantitation. Quick SNP

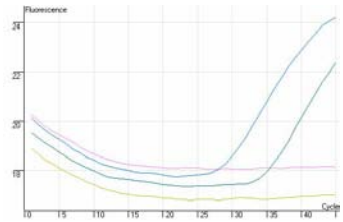
profile methods developed on such an instrument could be easily performed and would require no additional investment in equipment. TaqMan and molecular beacon based methods exist for detecting SNPs; however, at best two SNPs can be genotyped at once. Others (Hiratsuka et al, 2002; Pont-Kingdon and Lyon, 2003; Lareu et al., 2001) have used melting point methods to type SNPs. Such methods can use only one reporter dye per SNP; thus, theoretically four SNPs can be multiplexed making a simple, one tube profiling assay feasible). We have recently realized that it may be possible to multiplex 8 SNPs (two each color) as long as the melting points of the alleles of the two loci in the same color are sufficiently different. Lareu et al. (2001) did try something similar in their paper as their LightCycler™ instrument could only detect 3 colors and they had some overlap of peaks and dyes. Figure 26 demonstrates the FRET melting point assay method.

**Figure 26 – SNP FRET melt curve analysis method**

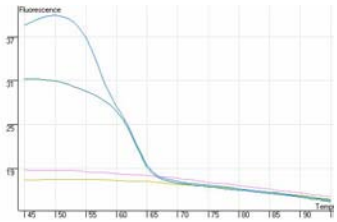


In this type assay, PCR is performed as usual using two primers flanking the SNP. Two probes are also present: one probe with a 3' dye label which covers the SNP and a second with a 5' dye and a 3' extra phosphate to prevent elongation. The first probe is a perfect match to one allele (allele A) but obviously has one mismatch with the other allele (allele C). When the two probes both bind to the PCR product, and the 3' dye is excited, it transfers energy to the 5' dye which produces light. Fluorescence by the second dye will only occur on (and will be proportional to) PCR product. Determination of which allele(s) is present depends on the melting temperature where the fluorescence is lost. Obviously, when the allele with the A is present, the first probe will melt off later than if the 1 bp mismatch G is present. This assay has a second variation where the 5' dye is replaced by a darkhole quencher. In this assay, the 3' dye is both excited and detected. PCR product is detected as a loss of fluorescence as the 3' dye is quenched by the black hole quencher. Melting thus causes an increase in fluorescence which can be detected.

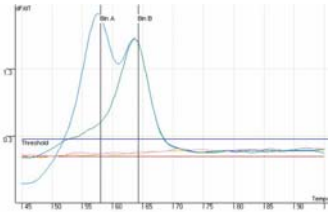
Figure 27 – Melt curve analysis (From Corbett Research "Mutation detection using FRET analysis" While two dyes are used here, fluorescence of only 1 dye is being detected (the 5' dye on the second probe); A. Fluorescence increase during amplification B. Raw data for melt C. Analyzed data for melt showing homozygote and heterozygote curves.



A.



B.



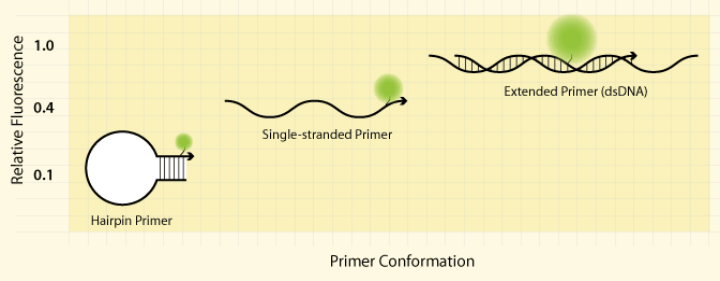
C.

Our long-term goal thus is to use this methodology to develop a simple, screening profile assay using 5 SNPs each with p≈q≈0.5 plus a gender (male/female) discriminator. This 6-plex

assay could be performed quickly on a 6 color real-time instrument and save much effort/expense by avoiding unneeded STR profiling of duplicate samples. Preliminary data also suggests that by running appropriate quantitation standards that this SNP assay could double as a means to quantitate samples. This assay would not be as sensitive as our *Alu* based assay but could be an effective use of time and of sample for those samples which do not require a very sensitive quantitation approach. We have written this goal as an aim in our current NIJ grant, "Improving the efficiency of DNA casework analysis through simple, effective, PCR-based screening methods" , (Grant # 2005-DA-BX-K003).

Goal 3 of this grant was to improve of the original *Alu* PCR Real-time PCR assay. We decided to try several new methods that we had heard about. The first was LUX (Invitrogen) (Figure 28).

Figure 28 -D-LUX™ detection technology uses one primer labeled with a single fluorophore and a corresponding unlabeled primer, both custom-synthesized according to the target of interest. Typically 20-30 bases in length, LUX™ Primers are designed with a fluorophore near the 3´end in a hairpin structure. This configuration intrinsically renders fluorescence quenching capability, making a separate quenching moiety unnecessary. When the primer becomes incorporated into the double-stranded PCR product, the fluorophore is de-quenched, resulting in a significant increase in fluorescent signal.




The advantage of the LUX system is that it only uses primers (one dye-labeled, one not) instead of two primers and a probe. Thus, it seemed simpler and also less expensive. However, the fluorescence intensity change as PCR proceeded was very minimal as compared to background noise (Figure 29) and the % stdev was very high (Table 17). Thus, we found the assay not viable for the low DNA concentrations isolated from forensic samples.

Figure 29 – Results with LUX primers (low RFUs)

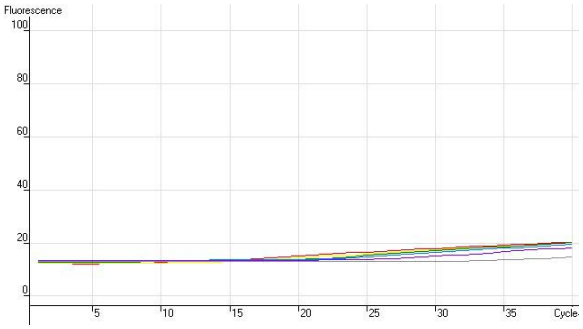



Table 17 – Results with LUX assay (high %stdev)

| Sample | Concentration | | | AVERAGE | STDEV | %STDEV |
|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | | | |
| #1 | 3.88 | 5.75 | 5.43 | 5.02 | 1.00 | 19.93 |
| #2 | 1.21 | 3.25 | 3.92 | 2.79 | 1.41 | 50.62 |
| #3 | 0.79 | 0.64 | 1.26 | 0.90 | 0.32 | 36.01 |
| #4 | 1.83 | 0.53 | 2.82 | 1.73 | 1.15 | 66.73 |
| #5 | 1.08 | 0.81 | 1.14 | 1.01 | 0.18 | 17.47 |
| #6 | 3.48 | 3.74 | 6.19 | 4.47 | 1.50 | 33.48 |
| #7 | 1.42 | 1.46 | 0.88 | 1.25 | 0.32 | 25.92 |
| #8 | 3.64 | 5.56 | 3.17 | 4.12 | 1.27 | 30.74 |

We also tried two different kinds of new proprietary probes from Stratagene, Keyhole and FLAP. It is unclear how these probes work; they were given to us on a test basis with no further information. The FLAP probes gave some inconsistent *Alu* amplification and no Y amplification (Figure 30) while the Keyhole probes did give some data but it was not as good as TaqMan (Figure 31).

Figure 30 – Results with FLAP probes A) *Alu* and B) *Y*

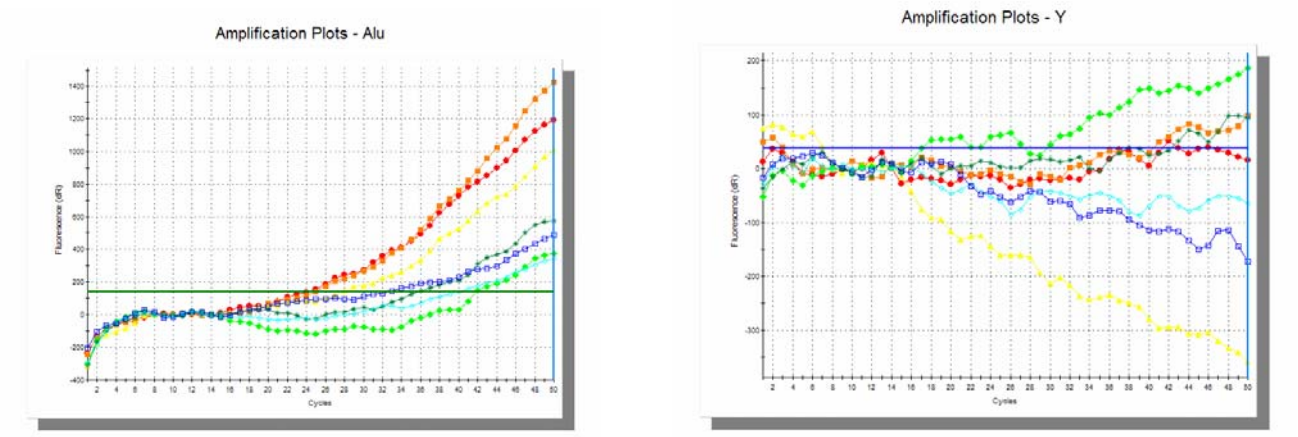
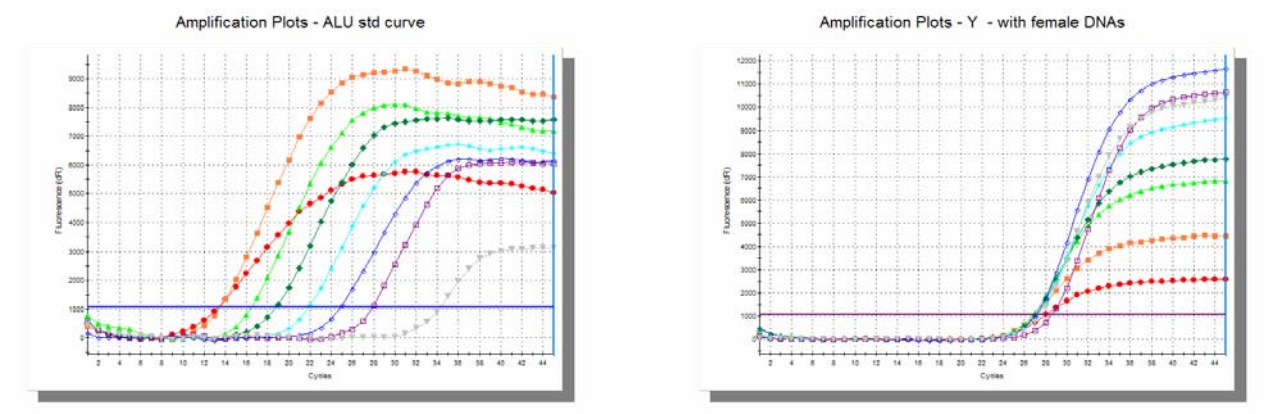


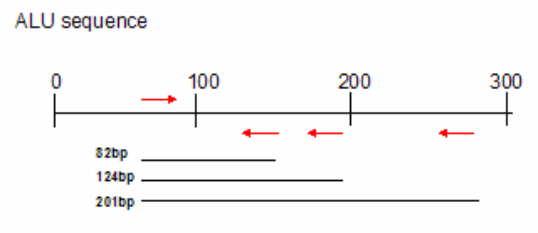
Figure 31 – Keyhole results A) *Alu* std curve amplification B) Y results from *Alu*/Y duplex showing effects of adding female DNA to a constant amount of male DNA (as seen before with various buffers – Figure 24)




Other improvements that we had put forth in the grant proposal were to develop a quantitation assay for the mini-STRs that would detect DNA degradation. Environmentally challenged forensic samples may often be degraded into small DNA fragments, resulting in a limited DNA profile using commercial STR profiling kits. For example, DNA degraded to an average size of 150bp will not allow amplification of a 320bp D18S51 allele. Because of this, forensic researchers have developed sets of primers to create smaller STR products (mini STRs) (Butler et al., 2003; Wiegand and Kleiber, 2001). Prior knowledge of the degradation state of a sample would allow the examiner to decide how best to proceed with the analysis of a sample. A precious sample could be conserved for an appropriate "mini STR" analysis or time and resources saved if it is determined that a sample is too degraded to profile. Current DNA quantitation kits will not accurately predict the DNA quantity needed for STR analysis when the DNA is degraded. The new ABI Quantifiler™ kit has a 62bp amplicon and, therefore, will not accurately predict if there is a sufficient quantity of large fragments to support a full amplification using commercial STR kits. We therefore wish to develop an assay to detect the

degradation state of the DNA. We will start with a multiplex PCR with several overlapping *Alu* amplicons (Figure 32).

Figure 32 – *Alu* amplicons for degradation studies

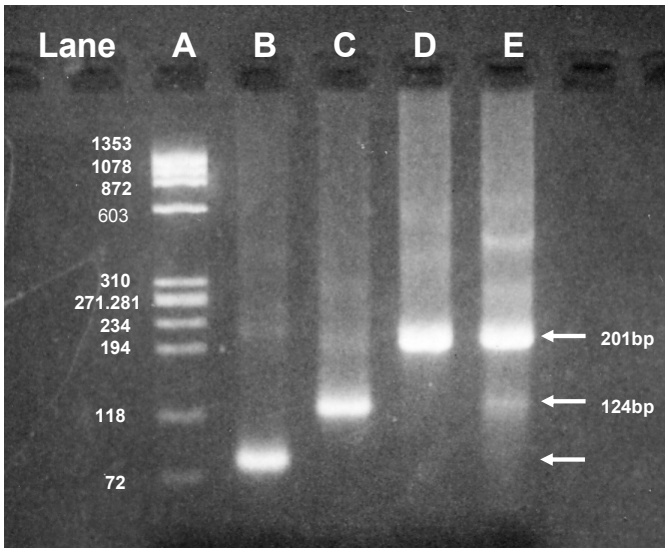


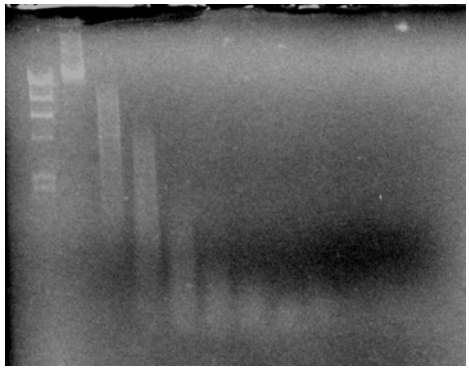

We have much experience with this sequence from our past development of human quantitation assays funded by NIJ grants. The longer amplicons will only be possible in non-degraded or slightly degraded DNA while the smaller amplicon will be amplifiable even in fairly highly degraded DNA. Thus, ratios of the different products should give a qualitative (or maybe even quantitative) measure of degradation state.

We performed some preliminary experiments using different sized *Alu* amplicons with a simple gel-based readout. Figure 33A shows a very quick demonstration of the three *Alu* amplicon on high-quality commercial DNA (giving 82bp, 124bp and 201bp products) while Figure 33B shows the results with DNaseI degraded DNA where the lower amplicon is lost with degradation and the smaller amplicon increases.

**Figure 33A – Gel of Alu PCR results for degradation studies**

verse primers



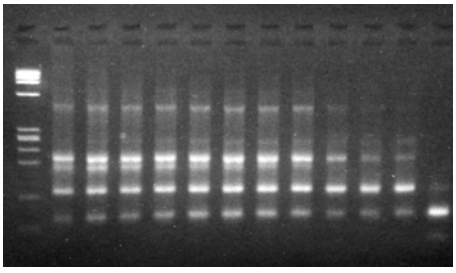



overlapping amplicons on same

Lane A – upper gel - λ HindIII,

Lane B – No digestion

Lane D – 1 min digestion



Lane I – 12 min digestion

Lane K – 24 min digestion

Lane L – 32 min digestion

Lane M – 48 minute digestion

Lane A B C D E F G H I J K L M

While a gel-based assay would work, a real-time assay would be much faster, more sensitive and less hands on. This assay will require effort in correct fluorescent probe design and also a great deal of optimization to obtain the appropriate ratio of products to allow the determination of the sample's degradation state. Very preliminary results show that ratios of the amplicon are easily manipulated by primer concentration, annealing temperature and extension time. We have written this goal as an aim in our current NIJ grant, "Improving the efficiency of DNA casework analysis through simple, effective, PCR-based screening methods" , (Grant # 2005-DA-BX-K003).

The last goal of this grant was dissemination of methods to the Forensic community. We approached this in several ways. One was talking about and demonstrating our various assays at meetings including the NIJ Grantees meeting, the New England SWGDAM meeting, the Association for Molecular Pathology meeting and the Promega meeting (Table 18).

Table 18 - Presentations on work from this grant

| **Individual** | **Meeting** | **Date** | **Type of Presentation** |
|---|---|---|---|
| Eric Buel | NIJ DNA Grantees' meeting | 6/01 | Talk |
| Eric Buel | NIJ DNA Grantees' meeting | 6/02 | Talk |
| Janice Nicklas | NIJ DNA Grantees' meeting | 6/03 | Talk |
| Janice Nicklas | New England SWGDAM meeting | 7/03 | Demonstration |
| Janice Nicklas | Promega, 14$^{th}$ Inter Symp on Human ID | 9/03 | Poster |
| Janice Nicklas | Assoc. for Molec. Pathology meeting | 11/03 | Poster |
| Eric Buel | Amer. Acad Forensic Sci meeting | 2/04 | Talk |
| Janice Nicklas | NIJ DNA Grantees' meeting | 6/04 | Demonstration |
| Janice Nicklas | NIJ DNA Grantees' meeting | 6/05 | Demonstration |
| Eric Buel | NIJ DNA Grantees' meeting | 6/05 | Talk |
| Janice Nicklas | Promega, 16$^{th}$ Inter Symp on Human ID | 9/05 | Poster |

The second approach to disseminating our results was to send protocols and kits to any forensic lab that requested them (Table 19).

Table 19 – List of labs to whom protocols and/or kits were sent.

| | Individual | Location | Date Sent | Our Action |
|---|---|---|---|---|
| **SYBR assay** | | | | |
| | Theresa Caragine | NY ME's Office | 9/02 | protocol, visit |
| | Robin | Orchid Cellmark | 9/03 | protocol, visit |
| | George Duncan | Broward, FL | 11/05 | protocol, kit |
| | Melissa Staples | NH | 6/05 | protocol |
| | Christine Waterhouse | Maine | 6/04 | protocol |
| | John Krebsbach | Albuquerque | 1/03 | protocol |
| | Abirami Chidambaram | Alaska | 1/03 | protocol |
| | Chris W. Beheim | Alaska | 1/03 | protocol |
| | Mark Timken | CA | ? | protocol |
| | Bruce McCord | FL | ? | protocol |
| **Gender assay** | | | | |
| | Daun Powers | Indiana | 11/05 | protocol, kit |
| | Theresa Caragine | NY ME's Office | 11/05 | protocol, kit |
| | Joanna Segulia | Mass | 11/05 | protocol, kit |
| | Margaret Kline | NIST | 12/05 | protocol, kit |
| | David Foran | MI State | 3/06 | protocol, kit |
| | Kristine Deters | MN | pending | |
| | Fenger/Chute | Marshall U | pending | |

The third was to attempt to find a company to produce our assays as kit(s) for sale to the forensic community. We forged a relationship with Stratagene and they were extremely interested in making a kit based on their proprietary methods. They loaned us a realtime MX3000 instrument and let us try their new FLAP and Keyhole probes as mentioned above. However, the person who we were dealing with, and who had the keen interest, left the company and the project and collaboration died as a result. We are still looking for a company with which to collaborate with but have not had success so far.

The last way to disseminate our work was to publish our results in forensic journals (Table 20).

Table 20 - Publications resulting from this work

Nicklas JA, Buel E. (2006) Simultaneous determination of total human and male DNA using a duplex real-time PCR assay. J Forensic Sci (in press)

Nicklas JA, Buel E. (2005) An Alu-based, MGB Eclipse real-time PCR method for quantitation of human DNA in forensic samples. J Forensic Sci 50(5):1081-90.

Nicklas JA, Buel E. (2003) Development of an *Alu*-based, real-time PCR method for quantitation of human DNA in forensic samples.J Forensic Sci 48(5):936-44.

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