Forensic Science International: Genetics 5 (2011) 185–193

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

An analysis of single and multi-copy methods for DNA quantitation by real-time polymerase chain reaction

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ARTICLE INFO

Article history:
Received 10 January 2010
Received in revised form 6 March 2010
Accepted 9 March 2010

Keywords:
Forensic science
Quantitation
Real-time PCR
DNA analysis
Plexor HY®
Quantifiler Duo®

ABSTRACT

The goal of this paper was to examine and compare two different commercially available approaches to the determination of the relative quantities of autosomal and Y chromosomal DNA using real-time PCR. One, Quantifiler® Duo, utilizes a TaqMan® assay with single copy probes for both autosomal human and Y quantification. The other method, Plexor HY® utilizes a primer quenching assay with multi-copy probes for its quantification of autosomal human and Y chromosomal DNA. To test these approaches we have utilized the NIST Human DNA Quantitation Standard Reference Material 2372, a set of three different NIST human DNA quantification standards, to examine the precision, accuracy and sensitivity of the real-time PCR assays. We also examined data from both systems utilizing casework samples. The results show that both systems produced linear estimates for DNA quantity over a broad range of input DNA. However we did observe some apparent copy number effects when comparing the three different NIST standards which we attributed to issues with sequence variations in the different standards. Overall, the single copy approach provided better accuracy while the multi-copy approach produced better sensitivity. Thus the choice of which system to use should depend upon the goals of the user.

1. Introduction

The determination of the quantity of DNA recovered from forensic samples is a critical step in the overall DNA typing process. A narrow range of input DNA from 0.5 to 2.0 ng is needed to produce optimal results with multiplex DNA typing kits. The Quality Assurance Standards for Forensic DNA Testing Laboratories requires human-specific DNA quantitation (Standard 9.4) [1]. This is due to extraction techniques that can recover human DNA as well as bacterial and other exogenous DNA. A number of procedures have been developed to permit human-specific quantification of DNA including slot blot techniques, liquid based hybridization assays, and real-time PCR (polymerase chain reaction), however, real-time PCR, due to its wide dynamic range and ease of automation, has become the most commonly used procedure for these assays [2]. Real-time PCR was first developed in 1993 by Higuchi et al. [3]. The process involves monitoring the PCR reaction with fluorescent probes that increase in intensity as the PCR reaction proceeds. At a predefined level known as the concentration threshold, Ct, a linear relationship can be established between the log of the concentration of DNA template and the number of PCR cycles. Generally quantitative PCR (qPCR) is utilized to detect variations in gene expression and to detect variants in genes. However in forensic science, the primary goal is to determine the quantity of recovered DNA. Early works by Nicklas and Buel, and Green demonstrated the efficacy of the technique, illustrating its wide dynamic range and minimal sample requirements [4–6]. Since then a number of procedures have been validated for the quantification of autosomal DNA and several commercial systems have appeared on the market. Real-time procedures may be based on the detection of fluorescent intercalating dyes, TaqMan® probes or primer quenching. Single or multi-copy targets may be used depending on the application. For example, utilization of highly repetitive multi-copy Alu inserts with Syber Green I intercalating dyes can yield high sensitivity (1 pg detection limits) as a single cell may contain 1000 s of copies of the target sequence [7]. TaqMan® and other probe-based techniques can be multiplexed, permitting the use of an additional internal template control to detect inhibition and ensure quality results [5].

Recently a number of real-time PCR approaches have been developed to determine the relative quantity of male DNA to autosomal (male and female) DNA in a sample containing a mixture of two or more individuals [6,8–10]. While the procedures vary in details, the overall goal is first to determine the amount of amplifiable autosomal DNA, secondly to ascertain the presence of male DNA and determine if sufficient male DNA is present to produce a useful STR (short-tandem repeat) profile, and lastly to

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doi:10.1016/j.fsigen.2010.03.002

detect the presence of inhibitors which might affect the output of the amplification and require further sample cleanup and reanalysis.

The answers obtained can be used to develop a strategy for downstream sample processing. For example, if a case involves a male suspect and a female victim, and little or no male DNA is detected, there may be no reason to proceed with STR typing. Alternatively if a large amount of male DNA is detected, autosomal STR typing may provide useful results even if a mixture is present. Lastly, if trace levels of male DNA are found in the midst of a large amount of female DNA, Y STR typing may be the most appropriate procedure to pursue. An additional consideration is the sensitivity of the test. If low levels of DNA can be detected, a laboratory may wish to proceed using low copy number protocols to make certain reliable data is reported [11,12].

There are presently two commercial systems available for concurrent estimation of human and Y DNA, Quantifiler® Duo (ABI, Foster City, CA) and Plexor HY® (Promega, Madison, WI) [8,10,13]. Both systems are based on fluorescent quenching to detect DNA. However the Quantifiler system utilizes a set of TaqMan® probes while the Plexor HY® system uses a labeled primer that is quenched during the extension phase, resulting in a loss of signal with increase in dsDNA. Both kits utilize specific probes for autosomal, Y and internal control DNA. The TaqMan® chemistry has been well characterized and involves the use of a specifically designed ssDNA probe that includes two dyes, a fluorescent reporter and a quencher. During the primer extension step, the 5′ exonuclease activity of the Taq polymerase digests the probe and releases the fluorescent dye from its quencher. Probes are multiplexed by using a different fluorescent dye with each probe [14].

The Plexor HY® chemistry utilizes two synthetic bases, isoguanine (iso-dG) and 5′-methylisocytosine (iso-dC) that will only bind to each other. During the PCR process a fluorescently labeled iso-dC is incorporated into the 5′ end of the primer. The reaction is set up such that the complementary strand will incorporate an iso-dG labeled with a quencher during the extension step. Thus as the amount of dsDNA accumulates the fluorescent signal decreases [15].

There are some important differences between the two approaches. Firstly, with TaqMan® probes, fluorescence increases as the reaction proceeds while fluorescence drops as PCR proceeds with Plexor HY® labeled primers. Secondly, the primer locations and gene copy numbers are different with the two approaches. The human probe in Quantifiler® Duo probes a single copy sequence with a target located in the RPPH1 gene on chromosome 14:14q11.2 and with an amplicon size of 140 bp. Quantifiler® Duo also probes the Y chromosome at SRY locus Yp11.3 with an amplicon size of 130 bp. Alternatively, Plexor HY® utilizes a multi-copy variant ($n = 10$) at locus RNU2 on chromosome 17 for autosomal DNA with an amplicon size of 99 bp and a multi-copy variant ($n = 20$) at locus TSPY for the Y chromosome component with an amplicon size of 133 bp [8,10]. Because there are 2 copies of the autosomal locus, there is an effective equivalence between the number of copies of the autosomal and the Y locus in Plexor HY®. Ignoring the effects of the detection chemistry, the major effect on the results when comparing the two kits should be the effect of the target copy number and the size of the amplicons [16,17].

In this study, our expectations were that the multi-copy system would provide better sensitivity and the single copy system would have better accuracy [4]. The recent release of NIST standards permits a comparison of the two approaches with respect to precision, accuracy and sensitivity and allows us to test these assumptions [18,19]. Thus the overall goal of this paper was to examine the two approaches with a view to defining their applicability to different forensic problems. We also utilized various casework samples to probe effects of environmental and other outside factors.

2. Materials and methods

2.1. Samples

Samples used in this study included a set of reference standards obtained from the National Institute of Standards and Technology (NIST) and a set of adjudicated casework samples obtained from the Broward Sheriff's Office Forensic Laboratory. The NIST Human DNA Quantitation Standard Reference Material 2372 standard consisted of three separate tubes labeled QSRM A, QSRM B and QSRM C. QSRM A is a single source male standard extracted from buffy coat white blood cells from an anonymous male at a concentration of 52.4 ng/μL. QSRM B is a pooled female sample extracted from buffy coat cells from multiple anonymous female donors at a concentration of 53.6 ng/μL and QSRM C is lyophilized human genomic extract and has both male and female donors with an unknown number of donors at a concentration of 54.3 ng/μL [18]. From this information it is reasonable to assume that QSRM A will give equal responses for both autosomal and Y DNA, that QSRM B will be negative for Y DNA and that QSRM C will give an unknown level of Y DNA somewhat less than the autosomal value. The NIST QSRM samples were diluted in TE buffer to the following concentrations: for QSRM A the dilutions consisted (in ng/μL) of 13.1, 6.55, 3.28, 1.64, 0.819, 0.409, 0.205, 0.102, 0.0512, 0.0256, 0.0128, 0.0064, 0.0032, 0.0016, 0.0008, 0.0004, and 0.0002. For QSRM B the dilutions consisted (in ng/μL) of 13.4, 6.70, 3.35, 1.68, 0.838, 0.419, 0.209, 0.105, 0.0523, 0.0262, 0.0131, 0.0065, 0.0033, 0.0016, 0.0008, 0.0004, and 0.0002. For QSRM C the dilutions consisted (in ng/μL) of 13.6, 6.79, 3.39, 1.70, 0.848, 0.424, 0.212, 0.106, 0.0530, 0.0265, 0.0133, 0.0066, 0.0033, 0.0017, 0.0008, 0.0004, and 0.0002. Three sets of samples (QSRM A 0.102, 0.0256, and 0.0064 ng, QSRM B 0.105, 0.0262, 0.0065 ng, and QSRM C 0.106, 0.212, and 0.0066 ng) for these studies were quantified four times each to examine reproducibility of the assays, the remainder were quantified singly.

2.2. Instrumentation

All of the Quantifiler Duo® and Plexor HY® experiments performed were run on an Applied Biosystems (AB) 7500 Real-time PCR Instrument (Applied Biosystems, Foster City, CA) and analyzed with Applied Biosystems Sequence Detection Software version 1.2.3. Promega samples were analyzed with the same Sequence Detection software followed by analysis with Plexor HY® Analysis Software version 1.5.4.18 (Promega, Madison, WI). All tests were prepared using AB 96-well Optical Reaction Plates.

Both positive (the standard dilution series) and negative controls were run on each plate.

Experiments that used Applied Biosystems Quantifiler Human® chemistry were run according to manufacturer's instructions [5]. An Applied Biosystems 7000 Real-time PCR instrument was used and these samples were analyzed with Applied Biosystems Sequence Detection System Software version 1.0.

2.3. Sample analysis

2.3.1. Quantifiler® Duo process

The Quantifiler® Duo standard curve was made from pooled human male genomic DNA at a concentration of 200 ng/μL provided by the manufacturer of the kit. A set of standards were prepared from this sample at concentrations of 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068 and 0.023 ng/μL as called for by the manufacturer.

Table 1
Comparison of amplicon size, location and copy number for Quantifiler Human®, Quantifiler® Duo, and Plexor HY® real-time PCR quantitation kits. Each kit utilizes one or more target sequences and an internal control sequence.

Kit	Gene	Target location on the chromosome	Copy number	Amplicon length
Quantifiler Human®	Human telomerase reverse transcriptase gene	5p15.33	1	62 bases
Quantifiler Y	Sex-determining region Y gene	Yp11.3	1	64 bases
Quantifiler IPC	synthetic sequence		–	79 bases
Quantifiler® Duo Human Target	Ribonuclease P RNA Component H1 (RPPH1)	14q11.2	1	140 bases
Quantifiler® Duo Male Target	Sex-determining region Y (SRY)	Yp11.3	1	130 bases
Quantifiler® Duo IPC	synthetic sequence		–	130 bases
Plexor HY® Human Target	Human RNU2 locus (Multi-copy)	17 Chromosome	10	99 bases
Plexor HY® Y Target	testis-specific protein, Y-encoded (TSPY) locus (Multi-copy)	TSPY/DYZ5 Y Chromosome	20	133 bases
Plexor HY® IPC	Novel DNA sequence		–	150 bases

Sample preparation and PCR reaction setups were prepared using the manufacturer's protocols [20]. The PCR thermal cycler protocol was set to the following parameters: stage one was 50 °C for 2 min, stage two was 95 °C for 10 min, stage three was performed with 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Analysis was performed with a threshold setting of 0.20. A best fit line was calculated by the software which uses the Ct values of the standards plotted against their starting quantity. The regression line formula was $Ct = m[\log(Qty)] + b$, where m was the slope, b was the y-intercept and Qty was the starting DNA concentration. These values, along with the correlation coefficient (R2) and the standard error of the y estimates on the regression line were used to determine the quality of the standard curve.

2.3.2. Plexor HY® process

The Plexor HY® standard curve was made from the manufacturer supplied tube of pooled human DNA at a concentration of 50 ng/μL. A set of standards were prepared from this sample at concentrations of 50, 10, 2, 0.4, 0.08, 0.016, and 0.0032 ng/μL. Sample preparation and PCR reaction setups were prepared using the manufacturer's protocols [17]. The PCR thermal cycler protocol was set to the following parameters: stage one was 95 °C for 2 min, stage two was performed with 40 cycles at 95 °C for 5 s and 60 °C for 35 s, stage three was 95 °C for 15 s followed by 60 °C for 1 min and finally 95 °C for 15 s. The sample analysis was performed with a threshold setting of 0.20, at the end of the geometric phase of the PCR process. Two files were generated from this analysis, the amplification (Delta Rn) and the dissociation (Raw and Derivative Data). These two files were imported into the Plexor HY® analysis software where analysis was continued.

Thermal melt curves were automatically generated during analysis with the Plexor HY® software.

2.4. Analysis of casework samples

Casework samples and controls prepared from buccal swabs were digested and extracted using the standard phenol chloroform iso-amyl alcohol process [21]. The Identifiler™ STR reactions were prepared as standard 25 μL reactions; the samples were run on Applied Biosystems 3130 and 310 Genetic Analyzers and analyzed with GeneMapper™ ID version 3.2. Alleles were called if they appeared above the laboratory generated stochastic threshold of 150 relative fluorescence units (rfu).

3. Results and discussion

Table 1 provides a comparison of the Quantifiler Human®, Quantifiler Y®, Plexor HY®, and Quantifiler® Duo commercial forensic DNA quantification kits for real-time PCR. Specific genes, chromosomal target location, and amplicon length are all listed [5,8,10]. As can be seen from Table 1 the different kits vary in amplicon size and target copy number. Generally speaking the target size determines how well degraded DNA will amplify [22]. Since standard STR kits produce amplicons ranging from 100 to 400 bp in size, it is reasonable to presume that kits with fragment sizes below 100 bp will overestimate the quantity of available DNA. The second generation kits, Quantifiler® Duo and Plexor HY® account for this by utilizing an allele size range of 99–140 bp in their kits.

3.1. Analysis of NIST standards

In the present study our goal was to examine the performance of Quantifiler Duo and Plexor HY when utilized with the newly developed NIST quantification standards [18]. For comparison purposes we also utilized a previously developed kit, Quantifiler Human®, which is commonly utilized to determine levels of autosomal DNA in the forensic community [5].

In our initial work we examined the neat standards using the three different kits and their respective DNA standards for curve creation, Quantifiler Human®, Quantifiler® Duo and Plexor HY®. Quantifiler Human® results were obtained from one plate with three replicates, Plexor HY® results were obtained from four plates with three replicates on each plate, and the Quantifiler® Duo

Table 2
A comparison of the three standards contained in the NIST DNA quantification reference SRM 2372 using Quantifier Human, Plexor HY® and Quantifiler® Duo. The results are given for replicate analysis of the three NIST ng standards. Data was analyzed using ABI 7000 (Quantifiler Human®) or ABI 7500 (Quantifiler® Duo). Results were the average of at least 3 different runs with three replicates each, except for the Quantifiler Human® results where the average of a single run with three replicates is given.

	NIST 2372 (ng/μL)	Quantifiler Human® avg result (ng/μL)	Avg Plexor HY® result (ng/μL)	RSD%	Auto/Y ratio	Avg Quantifiler® Duo result ng/μL	RSD%	Auto/Y ratio
QSRM A Auto	52.4	51.62	36.9 ± 8.2	22	0.59	74 ± 24	32	1.03
QSRM A Y			62.5 ± 9.4	15		72 ± 24	33	
QSRM B Auto	53.6	63.92	66 ± 13	20	N/A	71 ± 24	34	N/A
QSRM B Y			0	0		0	0	
QSRM C Auto	54.3	70.52	46 ± 11	24	0.75	44 ± 16	36	1.47
QSRM C Y			61.6 ± 7.5	12		30 ± 10	33	

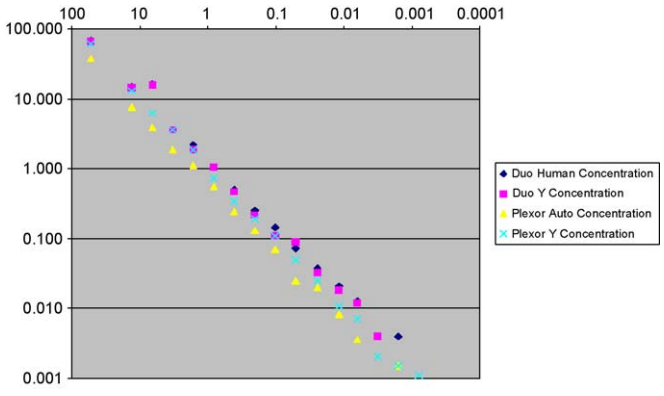

Fig. 1. A plot is the log of expected vs. actual concentrations for a serial dilution of QSRM A from NIST Standard Reference Material 2372 using Quantifiler® Duo (human and Y) and Plexor HY® (human and Y). The results show a linear relation for the two kits over 5 orders of magnitude. Data is plotted in units of ng/μL.

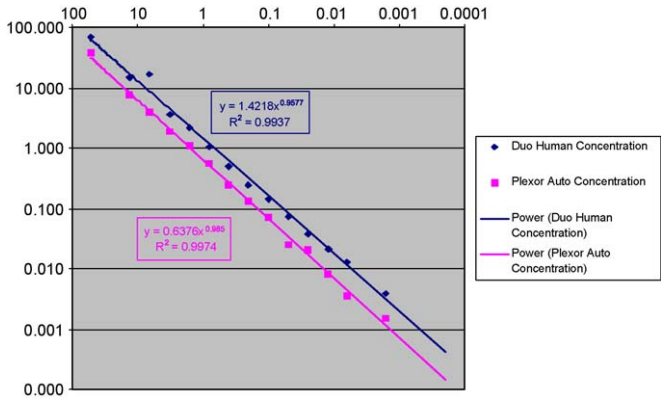

Fig. 3. A log/log plot of expected vs. actual concentrations for human results of Quantifiler® Duo and Plexor HY® using QSRM A from NIST Standard Reference Material 2372. The standard error for the y estimates were Plexor HY® = 0.07 and Quantifiler® Duo = 0.11 Note that values for the Plexor HY® estimate trend low perhaps indicating a copy number variant in the autosomal sequence for the NIST standard. Data is plotted in units of ng/μL.

results were obtained from three plates with three replicates each. The results are listed in Table 2. The data was surprising to us, with large relative standard deviations and inconsistent average values. The autosomal to Y ratio for standard A was near 1 as expected for the Quantifiler® Duo standard; however, the Plexor HY® data was less accurate giving a value of 0.59. Wider differences were seen with standard C. Precision was poor with all three kits, perhaps indicating that measurements at these rather large levels of input DNA (approximately 50 ng/μL) may produce fairly wide swings in quantitative values.

In an attempt to better define differences between the NIST standards and between the different kits, a complete analysis of the three standard quantitation reference standards (QSRMs) was performed using the Quantifiler® Duo and Plexor HY® kits. To do this, the QSRM samples were serially diluted as described in Section 2. Each diluted sample was amplified using real-time PCR protocols once with Plexor HY® and once with Quantifiler® Duo. For each set of dilutions the experimental values were plotted against the NIST values on a log/log plot. Error bars were determined from the standard error of the y estimates on the regression line. The overall data for QSRM A is shown in Fig. 1. As can be seen from Fig. 1 both Plexor HY® and Quantifiler® Duo produce a linear correlation between NIST values and experimental results over nearly 5 orders of magnitude (50–0.005 ng). Because the data is plotted on a logarithmic scale, small errors in the estimate can result in large deviations in concentration especially at higher levels.

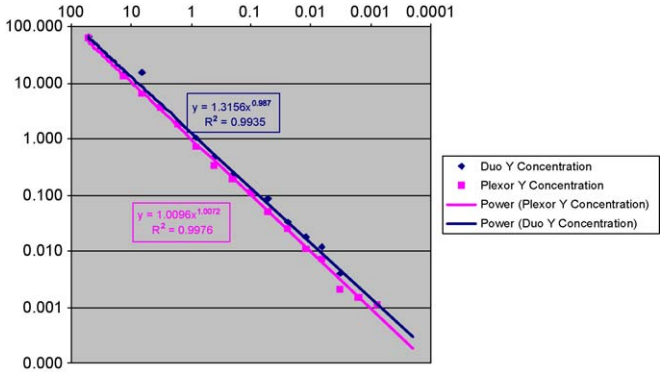

Fig. 2. A log/log plot of the expected vs. actual concentrations for male results of Quantifiler® Duo and Plexor HY® using QSRMA from NIST Standard Reference Material 2372. The standard error for the y estimates were Plexor HY® = 0.08 and Quantifiler® Duo = 0.11. Data is plotted in units of ng/μL.

The data also shows a negative bias for the Plexor HY® Human autosomal DNA sample. This was further explored in Figs. 2 and 3 which directly compare the Human and Y estimates between the two kits. From these results it appears that a mutation or other sequence effect in the autosomal chromosome of the QSRM A has resulted in an error in the estimation of the quantity of DNA for this sample when run by Plexor HY®. This can be verified by noting the fact that the *Y* concentration estimates for Plexor HY® and Quantifiler® Duo are nearly collinear (Fig. 2) while the same estimates for the autosomal DNA (Fig. 3) do not coincide. Note however that while a difference exists in the *Y*-intercept value, the slopes for the two estimates are nearly identical.

Experiments with the female reference sample QSRM B show collinear results with Quantifiler® Duo and Plexor HY®, however, excessive error in the low level data for Quantifiler® Duo tends to skew the calibration curve, Fig. 4. It should be noted that the manufacturer of Quantifiler® Duo does not recommend measuring DNA samples at such low values. Slopes and intercepts for the two lines would be nearly identical if these lower level samples were removed. These data are not unexpected as Plexor HY® should be more sensitive at lower levels since it uses multi-copy targets.

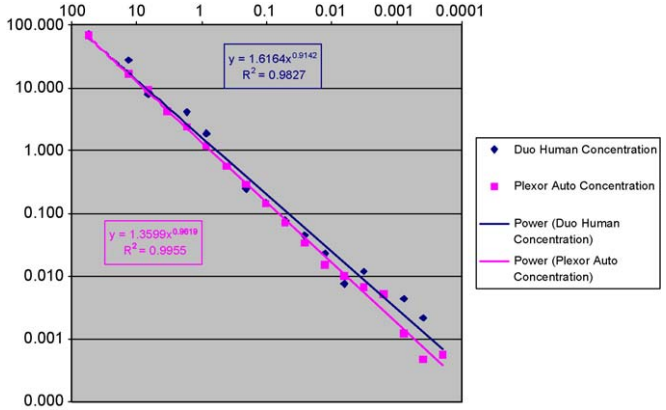

Fig. 4. A log/log plot of expected vs. actual concentrations for human results of Quantifiler® Duo and Plexor HY® using QSRM B from NIST Standard Reference Material 2372. This is a pooled female standard, so no Y concentration estimates are generated. The standard error for the y estimates were Plexor HY® = 0.11 and Quantifiler® Duo = 0.20. Note that concentrations used for the Duo estimate for values below 0.02 ng/μL are below the manufacturer's recommended levels and show lack of precision. If these points are eliminated the lines are virtually collinear. Data is plotted in units of ng/μL.

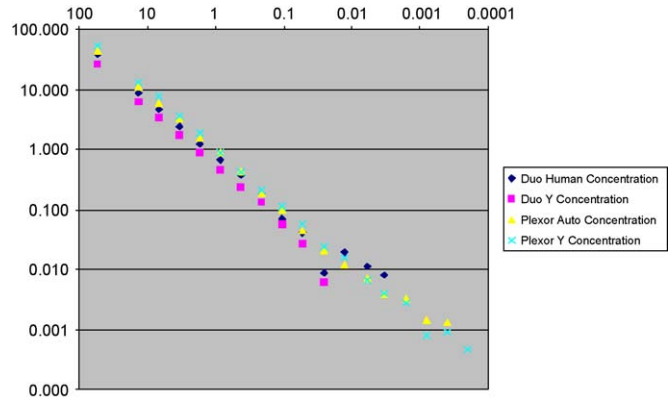

Fig. 5. A log/log plot of expected vs. actual concentrations for human results of Quantifiler® Duo and Plexor HY® using QSRM C from NIST Standard Reference Material 2372, which is an unknown mixture of multiple male and female DNA contributors. Note that concentrations used for the Duo estimate for values below 0.02 ng/μL are below the manufacturer's recommended levels and show lack of precision. If these points are eliminated the lines are virtually collinear. Data is plotted in units of ng/μL.

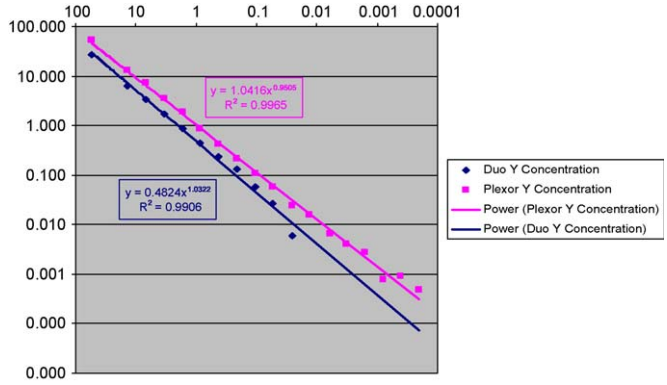

Fig. 7. A log/log plot of expected vs. actual concentrations for the Y chromosomal results of Quantifiler® Duo and Plexor HY® using QSRM C from NIST Standard Reference Material 2372, a mixed male/female standard. The standard error for the y estimates were Plexor HY® = 0.10 and Quantifiler® Duo = 0.11. Note that the differences in relative concentration between the two estimates, with Plexor HY® tending to read high when compared to Quantifiler® Duo. The figure also indicates that Plexor HY® has an expanded dynamic range for this sample.

Similar results are seen with QSRM C (Figs. 5–7). Note that concentrations used for the Quantifiler Duo are below 0.02 ng/μL, the manufacturer's recommended levels, and show a lack of precision. If these points are eliminated the lines become more collinear. The Y chromosomal results (Table 6) are different and show lower values for Quantifiler® Duo when compared to Plexor HY®. Without knowing the actual male to female ratio for this sample, we cannot give a strong conclusion about these results, however, the autosomal to Y ratio should be higher than one for a mixture of male and female DNA so we suspect an error in the Plexor HY® Y result. Instead the average of this ratio over all quantities of standard QSRM C measured is 0.75 for Plexor HY® and 1.45 for Quantifiler® Duo, Table 7.

To test if the relatively low autosomal/Y ratios from Plexor HY® were the result of specific issues with the NIST standards, we analyzed several single source male samples. The results, also shown in Table 7 reveal an interesting anomaly. For the four samples analyzed with Plexor HY®, all produced ratios greater than one while the NIST standards produced low values of 0.6 and 0.75. It should be noted that these results should not be considered unusual. The range given by Promega for a single source male sample is an Auto/Y ratio of 0.4–2 [10,13]. The lower accuracy for the Promega samples can be attributed to the multi-copy target. If less than the full set of 20 copies on a genome amplify, than this ratio may vary. Note that the results from Quantifiler® Duo were all close to one with the exception of the unknown mixture.

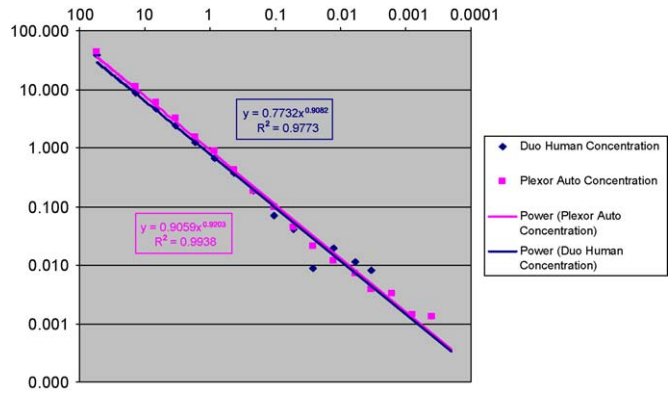

Fig. 6. A log/log plot of expected vs. actual concentrations for human results of Quantifiler® Duo and Plexor HY® using QSRM C from NIST Standard Reference Material 2372. This is an unknown mixture of male and female DNA. The standard error for the y estimates were Plexor HY® = 0.05 and Quantifiler® Duo = 0.11. Note that the lines are essentially collinear. Note that concentrations used for the Duo estimate for values below 0.02 ng/μL are below the manufacturer's recommended levels and show lack of precision. Data is plotted in units of ng/μL.

3.2. A comparison of real-time PCR sensitivity with STR results

In the next series of tests, the quality of the autosomal STR results was examined given the quantitative data from the tests on the NIST samples. Using the experimental values obtained for the individual dilutions of the standards, STR multiplex reactions were prepared using the Identifiler™ multiplex kit from Applied Biosystems. In this test we wanted to examine how useful the real-time result was in predicting the quality of the STR data at low levels. There have been numerous studies performed demonstrating that the data from real-time quantitative assays can be used to provide consistent quality when used as a guide for sample preparation with multiplex STR amplification [6,8,10]. We were interested in the ability of the real-time results to predict the quality of STR data when low levels of genomic DNA were detected. We also wanted to examine the effect of concentration on the autosomal/Y ratios. To perform these experiments STR amplifications were performed on the diluted NIST standards and the number of resulting alleles was determined. All results were obtained using 10 μL of input DNA. Data was not collected for DNA samples with levels above 0.2 ng/μL.

The results of this analysis for the NIST standards A and C are shown in Tables 3–6. The data shows a couple of interesting trends. In general once the concentration of DNA template falls below 3 pg/μL (equal to 30 pg input DNA) a sharp reduction in the quality of amplified DNA occurs, with most alleles dropping out. This has been noted in several previous papers as a design feature for these types of amplification kits [23,24].

The results for QSRM A with Quantifiler® Duo yield a fairly consistent autosomal/Y ratio of 1.02 ± 0.15 down to 0.006 ng/μL. Below that level, the ratio becomes unrecoverable due to low quantities of starting template. For Plexor HY®, the autosomal/Y ratio was 0.64 ± 0.09 over the same range. This lower accuracy is a likely consequence of the multi-copy target. Autosomal/Y ratios and amplification success was also measured with QSRM C using both Plexor HY® and Quantifiler® Duo (Tables 5 and 6).

Because this sample contains a mixture of male and female DNA, the ratio of autosomal/Y DNA is not expected to be 1.0. In

Table 3
Real-time PCR sensitivity vs. genotyping quality for QSRAM A: Plexor HY®. The results show the quantity of DNA determined for a serial dilution of the NIST standard. Data with a margin of error indicated are the average of 4 separate analyses. Following real-time quantification 10 μL of the solution was amplified to determine the quality of the resulting data. Overloaded samples were not reported. Grey areas indicate regions where amounts are unreliable due to low levels of input DNA. The results show a sharp drop in the number of amplified alleles below 30 pg. Note that with this system the loss of amplification at 30 pg coincides with the initiation of the grey zone at 3 pg for real-time amplification.

QSRM A (ng/μL)	Plexor HY® Quantity Auto (ng/μL)	Plexor HY® Quantity Y (ng/μL)	Ratio A/Y	Number of Identifiler™ alleles called out of 26 possible alleles. Amplified 10 μLof template DNA
52.4	38	62.3	0.61	
13.1	7.7	13	0.59	
6.55	3.9	6.4	0.61	
3.28	1.9	3.6	0.53	
1.64	1.1	1.9	0.58	
0.819	0.55	0.73	0.75	
0.409	0.24	0.34	0.71	
0.205	0.13	0.19	0.68	Overloaded
0.102	0.090 ± 0.017	0.14 ± 0.022	0.64	26
0.0512	0.025	0.05	0.50	26
0.0256	0.0293 ± 0.0063	0.038 ± 0.011	0.78	26
0.0128	0.0081	0.011	0.74	26
0.0064	0.0081 ± 0.0031	0.0082 ± 0.0013	0.99	20
0.0032	0.0001	0.0021	0.05	5
0.0016	0.0015	0.0015	0.00	6
0.0008	0	0.0011	0.00	2
0.0004	0.00073	0	0.00	0
0.0002	0	0	0.00	0

addition, a large number of alleles, one hundred eleven (111), were present in the electropherograms due to the fact that the sample contained DNA from multiple individuals. This situation provided an even better opportunity to test the relationship between amplification success and measured amount of DNA with the two kits. For Quantifiler® Duo, autosomal to Y ratios could be measured by qPCR to 30 pg/μL of input DNA and gave a result of 1.45 ± 0.09. This higher ratio was not unexpected given the presence of female and male DNA in the mixture. The Plexor HY® autosomal/Y ratios could be measured to a much lower level than Quantifiler® Duo, 2 pg, indicating a greater sensitivity for this kit. However, the ratio for this sample appeared to be unusually low at 0.75 ± 0.12. In general these tables illustrate an important point: the single copy procedure provides an accurate autosomal to Y ratio but cannot quantify DNA down to a level in which a negative amplification result will occur. The multi-copy method with its increased sensitivity can determine levels of DNA at which amplification will produce poor results, but yields a less accurate autosomal to Y ratio.

3.3. Casework samples

Forty-one adjudicated casework samples were analyzed with Quantifiler Human®, Plexor HY® HY and Quantifiler® Duo. The targeted amount of DNA for these samples was 1.0 ng of DNA template although certain samples contained lower levels of DNA.

Table 4
Real-time PCR sensitivity vs. genotyping quality for QSRAM A: Quantifiler Duo®. The results show the quantity of DNA determined for a serial dilution of the NIST standard. Data with a margin of error indicated are the average of 4 separate analyses. Following real-time quantification 10ul of the solution was amplified to determine the quality of the resulting data. Overloaded samples were not reported. Grey areas indicate regions where amounts are unreliable due to low levels of input DNA. This level is higher than in Fig. 3 due to the fact that Quantifiler Duo® has a single copy probe. However the A/Y ratio is more precise. The results show a sharp drop in the number of amplified alleles below 30 pg. The first three columns units are ng/μL.

QSRM A	Quantifiler® Duo Quantity Auto	Quantifiler® Duo Quantity Y	Ratio A/Y	Number of Identifiler™ alleles called out of 26 possible alleles. Amplified 10 μLof template DNA
52.4	68.7	66.5	1.03	
13.1	14.95	14.28	1.05	
6.55	16.58	15.77	1.05	
3.28	3.59	3.68	0.98	
1.64	2.24	1.85	1.21	
0.819	1.05	1.03	1.02	
0.409	0.504	0.473	1.07	
0.205	0.249	0.222	1.12	Overloaded
0.102	0.146 ± 0.018	0.134 ± 0.057	1.08	26
0.0512	0.073	0.088	0.83	26
0.0256	0.023 ± 0.013	0.0367 ± 0.0039	0.62	26
0.0128	0.021	0.018	1.17	26
0.0064	0.0057 ± 0.0059	0.0054 ± 0.0045	1.06	20
0.0032	0	0.004	0.00	5
0.0016	0.004	0	0.00	6
0.0008	0	0	0.00	2
0.0004	0	0	0.00	0
0.0002	0	0	1.05	0

Table 5
Real-time PCR sensitivity vs. genotyping quality for QSRAM C: Plexor HY® The results show the quantity of DNA determined for a serial dilution of the NIST standard. Data with a margin of error indicated are the average of 4 separate analyses. Following real-time quantification, 10 μL of the solution was amplified to determine the quality of the resulting data. Overloaded samples were not reported. Grey areas indicate regions where amounts are unreliable due to low levels of input DNA. The results show a sharp drop in the number of amplified alleles below 30 pg. The A/Y ratio for this sample should be greater than 1 due to the presence of female DNA. Note that with this system the loss of amplification at 30 pg coincides with the initiation of the grey zone at 3 pg for real-time amplification.

QSRM C	Plexor HY® Quantity Auto ng/μL	Plexor HY® Quantity Y ng/μL	Ratio A/Y	Number of Identifiler™ alleles called, unknown number of possible alleles. Amplified 10 μLof template DNA.
54.3	43.0	53.0	0.81	
13.6	11	13	0.85	
6.79	5.9	7.5	0.79	
3.39	3.2	3.6	0.89	
1.70	1.5	1.9	0.79	
0.848	0.89	0.88	1.01	
0.424	0.41	0.42	0.98	
0.212	0.18	0.21	0.86	111
0.106	0.115 ± 0.011	0.112 ± 0.010	0.90	96
0.0530	0.044	0.058	0.76	85
0.0265	0.0280 ± 0.0060	0.0260 ± 0.0025	0.88	56
0.0133	0.012	0.016	0.75	37
0.0066	0.0080 ± 0.0012	0.0076 ± 0.0011	1.07	11
0.0033	0.0039	0.004	0.98	1
0.0017	0.0032	0.0028	1.14	0
0.0008	0.0014	0.0008	1.75	0
0.0004	0.0013	0.0009	1.44	0
0.0002	0	0.0005	0.00	0

Both single source samples and mixtures were analyzed. The samples were statistically examined to show if variability exists between platforms or if the variability is normal.

Normal variability could be due to pipetting differences and other random errors. The sample types tested included bloodstains, vaginal swabs, sweat, and contact (or touch) samples. The statistical test employed for these data was the Friedman's Test [25]. This is a non-parametric (distribution free) test that can be used to compare observations repeated on the same subjects. A paired *t*-test would assume that the samples were random, normally distributed and have equal variances. This was not true in this study, therefore a non-parametric test, which makes minimal assumptions about the sample set which was used. Results were analyzed using the Quantifiler Human®, Quantifiler Y, Plexor HY® Human, Plexor HY® Y, Quantifiler® Duo Human, and Quantifiler® Duo Y results. The null hypothesis was that the distributions were the same across the repeated measures and the alternative hypothesis was that the distributions across the repeated measures were different. The test statistic for the Friedman's test was a Chi square with one degree of freedom [26]. If the *p*-value was small (<.05) then the evidence would reject the null hypothesis. The results were analyzed for forty-one samples run using Quantifiler Human®, Plexor HY® Human, and Quantifiler® Duo Human, Table 8. There was a significant difference among the three chemistries as shown by the Chi Square value of 22.47 which yields a *p*-value less than 0.001, $p < .001$. Therefore the null hypothesis was rejected indicating that there were significant differences between the distributions for the autosomal quantities

Table 6
Real-time PCR sensitivity vs. genotyping quality for QSRM C: Quantifiler Duo®. The results show the quantity of DNA determined for a serial dilution of the NIST standard. Data with a margin of error indicated are the average of 4 separate analyses. Following real-time quantification 10 μL of the solution was amplified to determine the quality of the resulting data. Overloaded samples were not reported. Grey areas indicate regions where amounts are unreliable due to low levels of input DNA This level is higher than in Fig. 3 due to the fact that Quantifiler Duo® has a single copy probe. A/Y ratio is higher than one due to the presence of female DNA in the mixture. The results show a sharp drop in the number of amplified alleles below 30 pg.

QSRM C	Quantity Auto ng/μL	Quantity Y ng/μL	Ratio A/Y	Number of Identifiler™ alleles called, unknown number of possible alleles. amplified 10 μLof template DNA
54.3	38.4	26.9	1.43	
13.6	8.75	6.20	1.41	
6.79	4.68	3.35	1.40	
3.39	2.39	1.68	1.42	
1.70	1.21	0.865	1.40	
0.848	0.648	0.451	1.44	
0.424	0.375	0.232	1.62	
0.212	0.185	0.129	1.43	111
0.106	0.050 ± 0.047	0.041 ± 0.039	1.26	96
0.0530	0.042	0.027	1.56	85
0.0265	0.008 ± 0.012	0.0059 ± 0.0082	1.50	56
0.0133	0.019	0	0.00	37
0.0066	0.0062 ± 0.0046	0.0031 ± 0.0062	0.00	11
0.0033	0.008	0	0.00	1
0.0017	0	0	0.00	0
0.0008	0	0	0.00	0
0.0004	0	0	0.00	0
0.0002	0	0	0.00	0

Table 7
Comparison of autosomal to Y ratios for NIST standards QSRM A, QSRM C and 4 male donors. The data shows a much wider range of values for Plexor HY®. For QSRM C which is an unknown mixture of male and female DNA, the autosomal to Y value (A/Y) should be above 1. The data for the NIST QSRM A and QSRM C results encompasses a wide range of input DNA values. The standard deviation for the auto to Y ratio was 0.16 for Quantifiler Duo® and 0.09 for Plexor HY®.

	Average Plexor A/Y	Average Duo A/Y	Expected
QSRM A	0.64	1.03	1
QSRM C	0.75	1.45	Unknown mixture
Male 1	1.95	1.1	1
Male 2	1.90	1.08	1
Male 3	1.80	1.05	1
Male 4	1.33	1.05	1

Table 8
Selected adjudicated casework samples. Samples were analyzed using Quantifiler, Plexor HY®, and Quantifiler® Duo, using the manufacturers suggested protocols. The last six columns units are in ng/μL.

Sample #	Type of sample	Quant Human	Quant Y	Plexor Human	Plexor Y	Duo Human	Duo Y
1	Male EZ1 aged bloodstain	0.105	0.0336	0.044	0.038	0.045	0.026
2	Male EZ1 aged bloodstain	0.987	0.515	1.7	1	0.794	0.65
3	Female EZ1 aged bloodstain	1.14	0	0.61	0.0001	0.917	0
4	Male EZ1 aged bloodstain	2.56	2.14	2.7	3.8	2.54	2.44
5	Female EZ1 aged bloodstain	1.15	0	0.55	0.0001	1.09	0
6	Female bloodstain on blue denim	0.961	0	1.24	0.0003	0.483	0
7	#86 diluted 1:10	0.225	0	0.069	0.0007	0.077	0
8	Male bloodstain with LCV	0.281	0.09	0.17	0.23	0.15	0.118
9	Male bloodstain on leather	0.68	0.155	0.54	0.61	0.312	0.282
10	Male bloodstain w/orange print powder	0.104	0.117	0.19	0.26	0.021	0.026
11	Sweat stain on swab from CC	0.053	0.007	0.091	0.043	0.057	0.02
12	Unknown	0.026	0.016	0.036	0.022	0.009	0.004
13	Inside shirt	0.066	0.017	0.066	0.076	0.045	0.035
14	Sample from fetus	80.1	59.6	110	83	N/A	N/A
15	Hair brush from missing person	0.551	0.006	0.29	0.0065	0.284	0.005

among the various kits. A similar analysis was performed using the data from Quantifiler Y®, Plexor HY® Y and Quantifiler® Duo Y. Again, there was a significant difference among the three chemistries with a Chi Square value of 49.06. This result corresponds to a value of $p < .001$ also indicating that there are significant differences between the Y quantity distributions for the kits.

To determine the relationship between relative quantities determined by each qPCR kit we also used a comparison method known as the Wilcoxon signed rank test. This procedure gives a rank of 1, 2, or 3 to the result for each qPCR method. A one is assigned to the lowest qPCR result and a 3 is assigned to the highest. Then all the ranks are averaged and a mean rank is determined for each sample treatment. The ranks assigned to the different chemistries are provided in Table 9. The results for autosomal DNA quantities indicate a marked difference between Quantifiler® Duo and the other two quantitative methods, Plexor HY® and Quantifiler Human®. This may be the result of two separate effects; the shorter amplicon target of Quantifiler Human® and the multi-copy target for Plexor HY®. Shorter amplicons should amplify relatively more DNA if the sample is degraded. This is likely the major difference between the kits when casework samples are considered and may explain differences seen between autosomal estimates between Quantifiler Human® (amplicon size = 62), Quantifiler® Duo (amplicon size = 140) and Plexor HY® (amplicon size = 99). In addition, with Plexor HY®, the multi-copy target for the human chromosome may result in additional shifting due to the effect of sequence variations on the multi-copy loci. The data for the Y quantities were different than the human autosomal quantities. Here the ranks for Plexor HY® were nearly double that of the Quantifiler® Duo and the Quantifiler Human® value. This result again is likely due to the fact that Plexor HY® uses multi-copy targets. The difference in kit standards was not considered to be contributing to this difference due to experiments conducted but not included in this publication that showed no significant differences between the standard calibrators used to make the curves.

Table 9
Ranks of different treatments for casework samples as determined by the Wilcoxon signed rank test. Evaluations for autosomal and Y quantities were performed separately.

Real-time technique	Mean rank
Quantifiler Human®	2.40
Plexor HY® Human	2.18
Quantifiler® Duo Human	1.41
Quantifiler Y	1.41
Plexor HY® Y	2.84
Quantifiler® Duo Y	1.74

4. Conclusions

In these experiments we have compared the response of the Quantifiler® Duo and Plexor HY® real-time PCR quantification kits using NIST Human DNA Quantitation Standard Reference Material 2372. We also examined the response of the two kits to various adjudicated casework samples. The sensitivity study was the most informative during this research. Both kits showed good precision over a very wide range of concentrations.

However, the dynamic range was one order of magnitude better for Plexor HY®. This kit performed particularly well with low levels of the mixed standard QSRM C where it could reliably detect sample concentrations below 10 pg. For the single source QSRM A sample, both kits performed well at low levels. Overall precision for QSRM A was about the same for both kits with standard errors of the y estimates for Plexor HY® at 0.11 and Quantifiler® Duo at 0.20; the greater precision for Plexor HY® is mainly due to the fact that Quantifiler® Duo was examined at lower concentrations than

recommended in the manufacturers guidelines. The auto/Y values were far more accurate for Quantifiler® Duo. For QSRM A, a single source male, Quantifiler® Duo produced an autosomal/Y estimate of 1.03 ± 0.16 while for the same data Plexor HY® yielded 0.64 ± 0.09. The autosomal to Y ratio for Plexor HY® also appeared to be relatively low for QSRM C.

A statistical comparison of the casework samples demonstrated significant differences between the different kits, however it was not possible to tell if these differences were the result of the amplicon size or the chemistry. Since the amplicon sizes for Quantifiler, Quantifiler® Duo and Plexor HY® vary from 62 to 140 bp, significant differences might be expected when the kits are used for degraded DNA. This would be especially true for casework samples.

Overall we were impressed with the capability of both systems. Laboratories interested in accurate autosomal to Y ratios may prefer the Quantifiler® Duo system while those facilities interested in higher sensitivity may select the Plexor HY® system. Overall both produce satisfactory results for the majority of samples.

Acknowledgements

The authors would like to Paulette Johnson for statistical support, DeEtta Mills for essential suggestions, James Ongley for his dedication to further education, and the members of the Broward Sheriff's Office DNA Unit.

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