APPLICATION NOTES

Developmental Validation of a Real-Time PCR Assay for the Simultaneous Quantification of Total Human and Male DNA

Promega

Developmental Validation of the Plexor® HY System

Developmental Validation of a Real-Time PCR Assay for the Simultaneous Quantification of Total Human and Male DNA

By Benjamin E. Krenke, Nadine Nassif, Cynthia J. Sprecher, Curtis Knox, Melissa Schwandt and Douglas R. Storts
Promega Corporation

Abstract

Multiplex human short tandem repeat analysis demands reliable DNA quantification to consistently produce interpretable genotypes. The Plexor® HY System is a multiplex quantitative PCR assay to quantify total human and male DNA. We performed developmental validation of the Plexor® HY System to demonstrate the performance capabilities and limitations of the assay for forensic applications. Validation studies examined: a) human specificity, b) sensitivity, c) quantification of degraded DNA, d) impact of inhibitors, e) male/female mixture and Y-assay male specificity, f) reproducibility and concordance and g) population studies.

Introduction

Multiplex short tandem repeat (STR) analysis is the core technology in DNA-based human identification. These assays require a defined range of template quantities to produce optimal results. In addition to accurate sample quantification, assessment of sample quality and sensitive detection are necessary to determine how best to proceed with sample analysis.

Quantitative PCR (qPCR) has displaced hybridization-based methods for human-specific DNA quantification in forensic applications. qPCR has reduced the rate of false-negative STR results due to lack of sensitivity and increased the objectivity of data interpretation by providing a numerical output rather than requiring a visual comparison of band intensities. However, some current qPCR methods do not allow simultaneous quantification of total human and male DNA or do not have a level of sensitivity that consistently exceeds that of subsequent STR assays.

The Plexor® HY System[a–g] is a qPCR assay that simultaneously quantifies total human DNA and male DNA (1–3) using the Plexor® technology, which results in decreasing fluorescence as the amplification progresses (4–7). The triplex configuration allows co-amplification of a human autosomal sequence, a human Y-chromosomal sequence and a novel exogenous control sequence to quantify total human DNA and male DNA and provide an internal PCR control (IPC), respectively.

The autosomal primers are labeled with fluorescein and amplify a 99bp sequence from the human RNU2 locus. The RNU2 locus encodes a small nuclear RNA involved in pre-mRNA processing. This region is conserved among primates and organized as a tandemly repeated motif (~6kb each) on the long arm of chromosome 17 (8–15). The Y-chromosome primers are labeled with CAL Fluor® Orange 560 (Biosearch Technologies, Inc.) and target a 133bp sequence from the testis-specific protein, Y-encoded (TSPY) locus. The TSPY gene is involved in spermatogenesis and is conserved in primates (15–18). The TSPY locus is within the DYZ5 region, a 20kb repeated motif on the Y chromosome. The IPC primers are labeled with CAL Fluor® Red 610 (Biosearch Technologies, Inc.) and detect a novel IPC sequence, which is included as a template in all reactions. The amplified IPC product is 150bp. Data from the IPC amplifications are used to monitor amplification inhibition. A fourth dye, IC5, is included in all wells and used as a passive reference. Data from the three amplifications can be normalized to the passive reference signal to reduce the impact of instrument-specific signal fluctuation.

The findings presented here document the basic performance characteristics of the Plexor® HY System as part of a manufacturer's validation. Given these results, laboratories implementing the Plexor® HY System may consider omitting some of these studies from their internal validation. More information about performing internal validation of the Plexor® HY System can be found in the *Validation Guide for the Plexor® HY System* #GE295, which is available upon request or online at: **www.promega.com/plexorhy/**

Materials and Methods

DNA

The Plexor® HY Male Genomic DNA Standard, provided with the Plexor® HY System, was used to generate all standard curves. This DNA is a mixture of several human male DNAs and is not derived from cell lines. Except where noted, we generated standard curves by amplifying a fivefold serial dilution of the DNA standard from 16pg/µl to 50ng/µl. We purified male and female human DNA for use as unknown samples from liquid blood using organic extraction (19) or from buccal swabs. Buccal swabs were heated to

90°C for 30 minutes in 400µl of SV Lysis Buffer (Cat.# Z3052) containing 10mM DTT. Samples were centrifuged through a DNA IQ™ Spin Basket (Cat.# V1221) for 2 minutes at 14,000 × *g* in a microcentrifuge to collect the lysate. DNA was purified using the Wizard® SV Genomic System (Cat.# A2361) and the genomic DNA purification protocol from lysates using a microcentrifuge (20). Samples were eluted in 100µl of TE^{-4} buffer [10mM Tris-HCl (pH 8.0), 0.1mM EDTA].

Pig, mouse, rabbit, macaque, Japanese macaque, ferret, cheetah, reindeer, elephant, cat, warthog, straw-colored fruit bat, wallaby, mandrill, tamarin, red panda, rat, rhesus monkey, orangutan, hyena and *Saccharomyces cerevisiae* DNA samples were kindly provided by Roger Frappier of the Centre of Forensic Sciences. DNA from calf thymus (bovine), *Escherichia coli* (strain B), *Micrococcus lysodeikticus* and *Clostridium perfringens* was obtained from Sigma-Aldrich. Dog, chicken, rabbit, rat, human papilloma virus, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and hepatitis B virus DNA were kindly provided by Cecilia Crouse of the Palm Beach County Sheriff's Office. An isolate of *Candida albicans* was kindly provided by Dr. Kenneth W. Nickerson (University of Nebraska—Lincoln). Herring sperm DNA (Cat.# D1811) was acquired from Promega. In general these DNA samples were purified using organic extraction and quantified by spectrophotometric analysis using A_{260} detection (19) or comparison to DNA standards on an ethidium bromide-stained agarose gel (19).

Real-Time PCR Cycling and Detection

Unless noted, all samples were analyzed in duplicate, and the average results are reported. All testing of the Plexor® HY System was performed using the Applied Biosystems 7500 Real-Time PCR System. Where noted, additional testing was performed using an Applied Biosystems 7500 FAST Real-Time PCR System (in standard 7500 mode) or Stratagene Mx3005P® Quantitative PCR System. Cycling parameters were: 1 cycle at 95°C for 2 minutes; 38 cycles of 95°C for 5 seconds, then 60°C for 35 seconds (7500) or 40 seconds (Mx3005P®) with data collection during the elongation/extension step. Postamplification melt analysis was performed using the default dissociation analysis (7500) or a 48-step protocol that increased the temperature 0.6°C per step from 65°C to 92°C and had two data collection points per step (Mx3005P®). Instrument calibration (7500 only), setup and programming were performed as described in the *Plexor® HY System Technical Manuals* (2,3).

For concordance studies, the Quantifiler® human and human male DNA quantification kits (Applied Biosystems) were used as directed by the manufacturer.

Plexor® Data Analysis

The Plexor® Analysis Software interprets amplification data with decreasing fluorescence, generates standard curves and calculates DNA concentrations of unknowns. We used the forensic release of the Plexor® Analysis Software, version 1.5.4.10, to analyze all data reported. Following data collection on each instrument, raw data was exported from the instrument software, then imported into the Plexor® Analysis Software (2,3). The Plexor® Analysis Software generated the amplification threshold, cycle threshold (C_T), melt threshold, product melt temperature (T_m), standard curve and unknown sample quantification data. Following quantification of unknowns, we used an STR normalization module to a) compute sample input volumes required for autosomal STR and Y-STR amplifications, b) calculate necessary dilutions for concentrated DNA samples and c) flag low-quality data and inhibited amplifications.

Inhibitors

We examined the effect of three inhibitors: hematin, humic acid and calcium chloride. These inhibitors were added to samples prior to quantification and subsequent STR analysis. Porcine hematin (Sigma-Aldrich) was resuspended to 1mM in 1N sodium hydroxide. Humic acid (Sigma-Aldrich) was resuspended at 0.5µg/µl in water. Calcium chloride (Promega) was prepared at 100mM in water.

Degradation Assay

We treated DNA samples with DNase I (Promega Cat.# M6101) to simulate sample degradation prior to quantification and subsequent STR analysis. We assembled a 120µl reaction consisting of 1X reaction buffer, 1µg single-source human DNA and 1U DNase I on ice and removed 10µl of the reaction to serve as the "0" time point. The remaining reaction mix was incubated at 37°C, and 10µl aliquots were removed at 30 seconds, 90 seconds, 5 minutes, 10 minutes, 15 minutes, 30 minutes, 1 hour and 4 hours. To stop the reaction at each time point, each aliquot was added to 10µl of Stop Solution and incubated at 65°C. DNA samples were diluted 1:20 with water before analysis.

STR Analysis

We performed STR analysis to correlate changes in quantification results and the impact on genotyping. In general, peak height (yield) and the number of observed alleles were the primary considerations when interpreting STR data. We used autosomal quantification data ([AUTO]) to normalize DNA input into PowerPlex® 16 reactions (Cat.# DC6530) and Y-chromosomal quantification results ([Y]) to normalize DNA input into PowerPlex® Y System (Cat.# DC6760) reactions. In general, we amplified 0.5ng of DNA in a

25µl STR reaction using 32 cycles as described in the *PowerPlex® 16 and PowerPlex® Y Systems Technical Manuals*. After amplification, we prepared samples by combining 1µl of sample, 9.5µl of Hi-Di™ formamide (Applied Biosystems) and 0.5µl of Internal Lane Standard 600 (ILS600, Cat.# DG2611). Separation and detection of STR amplification products were performed using an Applied Biosystems 3130 or 3130*xl* Genetic Analyzer using a 10-second, 3kV injection or 5-second, 3kV injection, respectively. Data analysis was performed using the GeneMapper® *ID* software. We limited interpretation of electropherograms to peaks above a minimum detection threshold of 50 relative fluorescence units (RFU).

Results and Discussion

Species Specificity

DNA purified from forensic samples commonly contains a mixture of human DNA and contaminating DNA from bacteria, fungi, viruses or other organisms, and some samples may not include any human biological material. Consequently, STR genotyping assays for forensic use are generally reactive to human and primate DNA only (21,22). Similarly, the quantification system must not react to nonprimate DNA.

Six primate, 23 nonprimate mammal, two nonmammalian animal, two yeast, six prokaryotic and two viral DNA samples were analyzed using the Plexor® HY System. The DNA samples were at 1ng/µl, except for the Japanese macaque and orangutan, which were at 0.2ng/µl. A sample was considered "reactive" if ≥1pg/µl of DNA was detected in Plexor® HY assays using a human DNA standard curve with DNA concentrations ranging from 3.2pg/µl to 50ng/µl. Of the species tested, only higher primate samples were reactive (Table 1). Subsequent STR analysis of positive primate samples (1ng DNA per reaction) also demonstrated reactivity. As previously demonstrated, none of the primate profiles amplified at all STR loci, and many alleles were off-ladder or inconsistent with human alleles (21,22). Given the forensic irrelevance of primate reactivity, these results demonstrate that the Plexor® HY System is adequately species-specific for forensic use and does not yield quantitative results with nonprimate samples. Additionally, reactivity with higher primates suggests that primer-binding sites are well conserved among human populations.

Table 1. Species Specificity of the Plexor® HY System.

Species (Gender)	Plexor® HY Autosomal Results	Plexor® HY Y Results	Autosomal STR Results[1]
orangutan (male)	+	+	+
Rhesus monkey (male)	+	+	+
macaque (male)	+	+	+
Japanese macaque (male)	+[2]	–	+
mandrill (female)	+	–	+
tamarin (female)	–	–	N/A
nonprimate mammals [mouse (2 samples; male and female); rabbit (3 samples; male, female and unknown); pig (3 samples; one female and 2 males); cheetah (female); reindeer (female); elephant (female); cat (female); warthog (female); straw-colored fruit bat (female); wallaby (female); red panda (male); rat (2 samples; male and unknown); dog (unknown); hyena (male); bovine (unknown); ferret (2 samples; male)]	–	–	N/A
nonmammalian animals [chicken (unknown); herring (male)]	–	–	N/A
Saccharomyces cerevisiae, Candida albicans	–	–	N/A
Escherichia coli, strain B; *Micrococcus lysodeikticus; Staphylococcus aureus*; *Pseudomonas aeruginosa*; *Enterococcus faecalis*; *Clostridium perfringens*	–	–	N/A
human papilloma virus, hepatitis B virus	–	–	N/A

[1]N/A = Not applicable. STR amplifications were not performed.

[2]≤5pg/µl detected (minimal reactivity).

Sensitivity

The Plexor® HY System was designed to detect human DNA in a sample and allow an analyst to determine whether the amount of human DNA is adequate for STR analysis. Comparing the limit of detections (LOD) of the quantification and STR systems can verify the utility of a negative quantification result (i.e., a sample with a negative quantification result will not likely yield a genotype in subsequent STR analysis).

To examine sensitivity, we evaluated serial dilutions of five single-source, human male DNAs using the Plexor® HY, PowerPlex® 16 and PowerPlex® Y Systems. Quantification was performed using the Applied Biosystems 7500 and 7500 FAST real-time PCR systems and Stratagene Mx3005P® quantitative PCR system. For each sample, we created a threefold serial dilution from 69fg/µl to 50pg/µl. Quantification results were generated using 2µl of each sample, whereas 10µl of sample was analyzed using the PowerPlex® 16 or PowerPlex® Y System. As shown in Table 2, the Plexor® HY LOD with 2µl of sample is comparable to that of STR systems with fivefold more input material.

The Plexor® HY System consistently detected all samples at as little as 3.8pg of total input DNA, and the majority of samples were detected at levels threefold below this quantity (approximately 1pg). In subsequent STR analyses using conditions that maximize sensitivity (32 cycles, injection times that approach saturation with 0.5ng of input DNA and a

Table 2. Limit of Detection for the Plexor® HY System versus the PowerPlex® 16 and Y Systems.

DNA Concentration	Applied Biosystems 7500 Real-Time PCR System[1]		Applied Biosystems 7500 FAST Real-Time PCR System[1]		Stratagene MX3005P® Real-Time PCR System[1]		Autosomal STR[2] (PowerPlex® 16 System)		Y-STR[2] (PowerPlex® Y System)	
	AUTO Plexor® HY (Individuals Detected[3])	Y Plexor® HY (Individuals Detected[3])	AUTO Plexor® HY (Individuals Detected[3])	Y Plexor® HY (Individuals Detected[3])	AUTO Plexor® HY (Individuals Detected[3])	Y Plexor® HY (Individuals Detected[3])	Average % of Alleles Observed ±Std. Dev.[4]	Samples with a Profile	Average % of Alleles Observed ±Std. Dev.[4]	Samples with a Profile
50pg/µl	5/5	5/5	5/5	5/5	5/5	5/5	100	5/5 full	100	5/5 full
17pg/µl	5/5	5/5	5/5	5/5	5/5	5/5	99±2.0	3/5 full	100	5/5 full
5.6pg/µl	4/4	4/4	4/4	4/4	5/5	5/5	55±15	5/5 partial	82±7.0	5/5 partial
1.9pg/µl	5/5	5/5	5/5	5/5	5/5	5/5	6.4±5.5	4/5 partial	17±8.3	5/5 partial
0.62pg/µl	3/5	3/5	5/5	3/5	1/5	4/5	0.74±1.7	1/5 partial	6.7±7.0	3/5 partial
0.21pg/µl	1/5	2/5	0/5	1/5	0/5	1/5	0	0	3.3±7.5	1/5 partial
69fg/µl	0/5	0/5	0/5	0/5	0/5	0/5	0	0	0	0
no-template control	0/5	0/5	0/5	0/5	0/5	0/5	0	0	0	0

[1]Two microliters of each sample was quantitated.

[2]Ten microliters of each sample was amplified.

[3]An individual was considered detected if both duplicate amplifications had C_T values and the melt temperatures of the amplification products were similar to those of the DNA standards.

[4]STR alleles were considered observed if peak heights were above the 50RFU threshold in duplicate amplifications.

50RFU threshold), we obtained full profiles with 500pg using the PowerPlex® 16 System and 170pg using the PowerPlex® Y System. We observed partial STR profiles for all samples with 56pg using both STR systems and complete amplification failure for some samples with 19pg (PowerPlex® 16) and 6.2pg (PowerPlex® Y) of DNA. Notably, samples with the lowest DNA concentrations constitute low-copy-number (LCN) STR analysis. Because significantly less input volume is used in the qPCR assay, the use of multicopy targets for DNA quantification is crucial to match the LOD of STR assays, which amplify single-copy targets. The Plexor® HY System's high level of sensitivity allows the use of lower sample volumes to reduce sample consumption and should allow laboratories to predict that a sample with a negative quantification result will not yield an STR genotype. Moreover, the Plexor® HY System can accommodate up to 9.0µl of sample, further increasing sensitivity. Given the differences in STR amplification, analysis and interpretation between laboratories, practitioners implementing any new quantification system should perform similar studies using internally accepted STR analysis guidelines.

Sample Degradation

Many forensic samples are exposed to harsh conditions that can challenge the integrity of the DNA available for analysis. DNA degradation and the presence of PCR inhibitors (discussed below) can reduce the success rate of STR amplification. Treating samples with DNase I, although artificial, provides some controlled means of studying the impact of sample degradation. This method has been used to study STR analysis and has been subsequently applied to qPCR assays (23,24).

Aliquots of a DNase I digestion of a single-source human DNA were taken at various time points, and the amount of DNA was quantified using the Plexor® HY System. We used the quantification results to normalize DNA input amounts in PowerPlex® 16 and PowerPlex® Y reactions. A subset of results is shown in Table 3 and Figures 1 and 2. DNA was rapidly digested under the reaction conditions used, and quantification results suggested some degradation in the 30-second sample, significant degradation in the 90-second sample and complete degradation in the 15-minute sample. The Plexor® HY, PowerPlex® 16 and PowerPlex® Y Systems

Table 3. Quantification of DNase I-Digested DNA Using the Plexor® HY System.

Length of DNase Digestion	Quantification Results of the Plexor® HY Autosomal Target (pg/µl)	Quantification Results of the Plexor® HY Y Target (pg/µl)	Percent of Autosomal STR Alleles Observed[1]	Percent of Y-STR Alleles Observed[1]
0 seconds (no DNA degradation)	280	240	100	100
30 seconds (slight DNA degradation)	200	160	100	100
90 seconds (significant DNA degradation)	55	12	0	0
15 minutes (complete DNA degradation)	<1.0	<1.0	N/A[2]	N/A[2]

[1]Each autosomal and Y-STR amplification reaction contained 500pg of DNA, except for the Y-STR reaction with DNA treated for 90 seconds, which contained 180pg of DNA.

[2]N/A = Not applicable. STR amplifications were not performed.

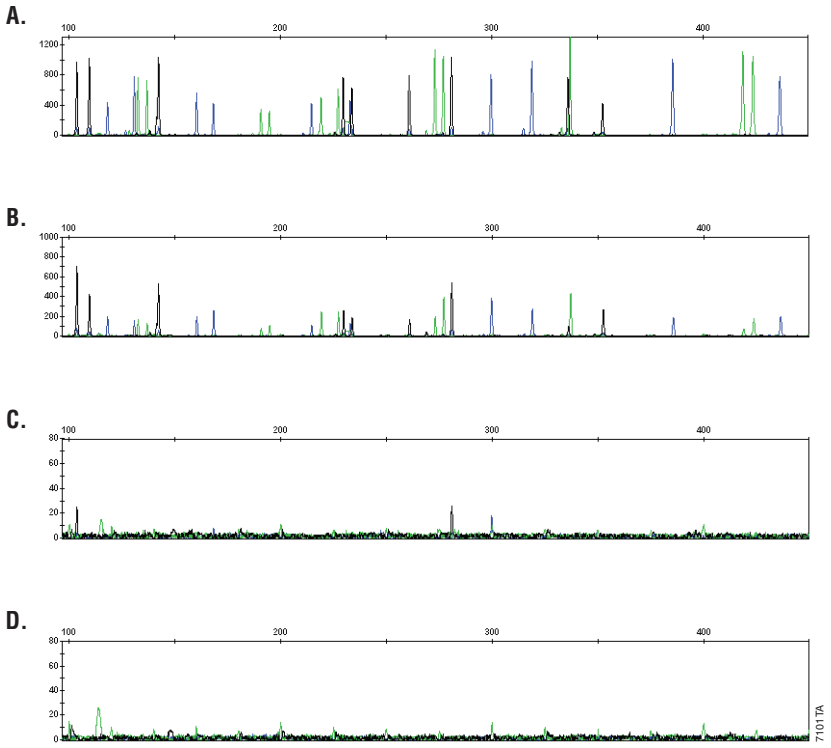

Figure 1. Autosomal STR analysis of degraded DNA. DNase I-treated DNA was amplified using the PowerPlex® 16 System and analyzed using an Applied Biosystems 3130 Genetic Analyzer. **Panel A.** An electropherogram showing the results of the 0-second DNase I-digested DNA control sample. **Panel B.** Results of a sample exposed to DNase I for 30 seconds. **Panel C.** Results of a sample exposed to DNase I for 90 seconds. **Panel D.** Results of a sample exposed to DNase I for 15 minutes.

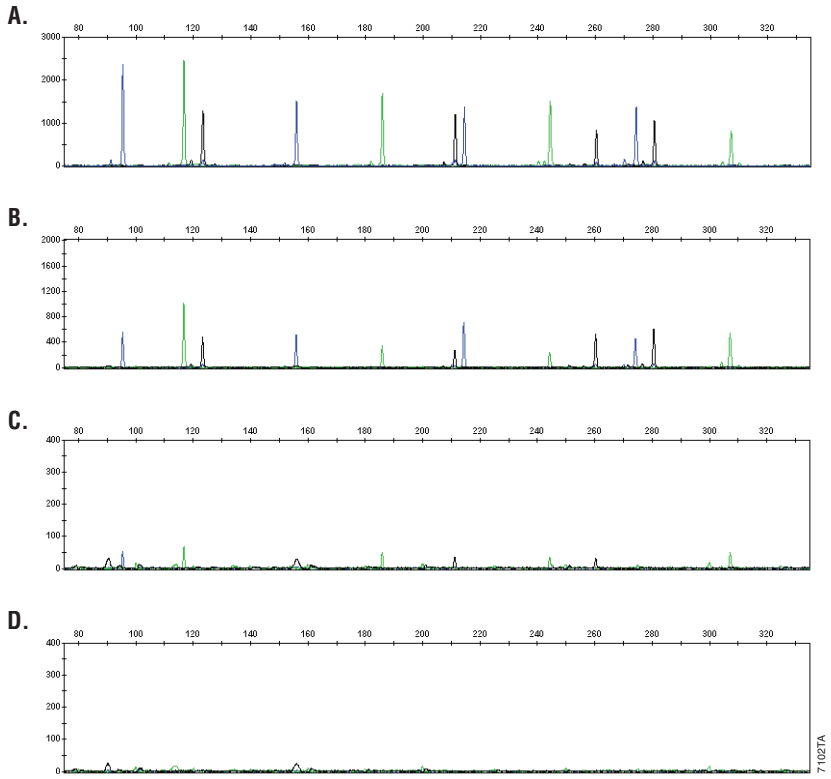

Figure 2. Y-STR analysis of degraded DNA. DNase I-treated DNA was amplified using the PowerPlex® Y System and analyzed using an Applied Biosystems 3130 Genetic Analyzer. **Panel A.** An electropherogram showing the results of the 0-second DNase I-digested control sample. **Panel B.** Results of a sample exposed to DNase I for 30 seconds. **Panel C.** Results of a sample exposed to DNase I for 90 seconds. **Panel D.** Results of a sample exposed to DNase I for 15 minutes.

all demonstrated changes in the results with the 30-second digestion sample compared to results with the 0-second sample. All three assays showed significantly reduced yield or amplification failure with the 90-second sample and amplification failure with the 15-minute sample. Overall, the Plexor® HY System was less affected by DNA degradation than the STR assays. It is important that the quantification assay not fail to amplify at a higher DNA amount than the STR assay to avoid falsely qualifying a sample as having no amplifiable DNA. These results suggest that Plexor® HY quantification will tolerate some DNA degradation. Lower quantification values due to DNA degradation will encourage an increase in sample input volumes in subsequent STR amplifications.

Inhibition

PCR inhibitors in a DNA sample can affect STR data quality and success rate. The same inhibitors can affect qPCR. An IPC target is included in all Plexor® HY reactions to indicate the presence of PCR inhibitors, which results in a delayed IPC C_T value. Because of its increased relative length, the 150bp IPC target in the Plexor® HY System is more susceptible to inhibition than the shorter autosomal (99bp) and Y-chromosomal (133bp) targets. Thus, the inhibitor concentration at which IPC amplification is affected is equal to or lower than that which will affect the autosomal and Y qPCR targets.

We titrated three inhibitors prevalent in DNA purified from blood, soil or bone (hematin, humic acid or calcium chloride, respectively) into Plexor® HY reactions and evaluated the results. We conducted inhibition studies at 0, 15, 20, 25 or 30µM final concentration of hematin; 0, 200, 300, 400 or 600ng of humic acid per reaction; or 0, 0.5, 1, 2 or 4mM final concentration of $CaCl_2$. Each reaction contained 2ng of a single-source, human DNA. Inhibition of the Plexor® HY System was defined as a delay of ≥2 cycles in the IPC C_T value. We observed inhibition at 25µM hematin, 300ng of humic acid per reaction or 2mM $CaCl_2$. Figure 3 shows

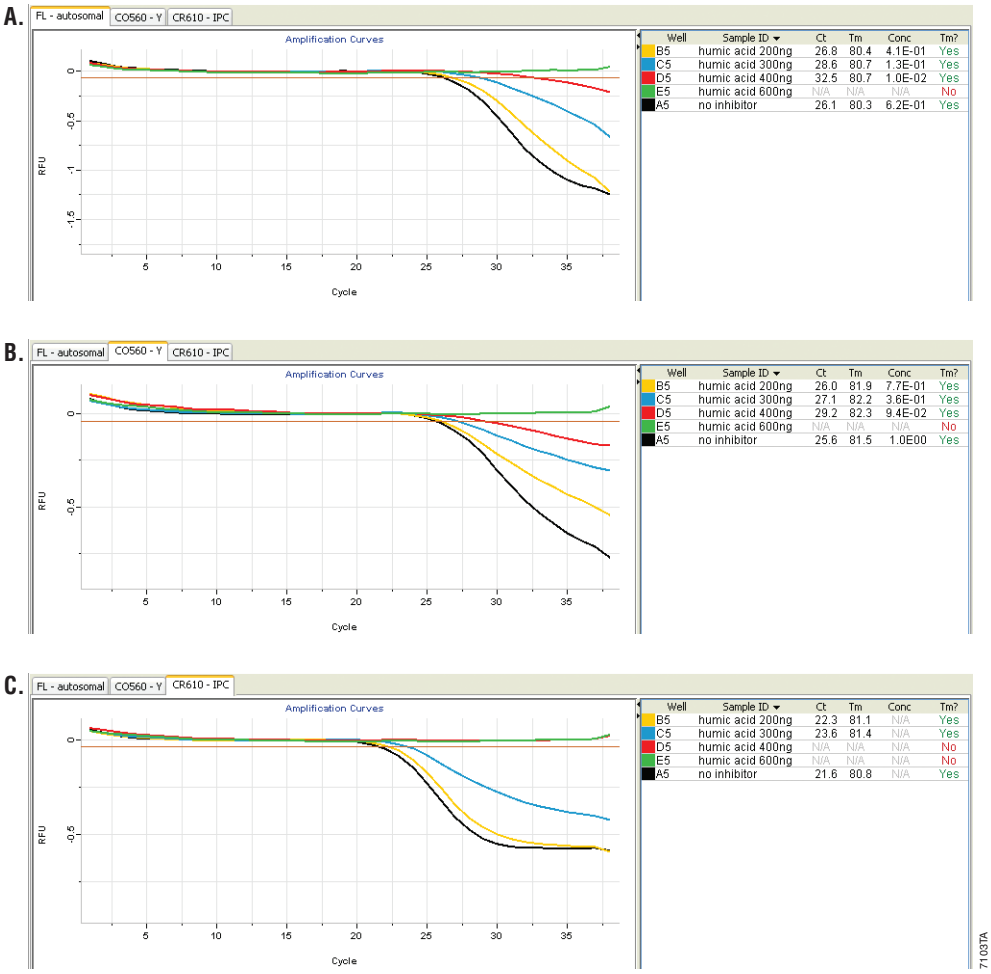

Figure 3. Humic acid titration in Plexor® HY reactions. DNA samples were amplified in the presence of 0ng (black), 200ng (orange), 300ng (blue), 400ng (red) and 600ng (green) of humic acid. Amplification curves are shown for Plexor® HY autosomal (**Panel A**), Y (**Panel B**) and IPC (**Panel C**) analyses of a 1ng/µl sample.

examples of Plexor® HY inhibition by humic acid. In all cases, inhibition of autosomal or Y amplifications at a given inhibitor concentration was accompanied by inhibition of the IPC amplification.

Samples that show IPC inhibition should be diluted or repurified before requantification. Failed or delayed IPC amplification for all DNA standards should prompt troubleshooting steps (amplification plate setup, instrument setup, etc.; 2,3). The level of sensitivity to inhibitors for the Plexor® HY IPC amplification closely correlates with that of the autosomal and Y qPCR amplifications, reducing the need for repurification and requantification of DNA samples. In contrast, using a quantification assay with a weak, inhibitor-sensitive IPC will lead to an excessive number of samples that require repurification or dilution, then requantification.

STR analysis of samples with delayed IPC C_T values showed significant amplification inhibition or failure (Figure 4). In general, amplification of the Plexor® HY IPC was equally or slightly more robust than PowerPlex® 16 and PowerPlex® Y amplifications for all three inhibitor models. Samples that cause IPC inhibition can be expected to cause inhibition of PowerPlex® 16 and PowerPlex® Y reactions if similar or greater volumes of input DNA are used.

Uninhibited IPC amplification should instill confidence in the *quantification* results for that sample. However, absence of IPC inhibition may not be a strong predictor of STR success. IPC amplification failure is predictive of STR amplification failure.

Mixture and Male Specificity

The increasing use of Y-STR analysis of forensic samples supports the need to detect and quantify the male component of male/female mixtures. In addition, comparing male and female DNA concentrations can suggest which type of STR analyses might provide useful male genotype information. Mixed samples that are primarily male may yield conclusive information about the male contributor with autosomal STR analysis, making Y-STR analysis unnecessary. When the male contributor is a minor component, Y-STR analysis is often performed in addition to autosomal STR analysis (21). When mixtures are more than ~95% female, autosomal STR analysis may not yield any useful information about the male contributor (20).

We created and analyzed five male/female mixture panels to characterize potential decision points for supplemental Y-STR analysis and omission of autosomal STRs. For each mixture, 0.25–1ng/µl of single-source, male DNA was quantified in the presence of increasing amounts of female DNA (approximate female:male ratios of 1:1, 2:1, 4:1, 8:1, 16:1, 32:1 and 64:1).

We used autosomal quantification results to normalize sample input for autosomal STR analysis (PowerPlex® 16 System). Figure 5 shows the correlation between the observed percent of unshared male alleles and Plexor® HY [AUTO]/[Y] ratios. These results show that the [AUTO]/[Y] ratio can provide predictive information about the likelihood of observing all male autosomal STR alleles in mixed samples ([AUTO]/[Y] <10) and not observing any male autosomal STR alleles ([AUTO]/[Y] >100). Laboratories may consider formulating general guidelines to add Y-STR analysis and omit autosomal STR analysis based on a sample's [AUTO]/[Y] ratio. These results will depend on a laboratory's STR analysis preferences and other laboratory-specific practices. Consequently, mixture experiments to establish decision points using [AUTO]/[Y] ratios should be repeated as part of an internal laboratory validation.

In addition to determining which STR system is appropriate to amplify mixtures with low mixture ratios, qPCR must detect male DNA in the presence of high levels of female DNA. To test male/female mixtures with extreme amounts of female DNA, we created a mixture series of two male DNAs with increasing amounts of female DNA. An average amount of ~0.8ng or ~0.06ng of male DNA was mixed with up to 1,000-fold or 10,000-fold more female DNA, respectively. Results showed some reduction in male quantification values as mixtures neared 1µg of female DNA, but all samples were detected with confidence (Figure 6). Reactions with 50ng/µl of only female DNA did not produce a positive Y C_T value (DNA concentration <1pg/µl, data not shown).

These results suggest that the Plexor® HY System can detect minimal amounts of male DNA in the presence of significant amounts of female DNA. Also, the [AUTO] and [Y] results can be used as a guide when selecting the appropriate STR assay.

Reproducibility and Concordance

We conducted a study to compare intra- and interrun reproducibility on Applied Biosystems 7500 and 7500 FAST Real-Time PCR Systems. We prepared a single-source male DNA at moderate concentration (~3ng/µl) and low concentration (~23pg/µl) and quantified 16 replicates of each concentration during two separate Plexor® HY runs. Table 4 shows an intrarun coefficient of variation (CV) of 20%. Interrun variation was higher, but the averages between data sets were well within twofold. In previous experiments, increased variation was observed near the system's limit of detection. Compared to values shown here, the 3.2pg/µl replicates had twofold or threefold greater intrarun variation for the Y and autosomal quantification values, respectively (data not shown).

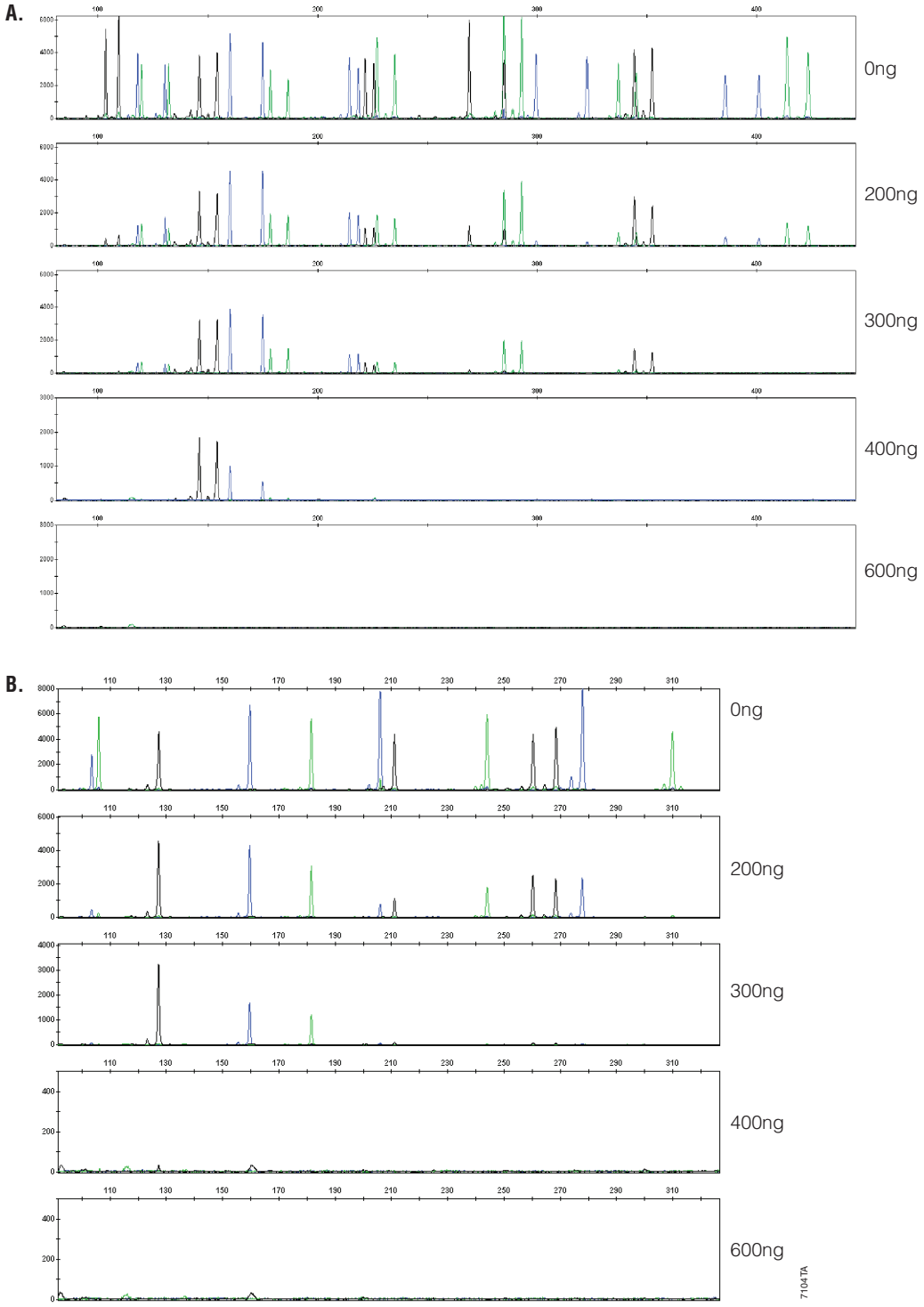

Figure 4. Humic acid inhbition of STR amplifications. PowerPlex® 16 (**Panel A**) or PowerPlex® Y (**Panel B**) amplifications of 1ng of DNA were performed in the presence of the indicated amounts of humic acid.

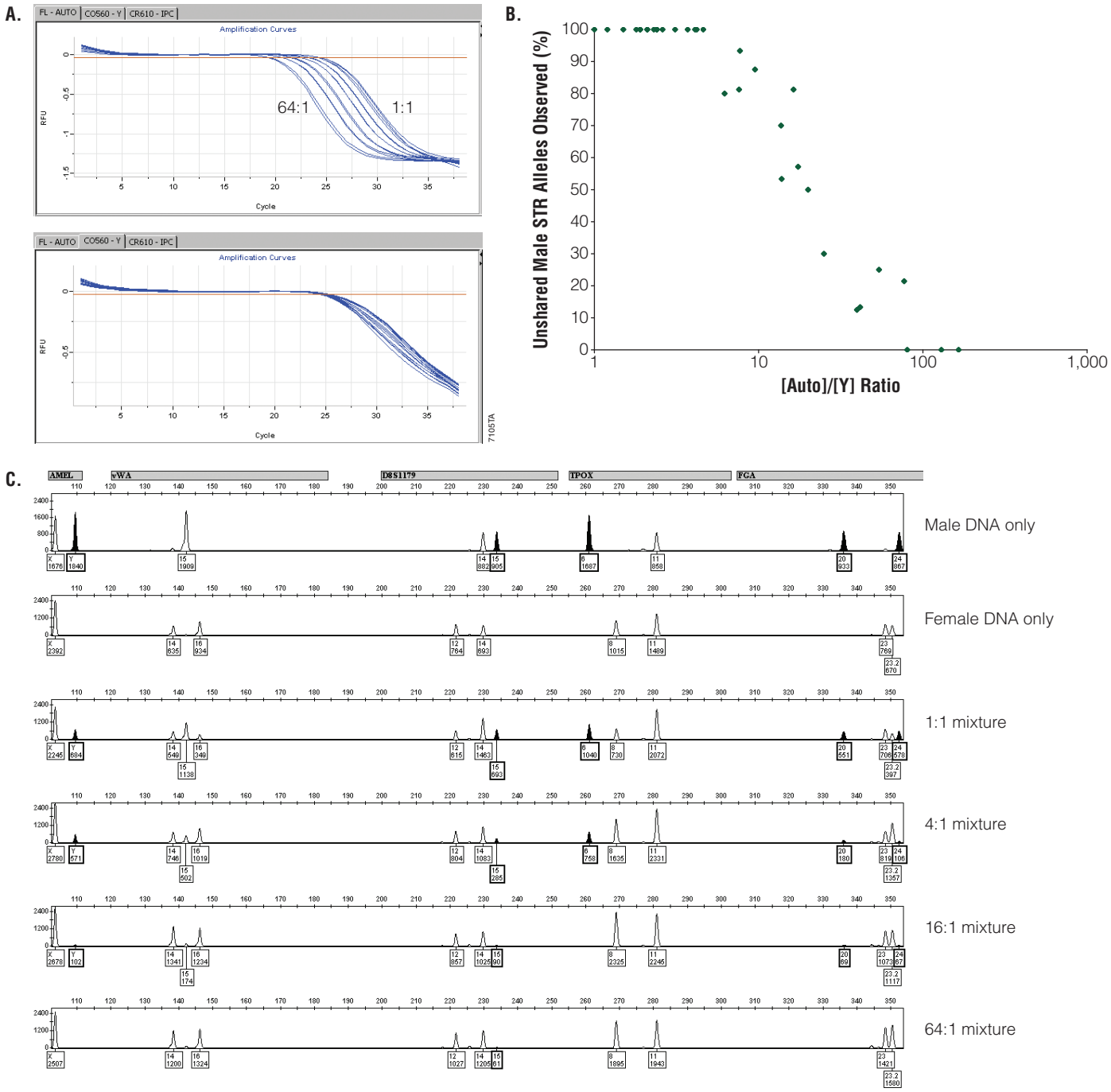

Figure 5. Relationship of Plexor® HY [AUTO]/[Y] ratio and ability to detect male alleles in autosomal STR analysis. Panel A. Amplification curves for a male/female mixture series. Mixtures shown include duplicate analyses of ~1ng/µl male DNA in a 1:1 to 64:1 female:male mixture. The top panel shows the autosomal amplification curves. The bottom panel shows the Y amplification curves. **Panel B.** Percent of unshared male autosomal STR alleles is plotted versus the [AUTO]/[Y] ratio observed for each sample (n = 34). **Panel C.** PowerPlex® 16 amplifications of a mixture series. Amplification of 0.5ng total DNA was performed to illustrate reduced amplification of male alleles as female DNA concentration increases. The TMR-labeled amplification products are shown for the male-only, female-only, 1:1 female:male mixture, 4:1 mixture, 16:1 mixture and 64:1 mixture amplifications. Highlighted peaks indicate male-specific alleles that are unshared and do not migrate in the stutter position of female alleles.

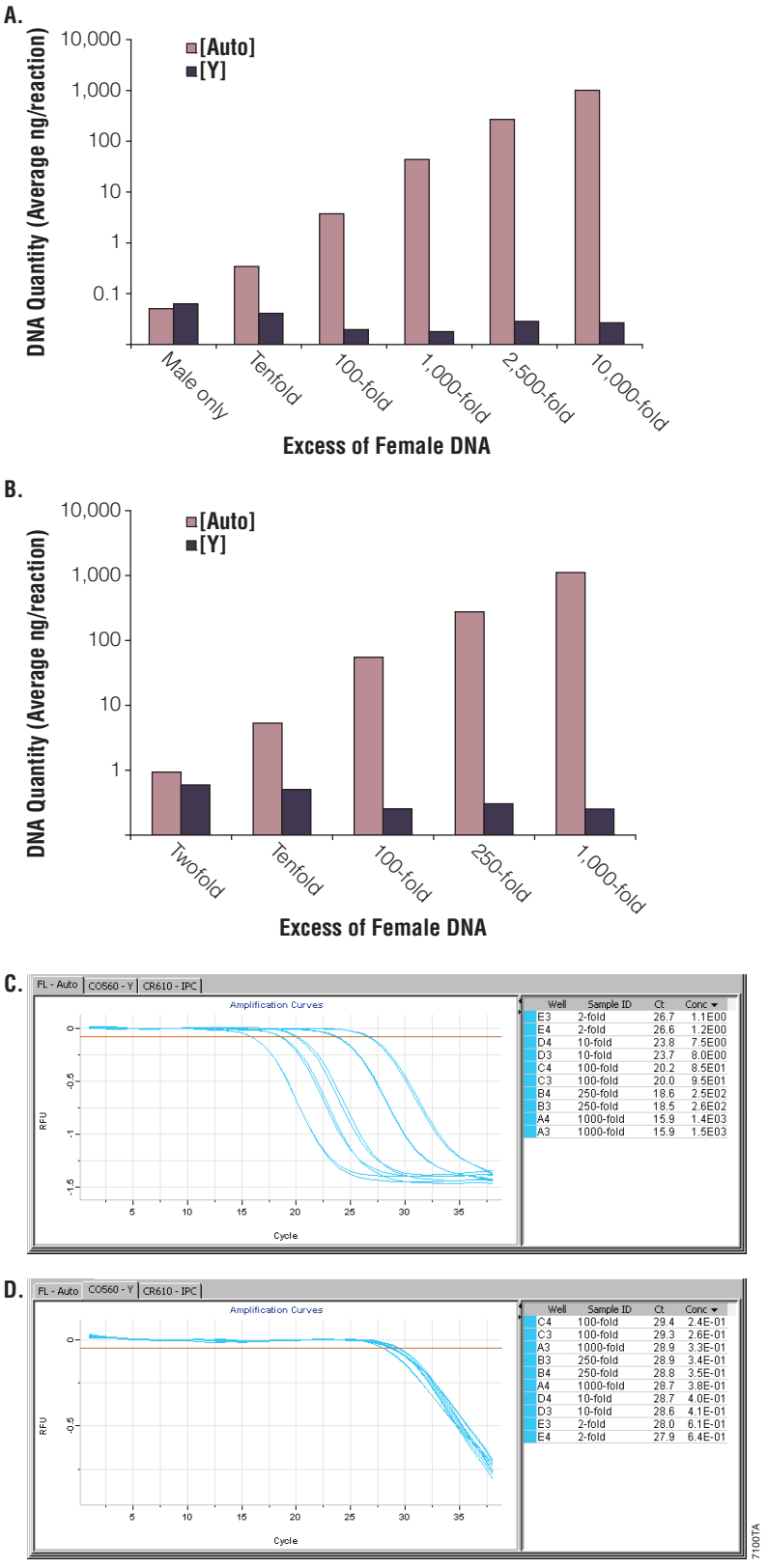

Figure 6. Detection of moderate- and low-concentration male DNA in the presence of increasing female DNA. Average amplification results for two mixture panels with increasing amount of female DNA and a constant amount of male DNA ~0.06ng (**Panel A**) or ~0.8ng (**Panel B**). Autosomal amplification curves for a male/female mixture series (**Panel C**). Y amplification curves (**Panel D**). Mixtures shown include duplicate analyses of ~0.8ng male DNA with an equal amount or up to 1,000-fold more female DNA.

Table 4. Intrarun and Interrun Variation of the Plexor® HY System.

	Applied Biosystems 7500 Real-Time PCR System				Applied Biosystems 7500 FAST Real-Time PCR System			
DNA Concentration	AUTO Average±CV Run #1	AUTO Average±CV Run #2	Y Average±CV Run #1	Y Average±CV Run #2	AUTO Average±CV Run #1	AUTO Average±CV Run #2	Y Average±CV Run #1	Y Average±CV Run #2
Moderate (~3ng/µl)	2.3±19%	2.5±6%	2.4±18%	3.4±16%	2.4±17%	2.5±11%	3.1±16%	3.2±17%
Low (~23pg/µl)	22±18%	22±19%	21±14%	27±18%	17±23%	20±17%	29±15%	28±15%

DNA Concentration	Average [AUTO]/[Y] Ratio±CV Run #1	Average [AUTO]/[Y] Ratio±CV Run #2	Average [AUTO]/[Y] Ratio±CV Run #1	Average [AUTO]/[Y] Ratio±CV Run #2
Moderate (~3ng/µl)	1.0±21%	0.8±15%	0.8±18%	0.8±16%
Low (~23pg/µl)	1.1±23%	0.9±23%	0.6±20%	0.7±20%

To compare results of different quantification methods, we analyzed twenty male samples with the Plexor® HY System and Quantifiler® human and human male DNA quantification kits. Autosomal DNA concentrations determined using the Plexor® HY System averaged 160% of those calculated using the Quantifiler® human kit. Y-chromosomal DNA concentrations were 120% of those determined using the Quantifiler® human male kit. This suggests that Plexor® HY quantification results are equivalent to values observed with the Quantifiler® kits. Any difference could be attributed to differences in the DNA standards.

Population Study

The use of multicopy targets provides the benefit of greater sensitivity. A multicopy target has many copies per cell compared to one or two for a single-copy haploid (e.g., Y) or diploid marker, respectively. However, copy number of these targets can vary between individuals, adding to the variation observed with any qPCR DNA quantification. We examined distribution of autosomal versus Y-chromosomal DNA concentration in four male populations, including African-American, Caucasian-American and Asian-American, and an undeclared group using 0.1–10ng DNA per reaction. The results are shown in Figure 7. The [AUTO]/[Y] ratio for 94% of the samples fell within the range of 0.4 to 2.0. Observation of one outlying sample ([AUTO]/[Y] > 4) indicates that rare but significant variants can occur. However, no false-negative individuals have been observed. These data highlight the fact that the [AUTO]/[Y] ratio of a sample is not intended to determine if a small amount of female DNA is present in a primarily male sample. This ratio is intended to indicate presence of a minor male component in a mixture sample.

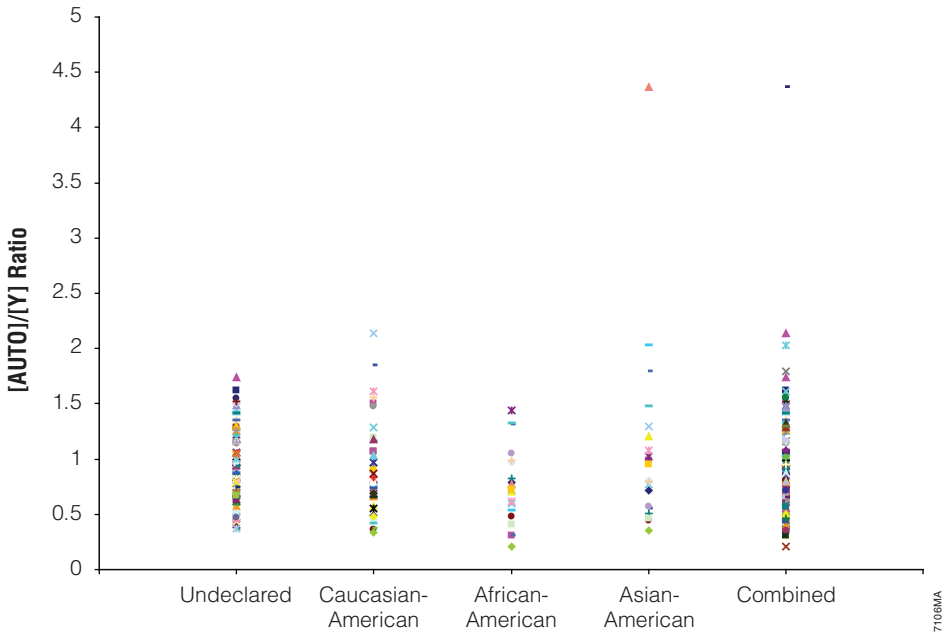

Figure 7. [AUTO]/[Y] ratios for four groups of male samples. For African-American, n = 20; Caucasian-American, n = 40; Asian-American n = 20 and undeclared, n = 80.

Conclusions

Quantification of both total human and male DNA in complex forensic samples provides critical information on how to proceed with sample analysis. Quantification must suggest an appropriate amount of sample to produce interpretable STR results. This information must be generated with minimal sample consumption and confidently identify samples with inadequate amounts of DNA for STR analysis. Quantification must not be significantly affected by the presence of contaminating DNA, and the quantification system must provide adequate controls to flag partial and complete inhibition that affect quantification results.

The Plexor® HY System is a primate-specific DNA quantification assay with a level of sensitivity that exceeds that of subsequent STR analyses. We reliably and simultaneously quantified total human and male DNA from single-source and mixture samples. We observed good correlation between inhibition of autosomal and Y quantification amplifications and changes in the IPC C_T value.

Ordering Information

Product	Size	Cat.#
Plexor® HY System*	800 reactions	DC1000
	200 reactions	DC1001
Plexor® Calibration Kit, Set A*	each	DC1500
Water, Amplification Grade**	5 × 1,250µl	DW0991

***Not for Medical Diagnostic Use.**

****For Laboratory Use.**

References

1. Knox, C. and Krenke, B. (2007) Improved DNA analysis through real-time PCR analysis. *Forensic Magazine.* This can be viewed online at: www.forensicmag.com/articles.asp?pid=141
2. *Plexor® HY System for the Applied Biosystems 7500 and 7500 FAST Real-Time PCR Systems Technical Manual* #TM293, Promega Corporation.
3. *Plexor® HY System for the Stratagene Mx3000P® and Mx3005P® Quantitative PCR Systems Technical Manual* #TM294, Promega Corporation.
4. Moser, M.J. and Prudent, J.R. (2003) Enzymatic repair of an expanded genetic information system. *Nucleic Acids Res.* **31**, 5048–53.
5. Johnson, S.C. *et al.* (2004) A third base pair for the polymerase chain reaction: Inserting isoC and isoG. *Nucleic Acids Res.* **32**, 1937–41.
6. Sherrill, C.B. *et al.* (2004) Nucleic acid analysis using an expanded genetic alphabet to quench fluorescence. *J. Am. Chem. Soc.* **126**, 4550–6.
7. Frackman, S. *et al.* (2005) Plexor® technology: A new chemistry for real-time PCR. *Promega Notes* **90**, 2–4.
8. Hammarström, K. *et al.* (1984) Genes and pseudogenes for human U2 RNA. Implications for the mechanism of pseudogene formation. *J. Mol. Biol.* **179**, 157–69.
9. Van Arsdell, S.W. and Weiner, A.M. (1984) Human genes for U2 small nuclear RNA are tandemly repeated. *Mol. Cell. Biol.* **4**, 492–9.
10. Westin, G. *et al.* (1984) Clustered genes for human U2 RNA. *Proc. Natl. Acad. Sci. USA* **81**, 3811–5.
11. Lindgren, V. *et al.* (1985) Human genes for U2 small nuclear RNA map to a major adenovirus 12 modification site on chromosome 17. *Nature* **314**, 115–6.
12. Pavelitz, T. *et al.* (1995) Concerted evolution of the tandem array encoding primate U2 snRNA occurs in situ, without changing the cytological context of the RNU2 locus. *EMBO J.* **14**, 169–77.
13. Cuello, P. *et al.* (1999) Transcription of the human U2 snRNA genes continues beyond the 3´ box in vivo. *EMBO. J.* **18**, 2867–7.
14. Tyler-Smith, C., Taylor, L. and Müller, U. (1988) Structure of a hypervariable tandemly repeated DNA sequence on the short arm of the human Y chromosome. *J. Mol. Biol.* **203**, 837–48.
15. Pierce, K.E. *et al.* (2000) Real-time PCR using molecular beacons for accurate detection of the Y chromosome in single human blastomeres. *Mol. Human Reprod.* **6**, 1155–64.
16. Guttenbach, M., Müller, U. and Schmid, M. (1992) A human moderately repeated Y-specific DNA sequence is evolutionarily conserved in the Y chromosome of the great apes. *Genomics* **13**, 363–7.
17. Zhang, J.S. *et al.* (1992) Molecular isolation and characterization of an expressed gene from the human Y chromosome. *Hum. Mol. Genet.* **1**, 717–26.
18. Manz, E. *et al.* (1993) TSPY-related sequences represent a microheterogeneous gene family organized as constitutive elements in DYZ5 tandem repeat units on the human Y chromosome. *Genomics* **17**, 726–31.
19. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Laboratories Press, Cold Spring Harbor, New York.
20. *Wizard® SV Genomic DNA Purification System Technical Bulletin* #TB302, Promega Corporation.
21. Krenke, B.E. *et al.* (2002) Validation of a 16-locus fluorescent multiplex system. *J. Forensic Sci.* **47**, 773–85.
22. Krenke, B.E. *et al.* (2005) Validation of a male-specific, 12-locus fluorescent short tandem repeat (STR) multiplex. *Forensic Sci. Int.* **148**, 1–14.
23. Swango, K.L. *et al.* (2006) Developmental validation of a multiplex qPCR assay for assessing the quantity and quality of nuclear DNA in forensic samples. *Forensic Sci. Int.* **170**, 35–45.
24. Nicklaus, J.A. and Buel, E. (2006) Simultaneous determination of total human and male DNA using a duplex real-time PCR assay. *J. Forensic Sci.* **51**, 1005–15.

Promega Corporation • 2800 Woods Hollow Road • Madison, WI 53711-5399 USA • Telephone 608-274-4330 • Fax 608-277-2601

Printed in USA 12/07
15530-AN-GI
Part #AN157

Promega
www.promega.com