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TECHNICAL NOTE

CRIMINALISTICS

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Development of a Real-Time Method to Detect DNA Degradation in Forensic Samples*

ABSTRACT: Knowledge of the degradation state of evidentiary DNA samples would allow selection of the appropriate analysis method (standard short tandem repeats [STRs] vs. mini STRs vs. mtDNA). This article describes the development of a Plexor® technology/real-time PCR DNA degradation detection assay, which uses a common forward primer and two reverse primers (different fluorophores) to generate two *Alu* amplicons (63 and 246 bp). This very sensitive assay was optimized for reaction volume, cycle number, anneal/extend time, and temperature. Using DNA samples degraded with DNaseI, the ratio of the concentration of the short amplicon to the concentration of the long amplicon (degradation ratio) was increased versus time of degradation. Experiments were performed on a variety of environmentally degraded samples (age, sunlight, heat) and with seven commonly encountered forensic inhibitors. The degradation ratio was found to predict the observed loss of larger STR loci seen in the analysis of comprised samples.

KEYWORDS: forensic science, DNA degradation, real-time PCR, Plexor®, quantitation, *Alu*

Environmentally challenged forensic samples may often be degraded into small DNA fragments, resulting in a limited DNA profile using commercial short tandem repeat (STR) profiling kits. For example, DNA degraded to an average size of 150 bp will not allow amplification of an STR locus with an amplicon size larger than 150 bp. Because of this, forensic researchers have developed sets of primers to create smaller STR products (mini STRs) (1,2). Appropriate quantitation methods designed to examine the average base pair size of the sample would better predict the amount of template required to optimize the performance of regular STR kits or mini STR kits and would allow the examiner to decide which assay would be a more appropriate approach for analysis. A limited 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. Alternatively, if one DNA sample from a crime scene is degraded to a lesser extent, then testing that sample is preferable.

Current commercial DNA quantitation kits do not accurately predict the DNA quantity needed for STR analysis when the DNA is degraded although several noncommercial, laboratory developed tests have been published. Niederstätter et al. (3) developed a dual human total nuclear (*RB1* gene) and mtDNA quantitation assay. They used several different sized products for each locus to assess DNA degradation. When DNA is degraded, amplification of the larger PCR products will be decreased or fail relative to the smaller products. Swango et al. (4) have also developed an assay for degraded DNA using a small (*CSF1PO*) and large (*THO1*) genomic target and as well as an internal positive control. The ratio of the amplification seen for the two loci can be used to assign a degradation score. This assay has been validated for casework samples (5). Furthermore, Alonso et al. (6) developed a DNA degradation assay using two different sized mtDNA amplicons to assess the degradation state of extracted mtDNA.

The nuclear assays both use single copy genes for quantification and have limits of sensitivity of approximately 25 pg. We have previously developed several DNA quantification assays using a repeated DNA sequence (*Alu*), which allows DNA quantitation down to 4 pg (7,8). To develop an *Alu*-based assay to detect the degradation state of DNA samples, a multiplex PCR with two overlapping *Alu* amplicons using Plexor® technology was designed. The Plexor® technology relies on the use of two alternative base-pairs, isoC and isoG, which pair only to each other. The forward primer is designed with a 5′ isoC with attached fluorophore. During amplification, an isoG with attached quencher is incorporated and fluorescence is quenched owing to proximity of the fluorophore and quencher.

For the experiments reported here, a different fluorophore is placed on each of the specific reverse primers (FAM for long and Quasar 670 for short). The decrease in fluorescence for each fluorophore is monitored during PCR. Amplification of the longer *Alu* amplicon is possible only in nondegraded or slightly degraded DNA whereas the smaller amplicon is amplifiable even with highly degraded DNA. The ratio of the concentrations of the two products gives a quantitative measure of degradation state. The concentration of DNA measured by the long amplicon can be used to determine input DNA for STR analysis and the ratio predicts the amplification of high molecular weight alleles or "ski-slope" observed in STR analysis. This assay was tested on environmentally degraded samples and with various commonly encountered forensic inhibitors.

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Materials and Methods

DNA Sample Preparation

Degraded DNA samples were created by treating nine high concentration DNA samples with DNaseI (Promega, Madison, WI) for 0–128 min. Aliquots were taken at eleven intervals (0, 15, 30 sec, 1, 2, 4, 8, 16, 32, 64, and 128 min), to create DNA samples with various levels of degradation. DNA samples isolated from environmentally treated samples (sunlight or 37°C for varying time intervals) were extracted using the Maxwell®16 instrument (Promega). Samples were lysed in 1.5-mL microcentrifuge tubes by incubation for 30 min at 83°C, in 500 μL of stain extraction buffer (0.01 M Tris Base/0.01 M $Na_2EDTA\text{-}2H_2O$/0.1 M NaCl/10% SDS, pH 8.0 + 0.39 M DTT) and 12.5 μL of 1 mg/mL proteinase K (Sigma, St. Louis, MO). The solid material was moved into a "spin basket" and samples centrifuged at maximum speed for 2 min. The lysed sample was transferred into well #1 of the Maxwell® 16 and extracted using the Promega DNA IQ Reference Sample Kit™ for Maxwell®16. Samples were eluted with 300 μL of TE^{-4} (Tris–EDTA, pH 7.5). Other control DNA samples were isolated using a phenol/chloroform organic extraction method (9,10).

Inhibitors

All chemicals used for the inhibitor studies were purchased from Sigma and prepared as follows: hematin (100 mM in 0.1 M NaOH), indigo (100 mM in 2% Triton ×100), humic acid (1 mg/mL in DIH_2O), tannic acid (1 mg/mL in DIH_2O), calcium hydrogen phosphate (100 mM in 0.5 M HCl), melanin (1 mg/mL in 0.5 M ammonium hydroxide), and collagen (1 mg/mL in 0.1 M acetic acid).

PCR Primers

Primers (Biosearch Technologies, Novato, CA) were chosen as shown in Fig. 1. The sequences of the primers were the following: *Alu* short reverse primer—5′ Quasar 670-isoC-CTCGTGATCC GCCCGCCTC 3′ (amplicon size, 63 bp), *Alu* long reverse primer—5′ FAM-isoC-GAGTGCAGTGGCGGGATCTC 3′ (amplicon size, 246 bp), and common forward primer—5′GGGCGCGG TGGCTCAC 3′. All primers were diluted to 200 μM stock in the MOPS/EDTA buffer supplied with the Promega Plexor® mastermix. Primers with different fluorophores were investigated. The combinations of long-FAM with short-CAL Fluor Orange 560 and long-CAL Fluor Orange 560 with short-FAM as compared to long-FAM with short-Quasar 670 were utilized. The degradation ratios for the long-560/short-FAM were much higher than for long-FAM/short-Quasar 670, whereas the ratios for the long-FAM/short-560 were about the same. This could be attributed to fluorescence efficiencies of the Quasar 670 in the Rotorgene affecting the Ct values. However, replicate values were widely different for long-560/short-FAM, suggesting that this combination may be subject to quite a bit of variability. Thus, the long-FAM/short-Quasar 670 was chosen for the final assay.

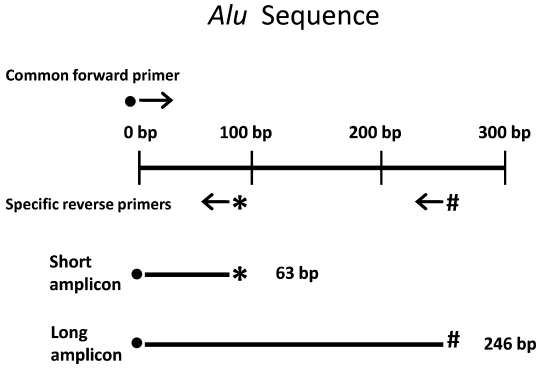

FIG. 1—*Diagram of the placement of the primers on the* Alu *sequence.*

PCR

For a 20 μL reaction, 6.72 μL deionized H_2O (supplied with kit), 10 μL Promega Plexor® qPCR System (A4011) Mastermix, 1.282 μL Primer/bovine serum albumin (BSA) mix [43.2 μL BSA stock (6.25 mg/mL), 5 μL of 200 μM forward primer stock, 1.5 μL of 200 μM short primer stock, 2.0 μL of 200 μM long primer stock, 76.5 μL deionized H_20] plus 2 μL of DNA sample were combined. A standard curve of 32 ng/μL down to 0.0078 ng/μL was made using Promega human genomic DNA (G1471 or G3041). PCR cycling conditions in a Qiagen RG6000 (Valencia, CA) were as follows: 95°C for 2 min, 32 cycles of 95°C for 5 sec, and then 60°C for 25 sec.

Data Interpretation

Results were analyzed using the Corbett (Qiagen) software or the Plexor® software supplied by Promega. The DNA concentrations for each sample were determined based on the short and long results for the standard curve and the short/long ratio calculated (degradation ratio). For nondegraded DNA, this ratio should be approximately 1.0. If the ratio is greater than 10, this indicates significant degradation.

Results

A single unlabeled forward and two labeled Plexor® System (Promega) reverse primers were designed to amplify two different sized *Alu* amplicons (63 and 246 bp) (Fig. 1). Figure 2 shows data for a dilution series of DNA for both the long (A) and short amplicons (B). The R^2 values are very good (0.99) although the efficiency values are both low (0.71/0.78); this is undoubtedly because the two amplicons are competing.

Validation of the assay requires that when DNA is diluted, the ratio of the relative fluorescence units (RFUs) (measured by the difference in the Cts) of the large to small should not change (11). This was tested by plotting the ΔCt versus DNA concentration, and Fig. 3 shows the slope of the line was between −0.1 and 0.1 for the ratio of the large to small RFUs over the concentration range tested (32–0.0078 ng/μL). The assay was optimized for volume (20 μL), cycle number (32 cycles), anneal/extend time (30 sec), and temperature (60°C) (data not shown).

Degraded DNA samples were analyzed with the assay, and a graph was made of the ratio of short to long concentration (degradation ratio) versus time of degradation. Table 1 shows the results for the DNaseI-degraded DNA samples. As can be seen, the ratio increases with degradation. The final ratio is variable for the different DNA samples, which may reflect their initial quality or concentration. While an attempt was made to have the initial DNA concentration the same for all, the DNA samples were of such high concentration and quality (extremely viscous) that determination of an exact concentration was difficult. The ratio also decreased for many of the samples as degradation time becomes very long; this is because of the fact that amplification of even the short product decreases as the size of the degraded DNA fragments falls below the size of the short amplicon.

Experiments were performed to test the assays on a variety of environmentally degraded samples (age, sunlight, and heat). Table 2 shows results for a number of degraded samples and control samples that were stored refrigerated or frozen. The samples (blood, saliva, or semen) had been kept either at room temperature, at 37°C, or taped to a window in sunlight for the indicated time. The data indicate that the samples kept at 37°C were the most degraded, and the controls showed the lowest level of degradation, as would be expected.

To test the reproducibility of the assay, another experiment took six samples (five from the above set and the 9947 control DNA from an Applied Biosystems STR kit; Foster City, CA) and amplified them in triplicate, once in an RG6000 and once in an RG3000 (Table 3). These results were generally quite consistent. For example, for sample 2–5, the long amplicon values were 0.11, 0.10, and

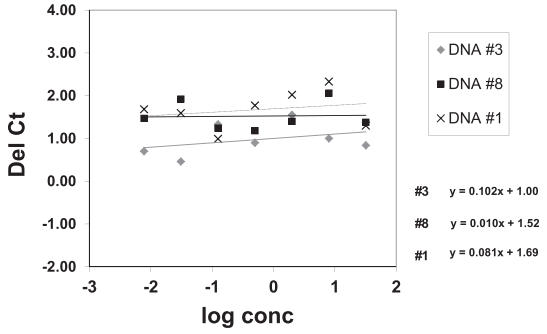

FIG. 3—*Delta Ct (Del Ct) for duplex assay – slopes are between −0.1 and 0.1 (results for three different DNAs shown).*

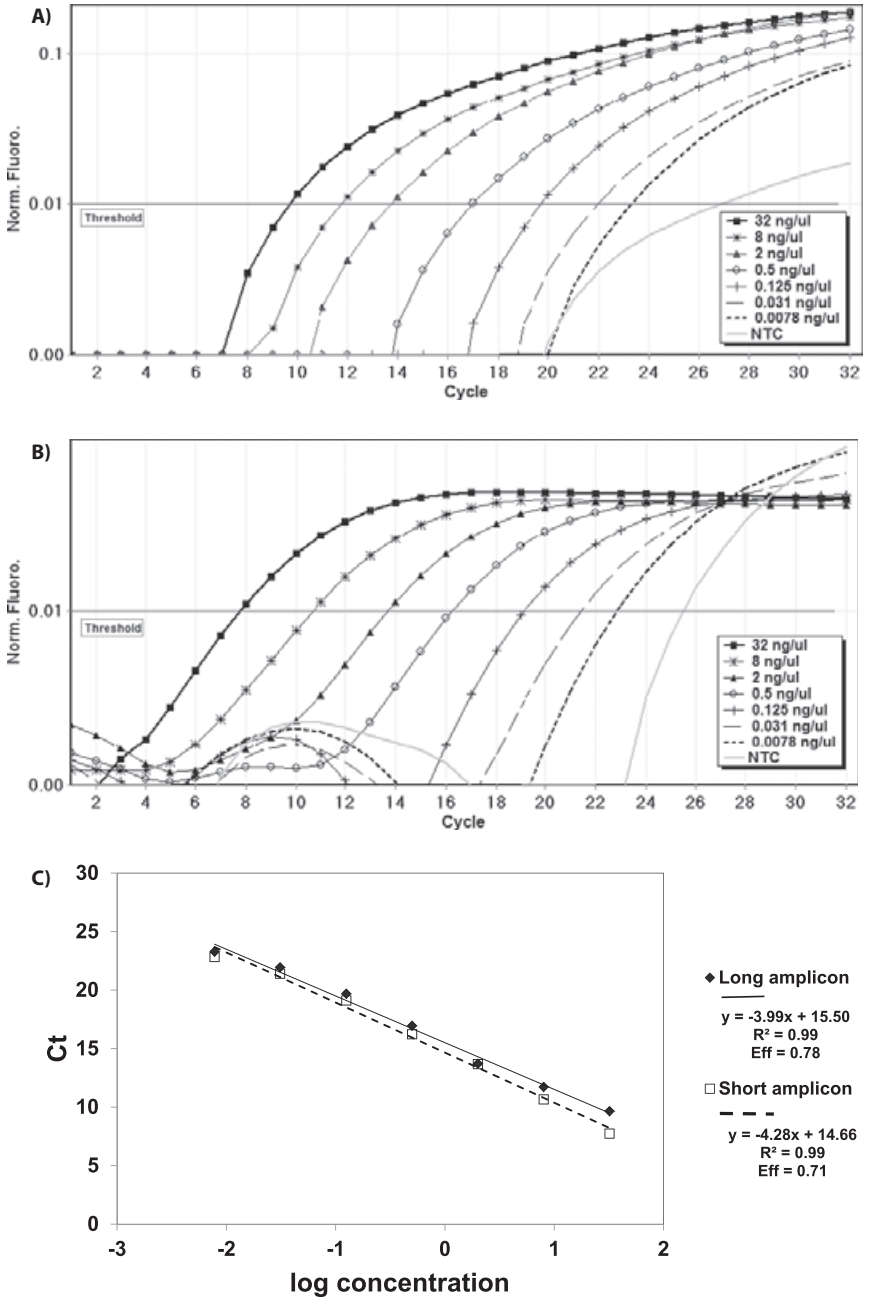

FIG. 2—*Analyzed data from the degradation assay on a dilution series of DNA; (A) long amplicon, (B) short amplicon, and (C) standard curve (concentration values are ng/μL).*

TABLE 1—*The degradation ratios for nine degraded DNA samples.*

	Degradation Ratio								
Degradation time (min)	#1	#3	#4	#5	#6	#7	#8	#9	#10
0	0.76	0.81	0.56	0.85	0.88	0.74	0.82	0.82	1.43
0.25	1.73	1.03	0.64	0.78	0.98	1.38	1.21	1.44	1.04
0.5	1.59	1.42	1.22	0.95	1.11	1.26	1.53	1.53	1.08
1	3.58	1.91	2.08	2.19	1.30	2.55	2.35	1.78	1.26
2	8.21	6.12	4.29	18.5	2.82	14.4	6.44	2.06	7.33
4	203	93.9	44.1	216	20.6	144	12.8	5.74	132
8	672	134	130	574	82.7	1075	47.2	16.9	948
26	649	483	171	523	329	317	119	52.6	834
32	123	498	157	ND	442	482	123	49.9	574
64	25.4	288	62	881	332	227	79.2	13.1	187
128	24.0	249	40.3	884	326	31.1	141	41.3	162

ND, not determined.

TABLE 2—*Degradation results for environmentally treated DNA samples and controls.*

Item No.	Storage	Age of Deposition (years)	Storage Condition	DNA Concentration Measured Using the Long Amplicon (ng/μL)	DNA Concentration Measured Using the Short Amplicon (ng/μL)	Degradation Ratio
1-1	Blood on cloth	5.1	Room temp	0.02	0.03	2.14
1-2	Blood on FTA®	5.1	Room temp	0.29	0.51	1.76
1-3	Blood on cloth	4.2	Room temp	0.18	0.22	1.24
1-4	Blood on cloth	4.2	37°C	0.11	0.78	7.21
1-5	Blood on cloth	2.7	Room temp	0.16	0.28	1.77
1-6	Blood on cloth	2.7	Sunlight	0.11	0.29	2.67
1-7	Blood on cloth	2.7	37°C	0.06	0.30	4.87
1-8	Blood on denim	2.4	Room temp	0.10	0.11	1.15
1-9	Blood on denim	2.4	Room temp	0.70	0.90	1.27
1-10	Blood on cloth	0.8	Room temp	0.29	0.28	0.97
1-11	Blood semen on cloth	2.6	Room temp	0.10	0.11	1.12
1-12	Blood/semen on cloth	2.6	Sunlight	0.06	0.19	3.45
1-13	Blood/semen on cloth	2.6	37°C	0.00	0.00	3.25
1-14	Control blood on cloth	NA	Frozen	0.16	0.11	0.73
1-15	Reagent blank	NA	NA	0.02	0.00	0.03
2-1	Blood on cloth	4.2	Sunlight	0.00	0.00	16.68
2-2	Buccal on FTA®	2.6	Room temp	0.07	0.04	0.54
2-3	Saliva on cloth	4.3	Room temp	0.02	0.22	11.43
2-4	Saliva on cloth	4.3	37°C	0.00	0.03	13.46
2-5	Saliva on cloth	1.9	Room temp	0.19	0.55	2.94
2-6	Semen on cloth	4.3	Room temp	3.17	6.14	1.93
2-7	Semen on cloth	4.3	37°C	2.26	14.29/	6.31
2-8	Semen on carpet	2.9	Room temp	0.40	0.65	1.62
2-9	Semen on cloth	2.9	Sunlight	0.64	2.40	3.76
2-10	Semen on cloth	2.8	Room temp	0.73	0.51	0.69
2-11	Control blood on cloth	NA	Frozen	0.31	0.29	0.96
2-12	Reagent blank	NA	NA	0.00	0.00	0.30
88	DNA from peripheral blood	NA	None	0.03	0.02	0.76
87	DNA from peripheral blood	NA	None	23.55	22.07	0.94
47	DNA from peripheral blood	NA	None	21.39	17.73	0.83
JLA	Blood, organic extraction	NA	None	0.15	0.23	1.55
1518	Blood, sucrose isolated	NA	None	2.36	3.82	1.62
1414	Blood, sucrose isolated	NA	None	14.85	16.81	1.13

ND, not determined.

0.10 and the short amplicon values were 0.32. 0.36, and 0.27 giving ratios of 2.86, 3.70, and 2.81 for a percent standard deviation of 16%. Most percent deviations were in the range of 2–16% although a few were higher (up to 62%). Taking the ratio of two numbers which inherently have variations of ~15% will give a higher standard deviations for the ratio.

Last, a set of five degraded samples and a control were chosen to run with five replicates on four occasions to test the reproducibility of the assay. The data were very similar to the above results. Standard deviations were between 0.12 and 2.89 (mean 1.24), and percent standard deviations were between 10.5 and 62.5% (mean 28.6%) (data not shown). The data were consistent with the control having an expected ratio of approximately 1 and the degraded samples having generally consistent values above 1.0 to approximately 10 for the most degraded sample.

This assay was also tested with the following seven different inhibitors commonly seen in forensic samples: hematin, indigo, melanin, collagen, calcium phosphate, tannic acid, and humic acid. These inhibitors were diluted and tested at five concentrations to span from no inhibition to severe inhibition (plus a no inhibitor control). Figure 4 shows some results from these experiments. Most inhibitors do not affect the degradation ratio when the assay is not inhibited to such an extent that PCR does not occur (Table 4). The exception is calcium phosphate that does

TABLE 3—*Degradation assay triplicates for six samples over 2 days on two real-time instruments.*

Sample	Real-time instrument	DNA Concentration Measured Using the Long Amplicon (ng/μL)			DNA Concentration Measured Using the Short Amplicon (ng/μL)			Degradation Ratio					
		Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Mean	SD	% SD
1-4	RG6000	0.07	0.06	0.06	0.36	0.46	0.41	4.90	8.06	6.78	6.58	1.59	24.16
1-4	RG3000	0.04	0.05	0.03	0.40	0.45	0.32	9.43	9.04	9.23	9.23	0.20	2.11
1-6	RG6000	0.03	0.03	0.02	0.15	0.11	0.11	4.29	3.81	4.54	4.21	0.37	8.81
1-6	RG3000	0.02	0.04	0.02	0.09	0.12	0.08	3.59	3.31	4.22	3.71	0.47	12.57
2-10	RG6000	0.37	0.35	0.29	0.63	0.47	0.48	1.71	1.37	1.67	1.58	0.19	11.74
2-10	RG3000	0.31	0.25	0.39	0.36	0.32	0.58	1.15	1.27	1.49	1.30	0.17	13.23
2-5	RG6000	0.11	0.10	0.10	0.32	0.36	0.27	2.86	3.70	2.81	3.12	0.50	16.01
2-5	RG3000	0.10	0.09	0.11	0.40	0.27	0.35	3.97	3.00	3.28	3.42	0.50	14.61
2-6	RG6000	1.77	2.02	1.43	6.53	4.23	9.69	3.69	2.10	6.77	4.19	2.37	56.71
2-6	RG3000	4.34	2.62	2.81	9.86	7.18	8.29	2.27	2.73	2.95	2.65	0.35	13.09
9947	RG6000	0.07	0.04	0.08	0.05	0.06	0.04	0.71	1.58	0.50	0.93	0.57	61.57
9947	RG3000	0.10	0.09	0.11	0.08	0.06	0.07	0.80	0.67	0.60	0.69	0.10	14.71

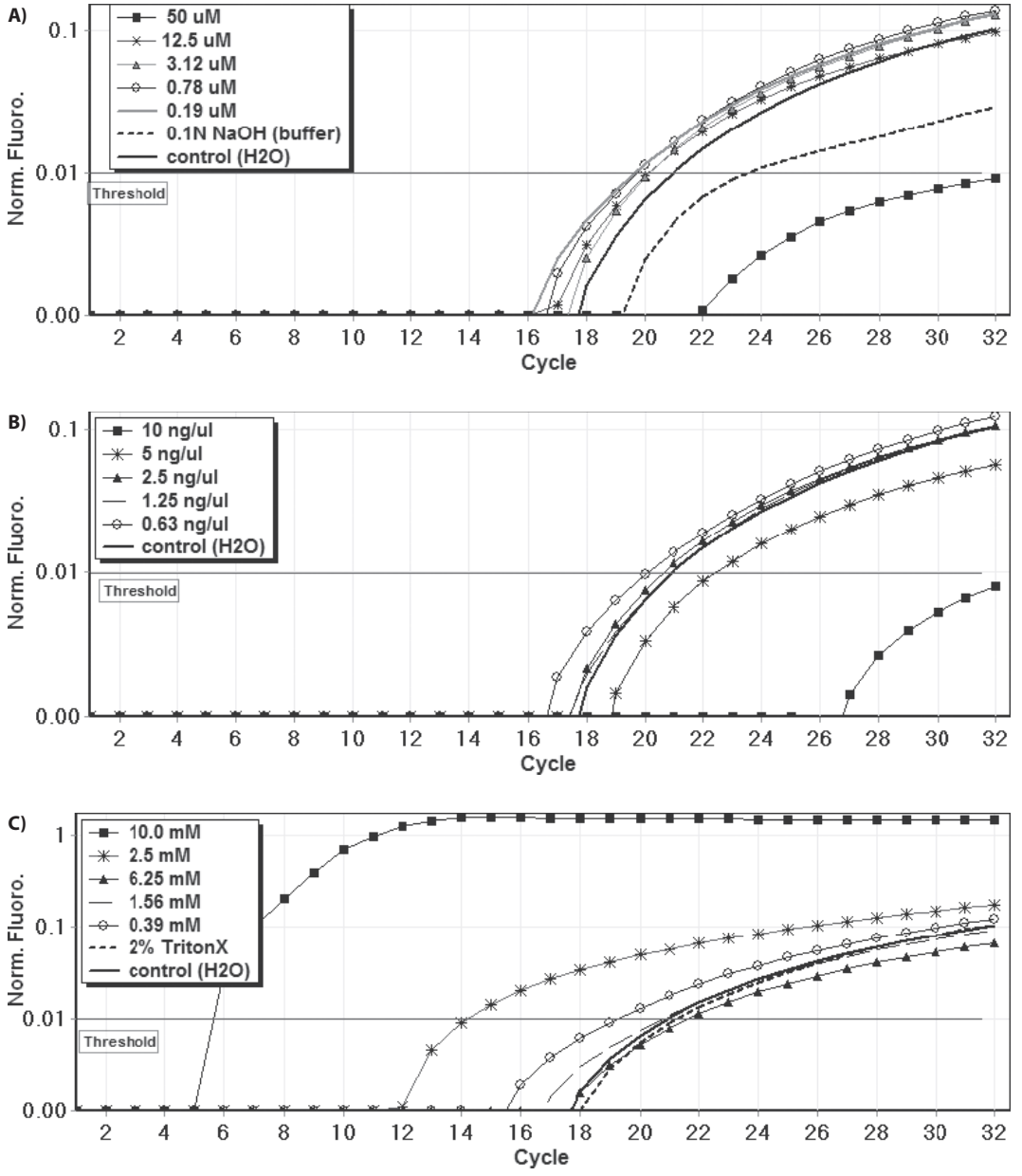

FIG. 4—*Selected effects of inhibitors on DNA degradation assay results. (A) Hematin on long amplicon results—inhibition at high concentrations and inhibition by the buffer, (B) tannic acid on long amplicon results—inhibition at high concentrations, (C) indigo on long amplicon results—the indigo dye interferes with detection and causes increased fluorescence readings (and therefore greatly decreased Ct values) at high concentrations.*

TABLE 4—*Effects of inhibitors on the DNA degradation assay.*

Inhibitor	Effects on Long Amplicon Amplification	Effects on Short Amplicon Amplification	Effects on Long/Short Amplicon Ratio
Hematin	50 μM total inhibition	50 μM minor inhibition	None until total inhibition
Indigo	False positive readings for 10 and 2.5 mM	False positive readings for 10 and 2.5 mM	Decreases ratio below 1.0 (owing to dye interference)
Melanin	Partial inhibition for 20 and 5 μM	Total inhibition for 20 μM	Only small increase in ratio above at 5 μM
Collagen	Total inhibition for 100 and 50 ng/μL	Significant inhibition 100 ng/μL	None until total inhibition
Calcium phosphate	Total inhibition above 0.63 mM	False positives 5 and 2.5 mM, slight inhibition 1.25 mM	Large increase in ratio
Tannic acid	Partial inhibition for 10 ng/μL	Partial inhibition for 10 ng/μL	None
Humic acid	Partial inhibition for 5 and 10 ng/μL	Slight inhibition for 5 and 10 ng/μL	Slight increase at high concentrations

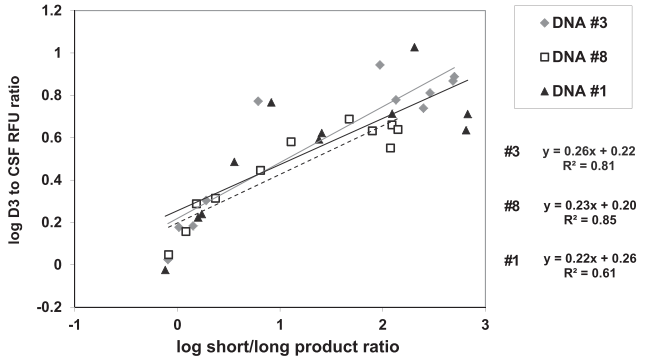

FIG. 5—*`Relationship of measured degradation ratio and ratio of RFUs from a small to a large STR product [D3S1358 (113–142 bp) to CSF1PO (281–317 bp)] from STR amplification (uses log ratios and only samples where the concentration was 0.1 ng/μL into STR amplification—i.e., 1 ng input).*

increase the degradation ratio and indigo that interferes with fluorescence detection.

To determine whether the calculated degradation ratio was predictive of STR success, degraded DNA samples were diluted to 0.1 ng/μL based on the quantitation determined by the long product. The DNA samples were amplified using the Applied Biosystems COfiler kit. All amplifications were successful in producing a full seven locus profile if 1 ng of input DNA could be added. However, considerable ski-slope was observed with the highly degraded samples. The ski-slope was proportional to the degradation ratio; that is, if the ratio of short to long product was high (DNA degraded), then the CSF (larger locus, ~300 bp) RFU was much lower than the D3 (smaller locus, ~120 bp) RFU (Fig. 5).

Discussion

This DNA degradation detection assay has the advantage of being able to detect very low levels of DNA because it is based on the very highly repetitive *Alu* sequence. The Plexor® technology that places the fluors directly on the primers, making for a simpler assay without probes, allows for the overlapping amplicon design. Using the two amplicons of significantly different sizes allows detection of degradation in the range of significance for STR analysis. Earlier commercial DNA quantitation assays had difficulty determining the correct DNA concentration for degraded DNA because of the choice of a very small amplicon (smaller than the size of STR amplicons).

With this assay, a degradation ratio over 2 indicates significant DNA degradation of the sample with the ratio increasing with increasing degradation. Use of the long amplicon concentration for STR input resulted in correct RFU values for STR analysis for most loci with the degradation ratio predicting how much ski-slope would be observed in the STR electropherogram. The assay was shown to be consistent and reasonably resistant to inhibitors.

Using this assay, samples of limited quantity and of a critical evidentiary nature may be screened prior to typing to determine the quality of the DNA. This assay may not be applicable to routine casework analysis, but some samples that may have been exposed to conditions known to degrade DNA could be candidates for this assay. With more validation, this assay should prove useful to the forensic community.

Acknowledgments

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