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Simultaneous Determination of Total Human and Male DNA Using a Duplex Real-Time PCR Assay

ABSTRACT: A single duplex assay to determine both the amount of total human DNA and the amount of male DNA in a forensic sample has been developed. This assay is based on TaqMan™ technology and uses the multicopy *Alu* sequence to quantitate total human DNA and the multicopy DYZ5 sequence to quantitate Y chromosomal (male) DNA. The assay accepts a wide concentration range of input DNA (2 μL of 64 ng/μL to 0.5 pg/μL), and also allows detection of PCR failure. The PCR product sizes *Alu* (127 bp) and DYZ5 (137 bp) approximate that of the smaller short tandem repeats (STRs) which should make the assay predictive of STR success with degraded DNA. The assay was optimized for probe/primer concentrations and BSA addition and validated on its reproducibility, on its human specificity, on its nonethnic variability, for artificial mixtures and adjudicated casework, for the effect of inhibitors and for state of DNA degradation. This assay should prove very usual in forensic analyses because knowing the relative amounts of male versus female DNA can allow the examiner to decide which samples may yield the most probative value in a case or direct the samples to methods that would yield the greatest information.

KEYWORDS: forensic science, human DNA, DNA quantitation, *Alu* sequences, polymerase chain reaction, real-time, male DNA

The application of real-time PCR for DNA quantitation has become an accepted technique surpassing the slot blot as the quantitation method of choice for forensic laboratories. Real-time PCR provides a rapid estimation of the DNA from extracted samples and streamlines the process of DNA analysis. As the demand for DNA analysis increases, forensic laboratories are obliged to investigate new methods that would further enhance the analytical tools and methods at their disposal. A method that would simultaneously quantitate the total and male DNA extracted would save time, in addition to facilitating the selection of probative samples for short tandem repeat (STR) typing.

The determination of the sex of the individual who donated a particular stain or the relative amount of male to female DNA contained in a sample can be critically important to deciding which analytical steps may be most appropriate. Blood stains could be easily screened to determine which were contributed by a male or female and only those of probative value selected for further analysis. Some of the serological testing done before DNA extraction to determine the presence of semen (prostatic antigen, P30) is very sensitive and can detect extremely low levels of semen; however, these tests are often not good predictors of STR typing success from a particular sample. In addition, the P30 test uses part of the evidence that is then lost to future DNA analysis. Skipping this step and proceeding directly to a quick DNA preparation followed by quantitation of the male DNA would address both the presence of male DNA and the quantitation. The identification of male DNA, in a vaginal swab for instance, may be enough evidence to address sexual assault issues for some jurisdictions and hence the determination of PSA or the identification of sperm may not be necessary. Knowing the amount or percentage of male DNA from a stain swab or differential extraction will indicate the probability of obtaining a full male STR profile, or whether Y STR analysis will be necessary. 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.

We have previously developed two real-time PCR methods to quantitate human DNA in forensic samples using the human *Alu* sequence (1,2). The use of a multicopy gene such as *Alu* (detection limit 0.5 pg) improves assay sensitivity compared with single copy gene assays (detection limit of about 25 pg). This paper reports the development of a duplex total human and male DNA quantitation assay using the human *Alu* sequence and a human Y chromosomal repeat (DYZ5). DYZ5 is a Y-specific sequence (Yp11.2) with a repeat of *c*. 20,300 bp (3). The testis-specific protein, Y encoded, genes are part of the DYZ5 repeat unit (4). There is one array of $\sim$ 540–800 kb and another minor block of 60 kb on the Y chromosome (3). The DYZ5 repeat is conserved in the great apes (5) but is not present in other mammals.

The duplex assay uses TaqMan MGB probes labeled with VIC and FAM dyes to detect the two amplified sequences, *Alu* and DYZ5, respectively. Studies were conducted to optimize probe/primer concentrations, mastermix and PCR conditions. The assay is reproducible and consistent from male to male across ethnic groups. The male specific assay has a $>$500,000-fold difference in detecting male versus female DNA and can detect down to 4.0 pg of male DNA.

Materials and Methods

TaqMan™ Primers/Probes

For a human specific sequence, the human *Alu* sequence (Ya5 subfamily) was chosen (6,7). Currently there are 2473 identified Ya5 sequences in the human genome, of which 75–80% are fixed (present in all individuals) and 20– 25% are polymorphic ((7), D. J. Hedges and J. A. Walker, personal communication). There

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are estimates of another ~ 557 polymorphic *Alu* sequences that are not yet discovered (D. J. Hedges and J. A. Walker, personal communication). 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 nonprimates. 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.

PCR Assay

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 premade 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], FastStart TaqMan Probe Master, Roche Applied Science, Indianapolis, IN]).

Real-time PCR for the assay was performed primarily in a Stratagene MX 3000P, 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 95°C for 15 min ("hotstart") followed by 45 cycles of 95°C for 30 s, 60°C 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.

DNAs Utilized

Promega Human genomic DNA: male (G1471, Madison, WI) was used for standards and Promega Human genomic DNA (G3041) and Promega Human genomic DNA: female (G1521) were used for controls. These three reagents are mixtures of DNA from several individuals. Animal DNA samples were extracted from buccal swabs of pets owned by Vermont Forensic Laboratory personnel or obtained from Dr. C. William Kilpatrick (University of Vermont). *Escherichia coli* DNA and *Clostridium* DNA were obtained from Sigma (St. Louis, MO). Herring sperm DNA was obtained from Gibco/BRL (Gaithersburg, MD). A male DNA sample (single source) was used for the DNAseI experiments.

Inhibitors

Bark and soil extracts were obtained from Dr. Gary Shuler and Katherine Woodard of the Washington State Patrol Crime Laboratory. Hematin (H3281, Sigma, La Jolla, CA) and indigo carmine (I8130, Sigma, La Jolla, CA) were used at final concentrations from 0 to 100 μM. For the inhibition assays, 1 ng of male DNA (total) was added to a 25 μL reaction (instead of the usual 20 μL reaction) to mimic the STR PCR reaction conditions.

STR Analysis

STR analyses were performed using the Applied Biosystems COfiler kit. Amplifications were performed as recommended by the manufacturer, with the exception that a 25 μL reaction was used (10 μL of 0.1 ng/μL sample added). The samples were run on an Applied Biosystems 3130 and analyzed using GeneMapperID 3.2 software (Applied Biosystems) according to manufacturer's recommendations.

Agarose Gel

PCR products were run on a 200 mL, 3% agarose (IBI, Shelton, CT, #IB70042) gel in 1% TAE (SIGMA, St. Louis, MO, #T9650) in a OWL (Owl Separation Systems Inc., Portsmouth, NH) Model A1 class II gel box for ~ 2 h at ~ 150 V.

DNaseI Digestions

Single source male DNA (5 μL of *c*. 200 ng/μL) was digested at 37°C for with RQ1 RNase-free DNase (Promega, Madison, WI, #M610A) in a 120 μL reaction volume containing 1X buffer, and 0.6 μL of a 1/10 dilution of the enzyme. At various times (0 sec to 128 min) after addition of the enzyme, 10 μL aliquots were removed and added to 1 μL of the STOP buffer that came with the enzyme. At the end of the experiment, all the samples were treated at 65°C for 10 min.

Results

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). Figure 1 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 137 bp male product is light with 0.0046 ng male DNA but clearly visible on the original gel).

Figure 2 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 < 4 pg/μL (2 μL of sample is added per reaction) with a tight grouping of Ct values for each sample. To show that the Y-assay was specific for male DNA, the assay was tested with both male and female DNA at 16 and 128 ng/μL (Fig. 3). In Fig. 3*a*, 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. 3*b*, 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.

The assay was optimized by changing several parameters. Primer/probe titrations were performed essentially as recommended by

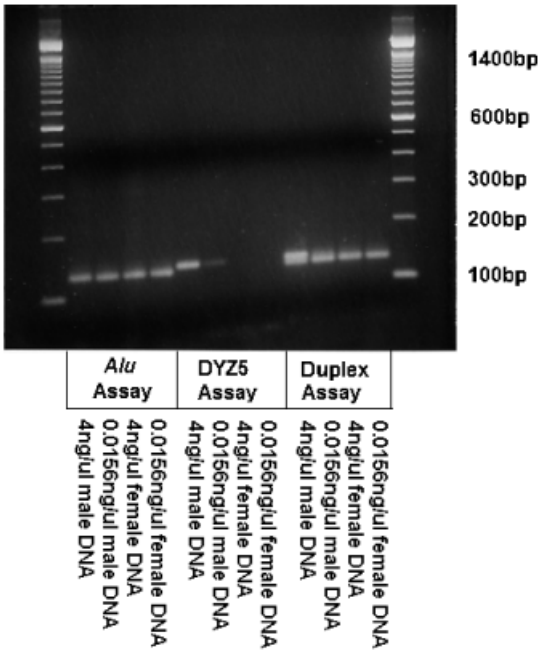

FIG. 1—*Gel electrophoresis of assay products. PCR was performed as described in "Materials and Methods" except that only 32 cycles were performed, no probes were used and three reactions were performed: only DYZ5 primers, only* Alu *primers and both pairs of primers. Male DNA (two concentrations of 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* φ*XHindIII as a size standard.*

Applied Biosystems (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 ng/μL) used in our previously reported SYBR Green *Alu* assay (1). 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).

A side-by-side comparison of six commercial mastermixes (QuantiTect Multiplex PCR kit [Qiagen], Brilliant Multiplex QPCR Master mix [Stratagene], QPCR Mastermix Plus [Eurogentec], Universal Mastermix [Applied Biosystems], Absolute QPCR mix [ABgene, Rochester, CA], FastStart TaqMan Probe Master [Roche Applied Science]) was also performed. 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 (0–51.2 ng/μL in 1:4 dilutions). The expected result was an incremental increase in amount of DNA (0.5–51.25 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. Considerable variation in results between mastermixes was observed (data not shown). 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 as the ingredients are proprietary but could include buffer composition (salts and Mg^{2+}), concentration of nucleotides, concentration of enzyme or extra 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.

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 1 shows the consistency in Ct for standard curve quadruplicate values on the same 96-well plate. As with our *Alu* SYBR® Green assay (1), 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 (8,9); thus, it is presumably due to ambient human DNA in the air and water (see "Discussion"). 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 with 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 2 shows the replicates (different days) of determined concentrations (ng/μL) for a set of 11 samples (five normal males, two males believed to be XYY based on Amelogenin ratios, a Promega stock mixture of male and female DNA diluted to ~4 ng/μL, the 9947 DNA from an Applied Biosystems STR kit [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 2a and 2b, 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 (4 ng/μL) and the neat solution of Applied Biosystems 9947 yielded results consistent with the stated concentrations. Table 2c gives the ratio of male DNA to total DNA which is *c*. 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 and the normal female). The percent standard deviations range from 5% to 30%.

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

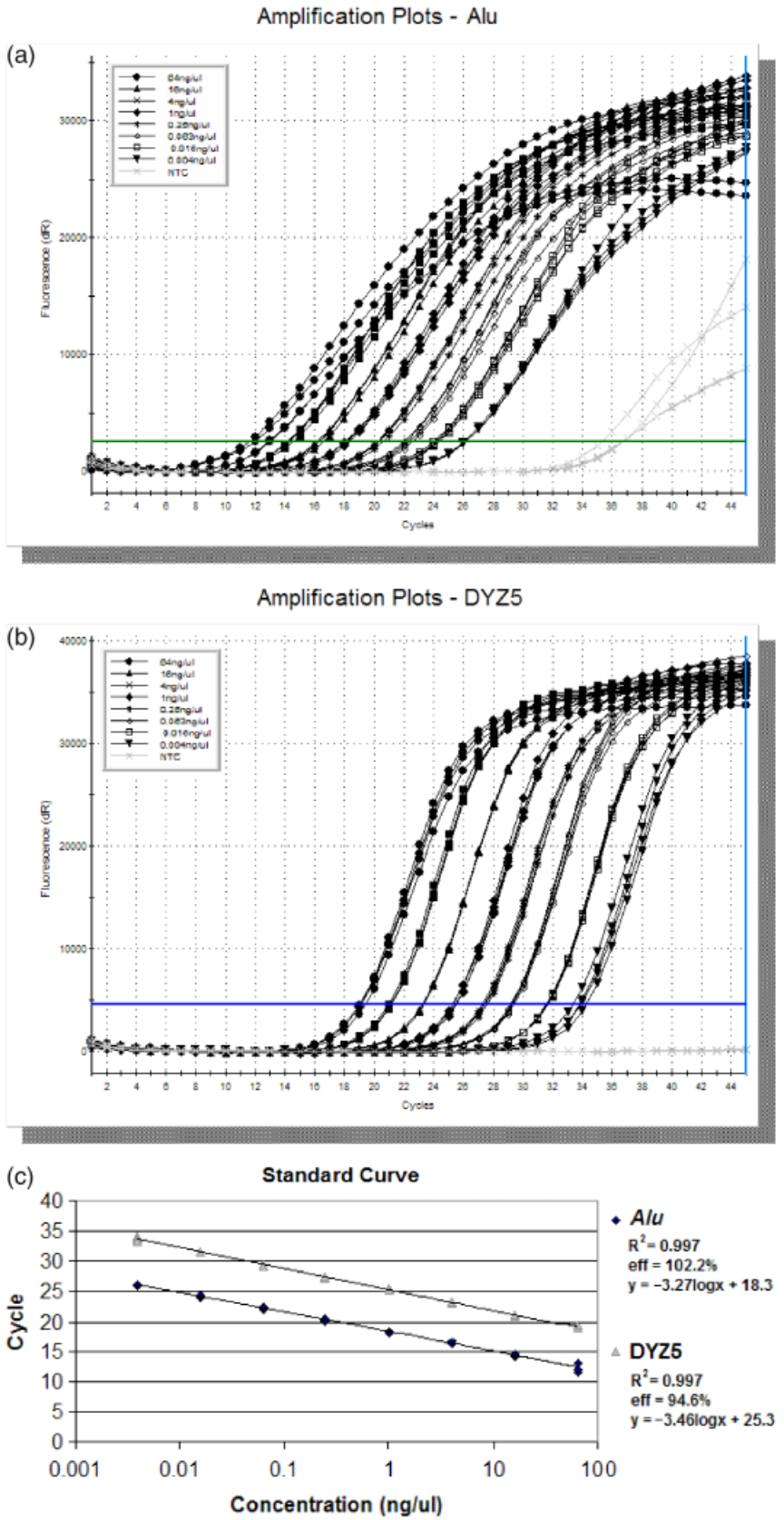

FIG. 2—*Duplex assay results run in quandruplicate. PCR was performed as described in "Materials and Methods." The standard curve dilution series samples were run in quadruplicate on the same plate. (a)* Alu *probe results, (b) DYZ5 probe results, (c) standard curve results. The DYZ5 FAM-labeled probe fluorescence was read in the FAM channel while the VIC-labeled* Alu *probe fluorescence was read in the HEX channel.*

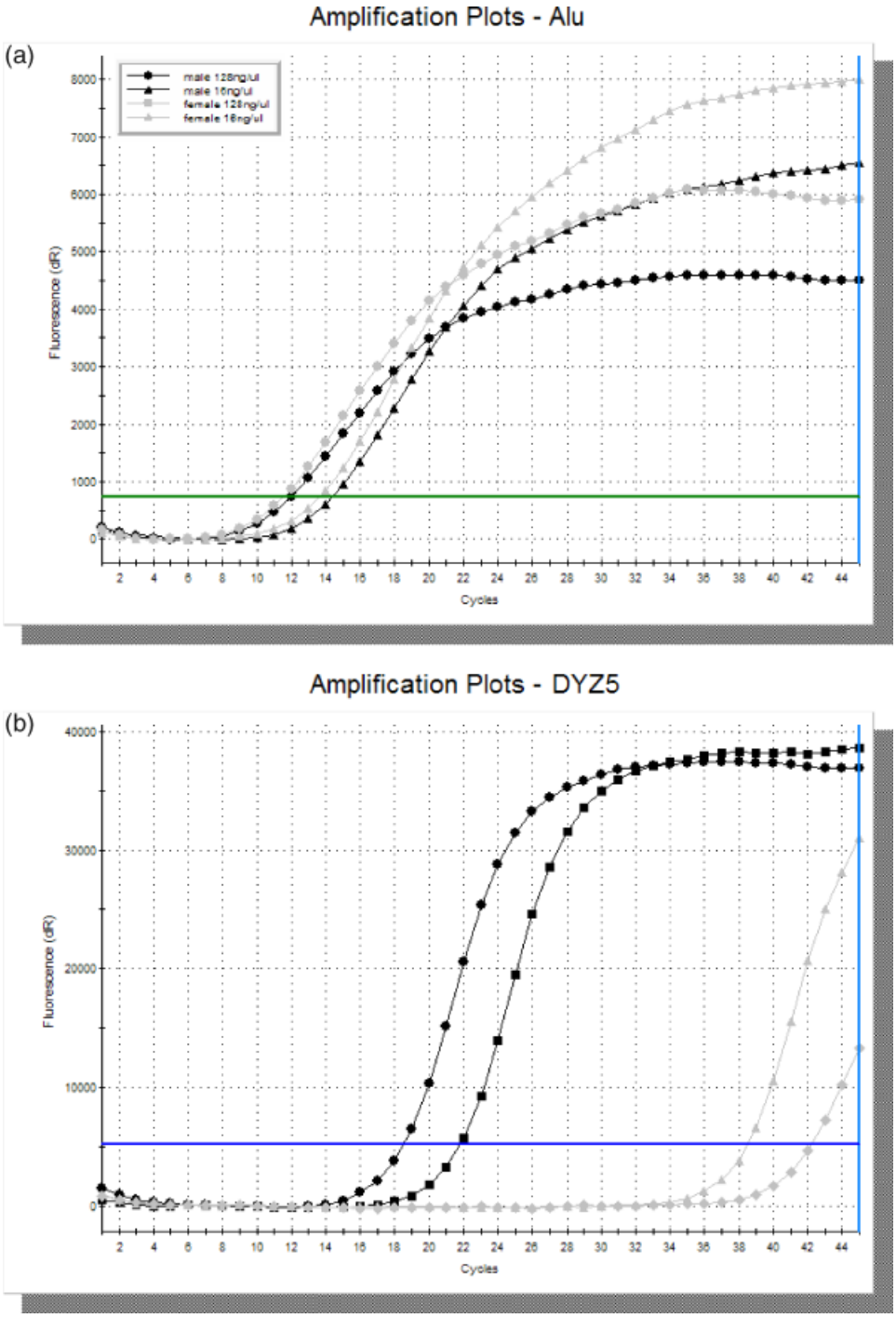

FIG. 3—*Duplex assay results on male versus female DNA. PCR was performed as described in "Materials and Methods." Male DNA (two concentrations of Promega human genomic DNA: male) and female DNA (two concentrations of Promega human genomic DNA: female) were used as samples. (a)* Alu *probe results, (b) DYZ5 probe results.*

the assay. Reactions with animal and bacterial DNA gave apparent concentrations between 0.0 (no Ct) and 0.00081 ng/μL 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 *c*. 1.0 while females should have a ratio of *c*. 0.0. Figure 4 graphs the results of these experiments for 54 males (19 Caucasians, five Native Americans, 14 African Americans, eight Asians, seven Hispanics, and one unknown). The DYZ5 or male DNA concentration (ng/μL) is graphed against the *Alu* or total DNA concentration (ng/μL). 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 (14 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 of the females had ratios of 0.00 and one had a ratio of 0.01).

TABLE 1—*Ct values for standard curve quadruplicates (a)* Alu *and (b) DYZ5.*

Standard Point	Repeat 1	Repeat 2	Repeat 3	Repeat 4	Mean	Standard Deviation	% Standard Deviation
(a)							
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
(b)							
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			

NTC, no template control.

TABLE 2—*Replicates of samples (a)* Alu*, (b) DYZ5, and (c) DYZ5:*Alu *ratio**.

Standard Point	Repeat 1	Repeat 2	Repeat 3	Repeat 4	Mean	Standard Deviation	% Standard Deviation
(a)							
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
(b)							
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	
(c)							
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	

Determined concentrations (ng/μL) for 11 samples.
*DYZ5 determined concentration: *Alu* determined concentration.

The assay was tested on 20 artificial mixtures. Table 3 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 (10 μL used for a 25 μL 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 4 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 5 shows the results with 32 casework samples. These ranged from standards to differentials to reagent blanks. Duplex assay results were compared with 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 only exception is for the second male standard where the DYZ5 probe only predicts 50% male rather than the expected 100%. The fact that the RFUs for the TPOX RFUs (Table 5, last column) were between 518 and 2639 show that the duplex assay correctly determined the DNA concentrations.

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 5 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. Table 6 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 as 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.

Lastly, the assay was used with DNaseI-treated DNA (single source male DNA) to simulate DNA degradation (Table 7). As expected, the Ct's 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 7. The *Alu* portion of the duplex assay generally correctly predicted input DNA for STR analysis (gave STR values between 150 and 5000 RFU) although some RFU values (such as the 2 min sample) are too low.

Discussion

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

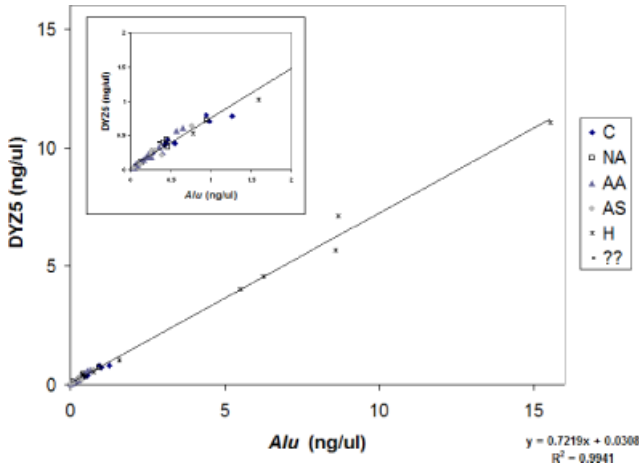

FIG. 4—*Plot of* Alu *and DYZ5 results for DNA samples from 54 male individuals of different ethnic groups. The inset is a blowup of the results for concentrations less than 2 ng/μL to show detail. A trendline is also graphed and the R^2 value (0.9941) and equation ($y = 0.7219x+0.0308$) given. The key for the ethnic groups is C, Caucasian; AA, African American; NA, Native American; H, Hispanic; AS, Asian; ??, unknown.*

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. 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 (10–12). 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 (13,14). As shown in Tables 1 and 2, the duplex assays compares favorably with these values.

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 $\sim$ 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 $0.8x$:$1.2x = 0.67$. Alternatively, if the *Alu* determined (total) concentration is 20% too low and the DYZ5 (male) concentration is 20% too high, the ratio will be $1.2x$:$0.8x = 1.5$. 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 ± 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 as the size of the major block appears to vary (3). 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 $\sim$ 38 cycles which is the Ct for the NTC (no added DNA) then some component is

TABLE 3—*Results on a series of artificial male DNA/female DNA mixtures.*

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.
STR, short tandem repeats.

TABLE 4—*Male:total (DYZ5/Alu) ratio* on serial dilution of selected male DNA/female DNA mixtures from Table 3.*

Mixture	Dilution							Mean	Standard Deviation	% Standard Deviation
	Neat	1:2	1:4	1:8	1:16	1:32	1:64			
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.

missing or there was inhibition. The assay, thus, has its own equivalent to an endogenous control.

This duplex assay can be compared with 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 (15) 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 and 61/64 bp, respectively. Each assay also contains an endogenous control. The use of single copy genes reduces sensitivity down to 23 pg (as compared with 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 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) reports results on six DNAs (with three 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

TABLE 5—*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 and COfiler analysis performed.

STR, short tandem repeats.

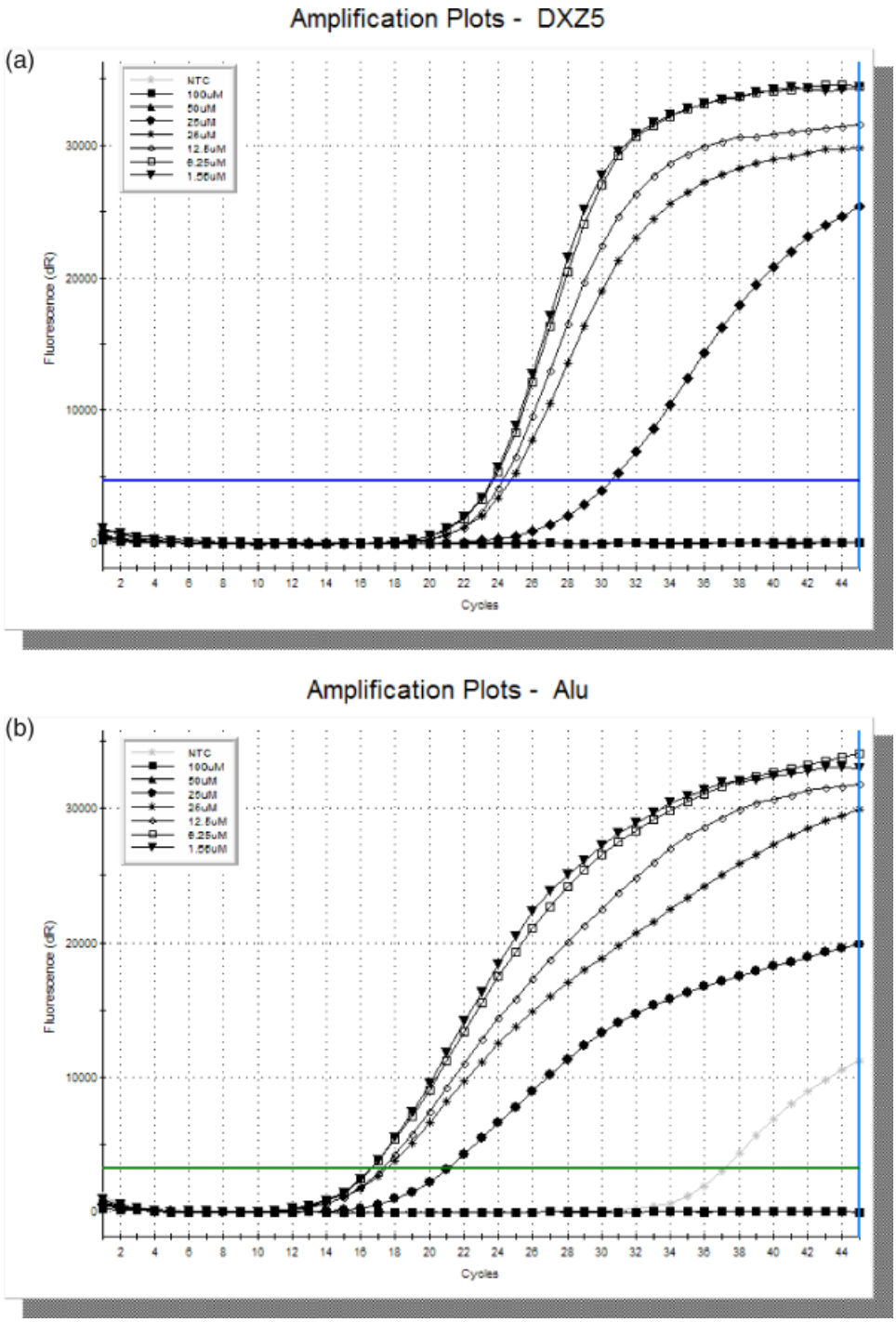

FIG. 5—*Duplex assay results on hematin containing samples. PCR was performed as described in "Materials and Methods" for the inhibitor experiments. (a) DYZ5 probe results, (b)* Alu *probe results.*

from 3.27% to 27.7%. These are also within the ranges reported by Bustin (13,14). 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 (16). 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, 79, 77, and

TABLE 6—*Results with inhibitors.*

Inhibitor	DYZ5ΔCt*	*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.
STR, short tandem repeats.

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 (17) 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 which does place it at the middle of the products of the STR loci. 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 would not discriminate between 20% and 40% male); however, the estimate will be sufficient to determine STR input DNA and to decide on autosomal versus 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 with 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.

TABLE 7—*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	1193, 1096
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

*Short tandem repeats (STR) results not performed as DNA < 0.1 ng/μL.

Conclusions

This sensitive duplex assay for both male and total human DNA quantitation should prove of value to the forensic community. The experiments performed here should meet the necessary validation requirements for forensic use. Multiplex real-time assays are clearly the future in forensic analysis. Real-time assays have also been developed for total nuclear and mitochondrial DNA (18), and the degradation state of DNA using small and large amplicons (19) by other laboratories. Our laboratory is also developing additional, multiplex assays for degradation state and other types of sample identification to speed forensic analysis.

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