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TECHNICAL NOTE

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Quantifiler™ Observations of Relevance to Forensic Casework*

ABSTRACT: The Quantifiler™ (QF) kit is regularly used by forensic scientists for DNA quantitation. We performed in-house validation studies which revealed some interesting observations. The QF standard displayed a two-fold difference between two different lot numbers which suggests that every standard should be tested prior to use. The Promega K562 DNA standard works well with the QF kit. *c.* 41% of samples that inhibited the internal PCR control (IPC) system within the QF kit still produced good Profiler Plus™ reactions. QIAquick® was effective at removing inhibitors. The presence of dyes within casework samples were observed not to inhibit QF amplifications. Template DNA greater than 100 ng/μL appeared to inhibit the IPC. Close to identical concentration results were obtained when alternative analysis settings were used. These validation findings will assist DNA processes involved in forensic casework.

KEYWORDS: forensic science, forensic DNA analysis, polymerase chain reaction, multiplex PCR, DNA quantification, real-time PCR, Quantifiler

The validation of a real-time polymerase chain reaction (PCR) quantification kit for use within the forensic community was published in 2005 by Green et al. (1). The Quantifiler™ (QF) Human DNA Quantification Kit by Applied Biosystems (ABI) incorporates a multiplex reaction where a portion of the human telomerase reverse transcriptase gene (hTERT), located on 5p15.33, together with a synthetic DNA sequence, are co-amplified (1). The synthetic DNA present within the master mix acts as the internal PCR control (IPC) to assess for any reaction deficiencies such as the presence of inhibitors (1,2). Both assays are distinguished from one another due to the presence of the FAM™ and VIC® dyes attached to the 5′ ends of TaqMan® probes specific to the hTERT and IPC templates respectively. A standard curve method of quantification is adopted using a standard DNA sample provided within the kit. The quick adoption of this methodology by the forensic community is evident from the NIST 2004 DNA quantitation study (3). It will likely continue to replace existing quantitation systems in forensic casework especially as a consequence of the projected discontinued manufacture of the QuantiBlot® (QB) Human DNA Quantitation Kit (4), from June 2007.

The use of real-time PCR for the quantification of DNA provides several advantages over the QB method. These include: higher throughput; increased sensitivity; a greater dynamic range; less sample required (no more than 2 μL); being based on the same principles as the techniques used for forensic genotyping, therefore, providing relevant information about the quantity of "amplifiable" DNA within a sample; data analysis being largely automated reducing individual interpretational differences which can lead to variable results; the presence of an IPC system which can provide an indication of which samples are inhibited, affected by a high DNA starting template and/or whether the reaction components or instruments have not performed satisfactorily, thus providing savings in down stream processes; being less labor intensive; being more suited to automated processes and accepted within the scientific community as an accurate, reliable, and reproducible method (3).

We have recently validated the QF kit for use in forensic casework. Most of our findings concur with that previously published (1). However, we identified a number of aspects and interesting results that have not been published previously which may be useful to the forensic community. This paper focuses on the following topics: DNA concentration estimation differences between alternative quantification methods, concentration differences between DNA standards from the same supplier as well as between different suppliers, the effectiveness of the IPC system within the QF kit, the success rate of alternative methods used to remove inhibitors that have affected the IPC, the effects of visible dyes in samples on the IPC system, the effect of high starting DNA template concentration on the IPC, and quantification results when using alternative analysis settings with the 7500 SDS Software (v1.3) from ABI.

Materials and Methods

Sources and Genomic DNA Extraction

Casework samples utilized in this study were derived from different sample types (blood, hair, cigarette butt, trace, seminal, and buccal swabs) and were extracted either organically (5,6) or by the Chelex® method (7). Human Genomic DNA from Roche Applied Science, Penzberg, Germany (lot number 11611420), was supplied at 200 ng/μL (100 μg in total) and had been isolated from whole human blood (pool of 80–100 donors of both sexes). High Molecular Weight K562 DNA from Promega Corporation, Madison, WI (system lot number 206778 and DD201A lot number 19714301) was supplied at 550 μg/mL (30 μg in total) and had been purified from a subculture of the human chronic myelogenous leukemia cell line.

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Quantification Using Spectrophotometry and QuantiBlot®

A GeneQuant Pro Spectrophotometer (v2.5) (Biochrom, Cambridge, UK) was used to determine the absorbance at 260 nm of each DNA sample tested. This result was then used in the following formula: amount (μg/μL) = 50 × A_{260} × dilution factor / 1000 (6). The use of the QB kit (colorimetric method) to quantify DNA was performed as per manufacturer's recommendations (8).

Quantification Using the Quantifiler™ Kit

Reactions were performed as recommended by the manufacturer (9) on an ABI PRISM® 7500 Sequence Detection System (ABI) in the 9600 emulation mode, where either the QF, Roche, or Promega DNA standards were used, using either manual or auto C_T and baseline analysis settings.

Genotyping Using AmpFlSTR® Profiler Plus™

Profiler Plus™ (ABI) reactions were performed according to the manufacturer's protocol (10) using a GeneAmp® PCR System 9600 (ABI). Samples were electrophoresed on an ABI PRISM® 3100 Genetic Analyser (ABI). GeneMapper™ ID software (ABI), version 3.2, was used for STR analysis.

Quantification Technique Comparison

Of 417 samples utilized in the in-house validation study (365 casework and 52 mock casework blood, hair, cigarette butt, trace, seminal, and buccal swab samples), 361 had previously produced quantitation results with the QB kit. All 417 were subsequently quantified using the QF kit (DNA standard lot number 0503012 [lot A]) and the QF kit in conjunction with the Promega K562 standard (QP) (system lot number 206778) instead of the QF standard supplied. Concentration percent differences were calculated when the quantification results from two methods were compared to each other. The lower concentration obtained for each sample from either method was divided by the higher concentration from the other method, and multiplied by 100. A mean of these calculations was then derived. These methods were compared to each other with respect to the concentrations obtained for either QB versus QF (n = 368), QB versus QP (n = 364), or QF versus QP (n = 369) samples. Samples which had generated the most divergent DNA quantifications results between the quantification methods, within each sample and extraction type and produced no undetermined IPC and hTERT results, were used in subsequent Profiler Plus™ amplifications (n = 38) to compare peak height (RFU) results. Of these samples, 29 were of single source (25 casework, four mock casework). The number of reportable alleles and potential stutter peaks above 40 RFU were also recorded for the remaining nine of these samples which were mixtures.

Standard Comparison Study

The following genomic DNA standards were tested with the QF kit: QF DNA standard lot numbers 0503012 (lot A), 0412010 (lot B), 0505013 (lot C), 0507015 (lot D), Roche DNA standard lot number 11611420, and Promega DNA standard system lot number 206778. All DNA standards were tested on the one plate to minimize instrument variation effects and the one master mix solution was used for each reaction. The DNA standards were tested at the following assumed concentrations based on product inserts: 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, and 0.023 ng/μL. All standards were tested in duplicate and four no template controls were also included. For both the Roche and Promega DNA standards, the 0.023 ng/μL concentration was not tested because their inclusion would have exceeded the capacity of the 96-well plate. QF standard lot A was used to generate a standard curve from which the concentration of the other standards on the 96-well plate were ultimately determined.

DNA Purification Methods

Four DNA purification methods were employed to remove inhibitors within casework samples. These were the phenol:chloroform purification (5,6), 20% Chelex® purification (7), QIAquick® (QIAGEN, Hilden, Germany) purification (performed according to the manufacturer's instructions [11]), and a TE wash using a Centricon® (Millipore, Billerica, MA). The TE-Centricon® method consisted of adding 2 mL of TE (Tris-EDTA, pH 7.5) into a Centricon® with the sample to be purified, and centrifugation at 3000×g for 5–10 min until the volume was reduced to approximately 50 μL. Of the 417 casework/mock casework samples, 25 of the 40 samples that produced undetermined IPC values were used in this study. Of these 25 samples, 60% were extracted using the phenol:chloroform extraction method (trace [n = 7], blood [n = 3], nonsperm fraction from seminal stain [n = 2], sperm fraction from seminal stain [n = 2], and cigarette butt [n = 1]) and 40% were extracted using the Chelex® method (trace [n = 8], sperm fraction from seminal stain [n = 1], and blood [n = 1]).

Effects of High Template Concentrations on the IPC

Twenty-five casework samples, not solely out of the 365 casework samples utilized in this study, that had previously been quantified as containing between 50 and 1250 ng/μL of DNA using the QB method were selected for this study. The DNA concentration in each sample was estimated by running neat as well as 1/10 and 1/100 dilutions using the QP method.

ABI PRISM® 7500 System SDS Software (Version 1.3) Analysis Settings

A comparison was made between the concentration estimations obtained when different analysis settings were used for the same run containing 72 casework samples and six mock casework samples. Concentration estimations obtained using the following analysis settings were compared: (i) 0.09 C_T and 3–15 baseline; (ii) 0.2 C_T and auto baseline; (iii) 0.2 C_T and 3–15 baseline; (iv) 0.09 C_T and 3–13 baseline; (v) 0.09 C_T and auto baseline; (vi) auto C_T (0.1 hTERT C_T, 0.05 IPC C_T) and auto baseline; and (vii) 0.2 C_T and 3–13 baseline. These analysis settings were applied to both the hTERT and IPC detectors. Percent coefficient of variation (%CV) calculations (standard deviation/mean × 100) were performed on the seven different analysis settings results.

Results and Discussion

The Comparison of Quantification Techniques

Three different DNA quantification approaches (QB, QF, and QP) were compared by determining the percent difference between the concentration estimations obtained from each method for the same sample set (Table 1). An alternative standard (from a different supplier) was used with the QF kit (i.e., QP) due to multiple observations throughout our validation indicating that the QF

TABLE 1—*DNA concentration percent differences in pair-wise comparisons of QB, QF (DNA standard lot A), and QP (DNA standard system lot number 206778). The percent a quantification test produced a concentration result greater than or less than another test is also indicated.*

Percent Concentration Difference		
QB vs. QF	QB vs. QP	QF vs. QP
55	52	69
QB > QF 31	QB > QP 61	QF > QP 99
QB < QF 69	QB < QP 39	QF < QP 1

standard was not supplied at the specified concentration. The use of an alternative standard with the QF kit has also been reported by others (12–14), although at scientific meetings only, where the latter also used the Promega K562 standard in conjunction with the QF kit. Only Nielsen and coworkers (13) described a problem with the Quantifiler™ DNA standard, however, they assumed it was caused by a mutation in the Quantifiler™ DNA standard hTERT gene, at the position in which the primer or probe binds.

The results presented in Table 1 suggest that the QF kit with DNA standard lot A overestimates the amount of DNA in samples compared to the QB kit. This would lead to less DNA being added into a subsequent PCR reaction for STR analysis which may result in lower peak heights for amplified DNA, and at the extreme, stochastic effects. Table 1 also suggests that when the Promega standard was used together with the QF kit (QP), the amount of DNA was more frequently underestimated relative to the QF and QB methods which would lead to more DNA than necessary being used in STR amplifications. The amount of DNA in casework samples was overestimated (in 99% of samples) when the QF kit was used with the QF standard compared to when using the Promega standard instead (Table 1). The greatest concentration percent difference was also observed between QF and QP (Table 1).

To investigate this observation further, peak height analyses (RFU) were performed from Profiler Plus™ amplifications on casework samples where the amount of DNA added to the amplification was determined from the actual quantification results obtained using the three methodologies described above. The 38 samples tested incorporated those exhibiting the most divergent DNA quantification results between the quantification methods. Of these samples, 29 were single source DNA samples, of which 27 had the desired IPC C_T results between 24 and 28, and two had IPC C_T values between 28 and 29. The remaining nine samples consisted of mixtures, all of which had IPC C_T results between 24 and 28.

Figure 1 presents the average Profiler Plus™ peak heights (RFU) for all ten loci for the 29 single source samples tested when using 1 ng of DNA as estimated by either the QF, QP or QB tests.

For the homozygote loci, it can be observed that QF generated an average peak height of 714 (RFU), which is lower than what is deemed the optimal range (1000–4000 RFU) validated at the Victoria Police Forensic Services Centre (VPFSC) (Fig. 1). When QF was used to quantify casework samples, 11 homozygote alleles were reported to be below 150 RFU (VPFSC determined cut-off for homozygote Profiler Plus™ alleles). In contrast, an average homozygote peak height of 2127 RFU was obtained using QP, with three peaks observed to be below the homozygote 150 RFU cut-off. Using QB, the average peak height of the homozygote alleles was 3240 RFU, although this is likely to be an underestimate since over-amplified peaks were observed at some loci. The peak heights of the heterozygote alleles (Fig. 1) have a similar trend to the homozygote alleles. The standard deviation error bar was also observed to be largest for the QB data which is not

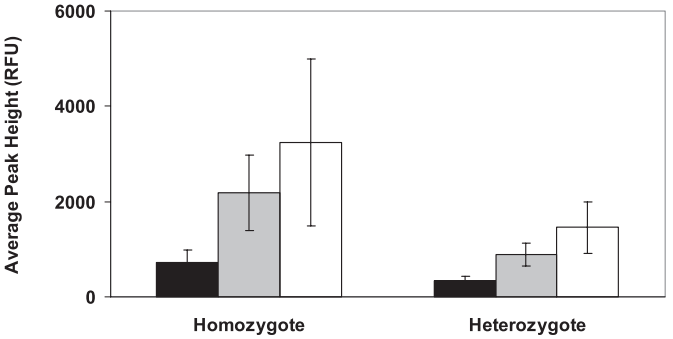

FIG. 1—*Homozygote and heterozygote average Profiler Plus™ peak heights (RFU) across all 10 loci, from 29 single source casework samples, using 1 ng of genomic DNA, as determined after quantification with QF (black bar), using DNA standard lot A, QP (grey bar), using DNA standard system lot number 206778 and QB (white bar). Y-axis bars within each plot represent the standard deviation.*

surprising since this method of quantification relies on more subjective analyst interpretation.

Table 2 presents the concentration and amplification results obtained from the Profiler Plus™ amplification of nine different mixture samples (eight casework and one mock casework) after QF, QB, and QP DNA quantification, presuming that the starting concentrations of the standards supplied in each test were as specified by the manufacturers. The Profiler Plus™ amplified and QP quantified samples produced more stutter peaks compared to samples quantified using the QF kit, similar to the QB results which in total produced more stutter peaks (Table 2). Also, the QF-quantified samples appeared to result in the amplification of fewer alleles. The most likely reasoning for this is that less template material than required was added into Profiler Plus™ amplification reactions due to the inaccurate estimation of DNA concentration. This appeared to be a result of the quantitation standard not being at the concentration specified by the manufacturer.

It is clear from above that a profiling result can be affected by the use of different quantification systems and the accuracy of the standards used.

TABLE 2—*The number of reportable alleles and stutter peaks for mixture samples amplified with Profiler Plus ™ using 1 ng of DNA according to quantification results from QB, QF (DNA standard lot A), and QP (DNA standard system lot number 206778).*

	Concentration Obtained (ng/μL)			Number of Reportable Alleles†			Number of Peaks in Potential Stutter Regions with Heights ≥40 RFU		
Sample	QB	QF	QP	QB	QF	QP	QB	QF	QP
1	0.25	4	2.74	22	2	21	11	0	3
2	0.125	2	0.92	24	19	21	14	1	10
3	0.25	0.47	0.26	24	22	24	10	1	9
4	0.125	0.66	0.55	17	21	32	2	1	3
5	0.75	3.17	1.41	40	22	35	11	0	2
6	0.06*	0.17	0.102	26	30	33	5	1	5
7	0.03*	0.34	0.21	0	22	20	0	0	2
8	0.03*	3.29	1.52	0	20	24	0	0	6
9	0.06	0.792	0.24	0	19	21	0	1	7
Total				153	177	231	53	5	47

*The presence of dyes which may have affected quantification estimations.

†VPFSC utilizes cut-offs for homozygotes (150 RFU) and heterozygotes (40 RFU).

TABLE 3—*DNA concentration results for the QF standard (lots A, B, C, and D) based on two separate spectrophotometer estimations and one QB estimation.*

	Quantifiler™ Standard Lot			
	A*	B*	C*	D*
	DNA Concentration (ng/μL)			
Spectrophotometry (1)	103.1	N/A	N/A	N/A
Spectrophotometry (2)	97.75	203.5	108.25	108.25
QuantiBlot®	100	200	N/A	N/A

N/A, data not available.
*Concentration at 200 ng/μL as specified on product inserts.

Standard Comparisons

As differences were observed between the QF and QP methods, as well as with the QF kit using a Roche DNA source (data not shown), further studies of the QF standard were performed. Table 3 presents the variability observed between the different QF standard lot numbers when two alternate quantification methods were used, particularly for lot A, originally used in our validation studies, which produced an average estimate of 100 ng/μL and not 200 ng/μL as specified by the manufacturer.

When QF standard lot A was used to generate a standard curve in a real-time PCR experiment, all other standards were quantified to have an apparently greater amount of DNA present than as specified by their manufacturer's product inserts (Fig. 2). The QF standards tested within the one run were believed to be at 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, 0.023 ng/μL, although as shown in Fig. 2, this did not always appear to be the case. Similar to the spectrophotometry and QB results, QF standard lot B contained approximately twice the amount of DNA compared to QF standard lot A at all concentrations tested. Both the Roche and Promega standards exceeded the amount of DNA present within QF standard lot A, where for the Roche sample, it was ~2- to 3.5-fold greater at all concentrations tested, and for the Promega sample, it was approximately three- to four-fold greater at all concentrations tested (Fig. 2).

This study did not test for DNA concentration variability between different Promega K562 or Roche DNA lot numbers due to the purchase of only one of these samples which was sufficient for our initial validation work. The Promega and Roche standards were also not tested by other quantification means, so their concentrations may or may not vary to that specified on their product inserts. One can therefore not eliminate the possibility that concentration differences may also occur with these and other DNA standards.

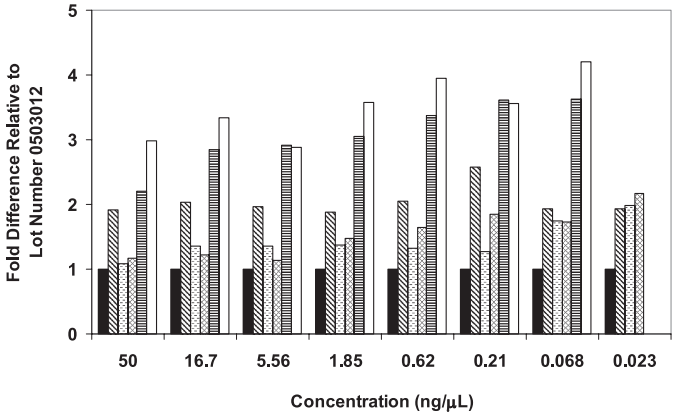

FIG. 2—*Concentration fold-differences between the different QF standards and the DNA samples from Roche and Promega when QF standard lot A was used to generate a standard curve. QF lot A, black bar; QF lot B, downward diagonal bar; QF lot C, dashed horizontal bar; QF lot D, outlined diamond bar; Roche, horizontal bar; Promega, white bar. Each bar represents the average result of two replicates.*

Given the above, we advise that checks for DNA concentration integrity (irrespective of the supplier) of all separate aliquots of DNA standards to be used with the QF kit are performed before being used for casework DNA estimations.

It must also be noted that no "gold" DNA standard currently exists. At present, the National Institute of Standards and Technology are developing a stable, homogeneous, and properly characterized DNA quantitation reference material which will be called the NIST Standard Reference Material (SRM) 2372 DNA Quantitation Standard (3). This standard will minimize variability and help to accurately determine small concentrations of DNA.

ABI have also expressed their concern about the variability observed between the Quantifiler™ kits and are conducting further tests (15). They have stated that: "The real-time PCR assay specification targets a specific Ct value with an allowable variance of 0.32 Ct" (15); "This specification results in a maximum allowable difference of 0.64 Ct between two qualifying lots of DNA standard with a resulting possible 1.56-fold concentration difference" (15); and "Laboratories obtaining two different lots of DNA standard may assess the differences between the starting DNA concentrations by evaluating the ΔCt value" (15).

The Effectiveness of the IPC System

Casework samples with an IPC result that indicated total inhibition (undetermined IPC C_T) were investigated further to establish how such samples should be dealt with in future processes. From the total of 417 samples screened with the QF kit, 40 produced undetermined IPC results. Of these, 29 also produced an undetermined hTERT result, indicating that both assays were inhibited and no quantification result could be obtained. All 40 samples with undetermined IPC C_T values had previously been screened with the QB method where all but one sample produced a quantifiable result. Of these 39 samples quantified with the QB method (which provides no indication of the presence of inhibition), 16 had produced full profiles after Profiler Plus™ analysis in prior casework.

For those samples which presented both undetermined hTERT and IPC C_T results (n = 29), 10 had previously given QB estimations and subsequent Profiler Plus™ profiles which suggests that QF PCR amplifications are more sensitive to inhibition than Profiler Plus™ PCR amplifications. Also, approximately 41% of these samples that appeared to be totally inhibited when amplified using the QF kit actually produced full STR data when amplified using the Profiler Plus™ kit. Therefore, with the inhibitors tested here, it can be inferred that Profiler Plus™ PCR amplifications are more robust than QF PCR amplifications.

If the IPC control is above C_T 28 (which the VPFSC utilizes to indicate inhibition), it could be argued that the sample should be purified before proceeding to STR analysis, although one could opt to proceed directly to STR analysis because a substantial amount of DNA may be lost during a purification process (16). On the other hand, if the IPC control is not working correctly in a test sample and the sample proceeds to STR analysis without a prior purification and then subsequently fails, this has laboratory time and cost consequences. These issues should be considered when developing laboratory procedures.

TABLE 4—*The IPC C_T and STR results of 25 casework samples, originally with undetermined IPC C_T values, after a purification using one of four methods. QB concentration results from prior casework have also been included.*

Purification Method	Sample Type	Original QB Result (ng/μL)	IPC C_T	Profiler Plus™ Result
QIAquick®	Seminal male	20	24.8	Full
	Trace	0.06*	35.99	PN
	Seminal female	0.06	25.03	Full
	Seminal female	0.06	26.04	Partial
	Trace	0.03*	24.84	PN†
	Trace	0.03	24.79	Partial
Organic	Trace	0.06	UD	PN
	Trace	0.03*	26.86	No Profile
	Trace	0.03*	UD	PN
	Trace	0.06	UD	PN
	Trace	0.06*	UD	PN
20% Chelex®	Trace	0.03	UD	PN
	Blood	0.03	UD	PN
	Blood	0.03	UD	PN
	Trace	1	UD	PN
	Trace	0.03*	UD	PN
	Cig. Butt	UD*	UD	PN
	Blood	0.03	UD	PN
	Seminal Male	0.125	UD	PN
TE-Centricon®	Blood	0.06	34.8	No Profile
	Trace	0.03*	UD	PN
	Trace	0.03	29.21	PN†
	Trace	0.03*	24.89	PN†
	Trace	0.125	UD	PN
	Seminal Male	0.06	UD	PN

UD, undetermined; PN, Profiler Plus™ not performed.
*Dyes present.
†Insufficient amount of DNA available for further testing.

The Effectiveness of Different Genomic DNA Purification Methods

Studies were performed to determine if four purification methods (QIAquick® [11], organic [5,6], 20% Chelex [7], and TE-Centricon® [17]) could remove inhibitors which affect the IPC assay. Twenty-five of the 40 samples with undetermined IPC C_T values were further purified and re-quantified with the QF kit (DNA standard lot A) and the IPC C_T values checked for inhibition. Table 4 shows that the QIAquick® method for removing inhibitors was the most successful of the methods tested with all samples producing IPC C_T results after the purification. Only one sample indicated that inhibition was still present (IPC C_T>28). For all samples in which enough DNA was available for STR analysis using Profiler Plus™, either a full or partial profile was obtained.

The QIAquick® method was later used to purify all samples that were unsuccessful with the organic, 20% Chelex®, TE-Centricon® as well as with the original QIAquick® purification methods and in all cases, satisfactory IPC C_T results between 24 and 28 were obtained (data not presented). The effectiveness of the QIAquick® method is likely due to the fact that this method involves the binding of DNA to a silica membrane and the washing away of all other impurities.

The Effects of Dyes on the IPC System

The presence of dyes on the IPC system was also investigated in our validation because as part of routine casework, a decision is made after an extraction whether a sample requires purification based upon visual confirmation of the presence of dyes which can act as potential PCR inhibitors (18). Aliquots (5 μL) were taken from 26 casework samples (separate to the 365 used in the previous studies) and these samples were quantified using the QF kit (DNA standard lot A), prior to any further purification steps. Four of the 26 samples displayed inhibition (IPC C_T>28 or undetermined). These findings suggest that a purification step may not be necessary prior to a QF reaction, even if the samples appear to contain dyes.

Effects of High Template Concentrations on the IPC

It has been stated that for samples "with extremely high concentrations of human genomic DNA (>10 ng/μL), competition between the human-specific and IPC PCR reactions appears to suppress IPC amplification for that sample" (9). However, two of the standards used to generate the standard curve described in the QF manual are >10 ng/μL (i.e., 16.7 and 50 ng/μL). So if these concentrations were to suppress the IPC, should they be included for quantification purposes?

This study was performed to establish at which DNA concentration IPC inhibition actually occurs due to the presence of a high template concentration. Twenty-five samples with high DNA concentrations (50–1250 ng/μL) estimated by the QB method were quantified again using the QP test. The IPC C_T versus the concentration results are presented in Fig. 3. For all samples, three DNA concentrations were tested (neat, 1/10, and 1/100) where dilution factors were also taken into consideration to present the final DNA concentrations shown in Fig. 3. Nine of the 25 samples screened using the QP test did not indicate the presence of a high starting DNA concentration, as opposed to the original QB results, possibly due to the significant amount of variation incorporated during subjective analyst interpretation with the QB method. Two of the nine samples below 50 ng/μL also appeared to be inhibited (undetermined IPC C_T values).

Figure 3 shows that as concentration increased with the neat samples, the IPC became increasingly suppressed (C_T value increased). The IPC was also undetermined in four samples at a concentration of ~100 ng/μL or greater. This could have been due to a high template concentration as well as PCR inhibitors. One neat sample also appeared to have an IPC C_T value lower than the expected for unknown reasons. In general, it can be seen that once the samples were diluted 1/10 and 1/100, there was no suppression of the IPC. Despite this IPC C_T difference between many of the neat and diluted samples, the final concentration results

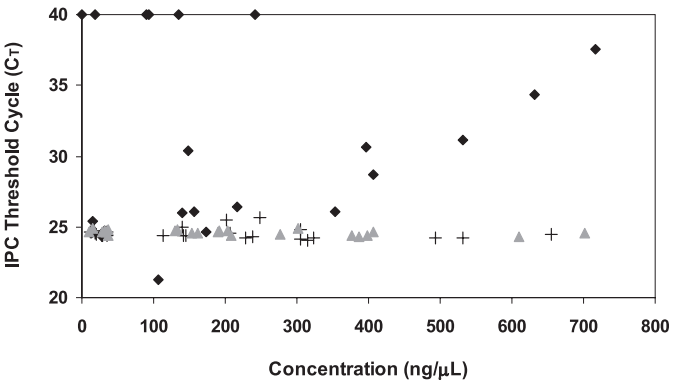

FIG. 3—*Testing of the IPC system in samples with high amounts of starting DNA. Samples with an IPC C_T value of 40 (maximum cycle number used) were specified as undetermined in the 7500 SDS Software (v1.3). The final corrected concentrations obtained (taking into account dilution factors) according to neat (black diamonds), 1/10 (crosses), and 1/100 (grey triangles) preparations have been plotted against the IPC C_T value.*

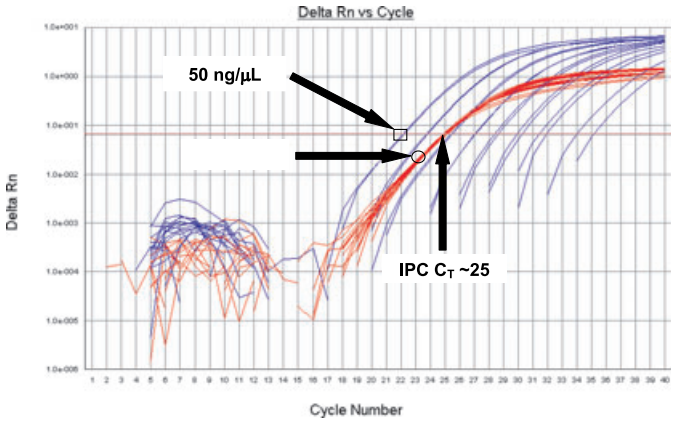

FIG. 4—*Amplification plots of the Promega K562 standard diluted to 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, and 0.023 ng/µL where the IPC assays are circled and indicated whereas the remainder of the amplification plots are for the hTERT assay. The average C_T value for the IPC is also presented as well as the 50 ng/µL standard (boxed).*

(extrapolated to the original neat sample) were similar, which suggests that the hTERT assays had not been affected as might have been expected. Overall, it appeared that the IPC became suppressed when there was >100 ng/µL of starting DNA. An experiment was performed to test the Promega K562 DNA sample at a high DNA concentration (550 ng/µL) and the IPC C_T value it produced in the absence of inhibitors. When run in replicates of four, the average IPC result was between 2 and 3 C_T values higher compared to those samples with a lower DNA concentration (I. Koukoulas, F.E. O'Toole, P.Stringer, R.A.H. van Oorschot, unpublished data).

Figure 4 presents the amplification plots of the Promega K562 standard diluted to 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, and 0.023 ng/µL (in duplicate). These estimations of concentration are based on the supposed concentration of the standard as detailed on the packaging labeling. It can be seen that the Promega standard at 50 ng/µL has a C_T value of ~22 for the hTERT assay. This appears earlier (as does the 16.7 ng/µL standard) than the IPC reaction which has a C_T value of approximately 25 (Fig. 4). Due to the higher concentration of the standard compared to the IPC, it could be expected to suppress the IPC due to competition for reagents. However, this is not the case because the higher concentration standards do not commence to reach the plateau phase until approximately cycle numbers 30–34 (Fig. 4). The plateau phase occurs when PCR reactions are exhausted of one or multiple reagents. Therefore, it appears that there are enough reagents for the IPC PCR reaction to occur when there is a starting sample template concentration of 50 ng/µL, as well as 16.7 ng/µL, for the hTERT reaction. However, it would be sensible to dilute apparently high concentration DNA samples and re-quantitate them to ensure that any unknown potential effects of the high DNA concentration on the quantitation reactions is reduced.

The Effects of Using Different Analysis Settings

The ABI PRISM® 7500 System SDS Software (Version 1.3) has the flexibility to use either automatic or manual analysis settings. In terms of analyzing data, the following three options exist: auto C_T, which automatically converts the baseline setting to auto also (i.e., a manual baseline setting cannot be used with an auto C_T setting); manual C_T with auto baseline; and manual C_T with manual baseline. With the auto baseline setting, each sample or amplification plot is given a different baseline range, whereas with the manual baseline setting, the identical baseline start and end is given to all amplification plots. One can either manually set a baseline start and end or use the default settings of 3 for the baseline start and 15 for the baseline end. If samples have a C_T value less than 15, which would likely be rare in forensic casework, the baseline end value will then need to be lowered.

Analysis settings were studied for the following reasons: ease of use for the caseworker—choosing the auto C_T setting would be the easiest option for all caseworkers; concerns over the variation that would be generated if each caseworker was required to set independent threshold and baseline values; to compare and to have an understanding of the differences between using the one baseline range for all samples (manual analysis) versus using different baseline ranges for each sample (automatic analysis). Also, current ABI publications (1,9,19) appear to have validated the manual threshold at 0.2 with an auto baseline and the manual threshold at 0.2 with a manual baseline from 3 to 15 (using the ABI PRISM® 7500 System SDS Software v1.2.3) and not the auto threshold and auto baseline settings for QF (19) despite this option existing within the software. Seven analysis settings were tested in order to generate results as performed by ABI as well as those performed by our group and a combination of the two. All possible combinations of different analysis settings were covered.

The standard deviations of all 76 samples estimated using the seven different analysis settings as well as the %CV values obtained were low i.e., the average standard deviation and %CV was 0.257 and 2.33 respectively (detailed data not presented). The largest %CV obtained was 16.6 (for a sample which gave a mean concentration of 0.00389 from the seven analysis settings), 67% of the samples had a CV less than 2.33%. Despite manual and auto analysis settings producing slightly different C_T results (data not presented), the concentration estimations were observed to be similar as anticipated. The use of different analysis settings can give slightly different C_T data, as a result of setting the threshold lower or higher; however, because any analysis setting change is relative to all samples, the overall concentration result should stay approximately the same. Therefore, any reasonable analysis setting can be used for the generation of concentration data (e.g., a threshold of 0.2 and not 2, to ensure that the threshold is set in the exponential phase of amplification).

It must be noted that when the auto analysis feature is selected (for both the threshold and baseline) it is necessary to check if the settings automatically chosen by the software are correct. This can be performed by selecting each detector separately (each dye layer) and then visually inspecting if the threshold was set in the exponential phase of amplification and that the baseline setting has produced typical amplification plots (similar to those in Fig. 4).

Conclusion

The results presented demonstrated that if the Promega K562 DNA standard (system lot number 206778), and any further lot numbers of the same DNA concentration, are used in conjunction with the QF kit, acceptable peak heights can be achieved in subsequent Profiler Plus™ amplifications of 1 ng of sample DNA, for both homozygote and heterozygote alleles in Profiler Plus™ amplifications as well as potentially other STR systems. This is not the case when QF standard lot A and any other lots with the same DNA concentration are used. These studies have also demonstrated that a two-fold difference in DNA concentration exists between lots A and B of the standard provided in the QF kit, which was supported by spectrophotometry, a QB experiment, and a real-time PCR experiment. Overall, DNA profiling results can be affected by

the DNA standard used. As suggested by ABI, an assessment should be made between two different standard lots by evaluating a ΔC_T value with a maximum allowable difference of ±0.32 C_T (15). We also recommend that any new standard be checked against a previously verified standard before the standard is used for casework sample DNA estimations.

It must be noted that it would be inefficient for a laboratory to have to adjust the total amount of DNA in STR reactions depending on the DNA standard used in a prior quantification. This would lead to different DNA volumes being added depending on the particular quantification test performed in order to obtain desirable STR peak heights after amplification. To prevent this, it would be appropriate to identify DNA quantitation standards with concentrations that differ from manufacturers' product inserts prior to the commencement of PCR amplifications.

Studies on the effectiveness of the IPC demonstrated that Profiler Plus™ reactions appeared to be more robust than QF reactions since Profiler Plus™ profiles resulted from samples that caused inhibited QF reactions. Therefore, the presence of inhibition within samples may not necessarily result in inhibited STR amplifications. The IPC is still useful in that when samples present both undetermined hTERT and IPC results, it would suggest that factors such as PCR inhibition may have masked the presence of DNA.

Of the four purification methods studied, the QIAquick® method was the most successful. Also, 85% of samples that would have required purification based on the visual confirmation of dyes in fact did not present any inhibition within QF reactions. Skipping an unnecessary purification step could save the laboratory time and money. It was also observed that a template DNA concentration greater than 100 ng/μL appears to inhibit the IPC.

When using the ABI PRISM® 7500 System SDS Software (Version 1.3), data can be analyzed using the auto analysis settings for both the threshold and baseline as similar DNA concentration estimations were obtained when using alternate analysis settings. The advantage of the auto analysis option is that it is the easiest to perform and whether correct settings were made can be easily checked.

The use of QF to quantify DNA for down stream processing of forensic casework samples has improved our ability to more accurately determine and predict STR amplification success. It can also estimate a wider range of DNA concentrations, it utilizes less DNA, and requires less user time and effort than other quantification methods.

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