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Technical note

Assessing the performance of quantity and quality metrics using the QIAGEN Investigator® Quantiplex® pro RGQ kit

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ABSTRACT

The Quantiplex® Pro RGQ kit quantifies DNA in a sample, supports the detection of mixtures and assesses the extent of DNA degradation based on relative ratios of amplified autosomal and male markers. Data show no significant difference in the accuracy and sensitivity of quantification between this and the Promega PowerQuant® System, both detecting the lowest amount of DNA tested, 4 pg. Laboratory controlled mixed male:female DNA samples together with mock sexual assault samples were quantified across a range of mixture ratios. Analysis software detected mixed DNA samples across all ratios for both quantification kits. Subsequent STR analysis using the Investigator® 24Plex QS Kit was able to corroborate mixture detection down to 1:25 male:female DNA ratios, past which point mixtures appeared identical to single-source female samples. Analysis software also detected laboratory degraded DNA samples, with data showing a positive trend between the Degradation Index (DI) and length of time of sonication. When used on ancient remains the assay was able to triage samples for further analysis, and STR profiles were concordant with DNA quantification results in all instances. STR analyses of laboratory-controlled sensitivity, mixture, and degradation studies supports the quality metric obtained from quantification. These data support the use of the Quantiplex® Pro RGQ kit for sample screening and quantification in forensic casework and ancient DNA studies.

1. Introduction

In forensic analysis, the quantification of DNA recovered from a crime scene sample is used to inform downstream processes such as STR profiling [1]. Currently, the standard method for quantifying DNA from forensic samples is quantitative PCR (qPCR), which monitors the amplification of specific regions of DNA by fluorophore excitation in real-time [2]. By comparing the resulting fluorescent output to the signal obtained from a series of DNA standards of known concentration, the unknown sample(s) can be accurately quantified [3]. If a high concentration of DNA is recorded, the sample is diluted prior to use in the STR amplification step. If a low concentration of DNA is recorded, DNA can be concentrated down or a larger volume of the sample can be used for STR amplification. By doing so, the DNA samples analysed should match the recommended input concentrations based on the manufacturer's instructions for the STR kit. Following these criteria, an informative STR profile of good quality is generated. Early examples of commercial qPCR kits include Quantifiler™ Duo (Applied Biosystems) [4] and Plexor® HY (PROMEGA) [5], both of which quantify total autosomal human and male (Y-specific) DNA to allow identification of sex, putative identification of male:female mixtures and to facilitate the quantification of male component for Y-STR testing. The use of such qPCR kits forms an important part of the forensic workflow although they do not always successfully detect degraded autosomal DNA samples and/or mixed male:female samples.

The detection of degraded autosomal DNA can be problematic at the quantification step as the fragmentation of DNA causes a shift in the ratio of high molecular weight (HMW) DNA to low molecular weight (LMW) DNA [6] due to preferential degradation of larger DNA fragments. As degradation increases the probability that the sample contains an adequate amount of DNA of sufficient length for STR profiling decreases [7]. Furthermore, quantification tends to underestimate the true amount of DNA present [8] and simply increasing the amount of this fragmented DNA in the STR amplification step may not result in an STR profile. By identifying that the sample contains degraded DNA at the quantification stage, forensic providers can select the most

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appropriate course of action, i.e. traditional STR profiling vs mini-STR profiling or massively parallel sequencing [9]. The detection of mixed male:female samples can also be complicated at the quantification step. If the sample is of known female origin but shows male-specific DNA amplification, it can be inferred that there is a male contributor present in the female sample. However, mixture detection becomes more difficult if the sex of the sample donor is unknown, as under these conditions a mixed male:female sample can look similar to a single source male sample with imbalanced amplification of autosomal and male-specific markers, an outcome observed when analysing low template DNA samples due to stochastic amplification. By identifying that the sample contains mixed male:female DNA at the quantification stage, forensic providers can select the most appropriate course of action, i.e. autosomal STR profiling vs Y-STR profiling. To support such decision making, commercial qPCR kits such as the Quantifiler™ Trio (Applied Biosystems) [10], PowerQuant® (PROMEGA) [11], and Quantiplex® Pro (QIAGEN) [12] have been developed that can now detect degraded autosomal DNA and/or mixed male:female samples [13].

One question that these current qPCR kits do not answer is whether the male component in a mixed male:female sample is degraded, instead giving a single autosomal measure of degradation. It becomes important to understand the amount of degradation in each component as such samples are common in sexual assault casework, where swabs from a female victim may be taken weeks after the assault or be subject to lengthy storage time prior to analysis [14]. To support analysis, QIAGEN have recently developed the Quantiplex® Pro RGQ kit [15] which assesses the quantity and quality of DNA through the amplification of both long and short autosomal and male DNA fragments. In this paper, we assess the performance of the Qiagen Quantiplex® Pro RGQ kit by running a series of small validation studies following SWGDAM guidelines [16]. Studies assessing accuracy, limit of detection, and capacity to detect sample mixtures and degraded DNA across laboratory-controlled DNA samples were compared against the Promega PowerQuant® System [11]. In addition, the ability of the Quantiplex® Pro RGQ kit to detect male:female mixtures from sexual assault casework and degraded DNA extracted from ancient bone samples was assessed. Representative samples quantified with the Quantiplex® Pro RGQ kit were subject to STR analysis using the Qiagen Investigator® 24plex QS Kit [17] to verify quantification results.

2. Materials and methods

Four key validation parameters of the Quantiplex® Pro RGQ kit were assessed: standard curve reproducibility, sensitivity, mixture analysis, and degradation analysis. All of these parameters were compared against the PowerQuant® System as a commercial equivalent using control DNA of known concentrations under laboratory-controlled conditions. A further two studies were performed using only the Quantiplex® Pro RGQ kit that used casework samples for mixture and degradation analysis. All DNA samples were either sourced commercially or obtained from volunteers with ethical approval.

2.1. Quantification accuracy studies

The first set of studies assessed standard curve reproducibility and sensitivity. **Study one** assessed the manufacturers' claim that either a four or seven point standard curve would provide reliable quantification for both quantification kits. In this study, three independent replicate standards curves were generated for each kit using four standards (Quantiplex standards – 50 ng/μL, 1.8519 ng/μL, 0.0686 ng/μL, and 0.0025 ng/μL. PowerQuant standards – 50 ng/μL, 2 ng/μL, 0.08 ng/μL, 0.0032 ng/μL) and seven standards (Quantiplex standards – 50 ng/μL, 10 ng/μL, 2 ng/μL, 0.4 ng/μL, 0.08 ng/μL, 0.016 ng/μL, and 0.0032 ng/μL. PowerQuant standards – 50 ng/μL, 10 ng/μL, 2 ng/μL, 0.4 ng/μL, 0.08 ng/μL, 0.016 ng/μL, and 0.0032 ng/μL). Regression analysis for each data set were performed, both within and between kits and results compared between four and seven standards. **Study two** assessed the quantification sensitivity of autosomal and Y-target DNA by the Quantiplex® Pro RGQ, and PowerQuant® using commercially sourced female and male DNA of known concentration (PROMEGA Corporation; G1471 and G1521, respectively). Six independent replicates at each input concentrations (250, 50, 10, and 2 pg/μL) were quantified. Box-and-whisker plots were generated by comparing observed quantification values against the expected quantification value.

2.2. Laboratory controlled mixture and degradation studies

This second set of studies used previously quantified volunteer DNA to prepare a series of male:female mixtures and male degraded samples from commercially-sourced DNA (PROMEGA Corporation G1521). **Study Three** assessed the ability of Quantiplex® Pro RGQ and PowerQuant® kits to detect male DNA contribution to a majority female DNA sample. Using genomic male and female DNA at 0.5 ng/μL concentrations, male:female mixtures were prepared at 1:1, 1:25, 1:50 ratios. Unmixed male and female samples were also run at 0.5 ng/μL. Three independent replicates at each mixture ratio were quantified with both kits. Ratios of male:female DNA quantification values were calculated and compared to the manufacturers default mixture detection metrics (Quantiplex® Pro RGQ 1:2 Y:autosomal mixture flag; PowerQuant® 1:2 Y:autosomal mixture flag). **Study Four** used DNA degraded under laboratory controlled conditions. Male DNA was diluted to 5 ng/μL in amplification grade water in a total volume of 120 μL. This DNA sample was sonicated at 35 kHz for 30 min using a Bandelin Sonorex RK 31 sonicator. Twelve microlitres of DNA was removed from the sample prior to sonication (t = 0) and again at 1, 10 and 30 min into the sonication procedure. Degradation of samples was confirmed by running 5 μL of sonicated DNA on a 2% TBE ethidium bromide stained agarose gel. Degraded DNA samples were diluted to 0.25 ng/μL and 25 pg/μL and quantified using both kits. Ratios of HMW and LMW DNA were calculated and compared against the manufacturers default Degradation Index (DI) metrics (Quantiplex® Pro RGQ 1:10 HMW:LMW degradation flag; PowerQuant® 1:2 HMW:LMW degradation flag).

2.3. Mock casework mixture and degradation studies

Two additional studies were carried out using only the Quantiplex® Pro RGQ kit. These studies used DNA extracted from mock casework samples as an assessment of how the kit performs when using DNA collected in the field. **Study Five** prepared mixed, mock sexual assault samples by spiking male volunteer seminal fluid onto female volunteer buccal swabs. Seminal samples and buccal swabs were collected following standard methods and were stored in the fridge overnight prior to spiking. Three independent replicate samples were prepared for each dilution series to represent a range of mixtures (neat seminal fluid at 50 μL, 5 μL, 1 μL, 0.1 μL spiked onto a female buccal swab). After spiking, the swabs were subject to RSID testing [18] and DNA extraction was performed on the remaining solution using the DNeasy Blood and Tissue kit (QIAGEN) [19] before undergoing quantification to assess the kits ability to detect mixtures. **Study Six** assessed the utility of the DI with ancient DNA (i.e. naturally degraded) samples obtained from the Late Medieval (13th-15th century CE) archaeological site of Poulton, located approximately eight km south of Chester (Cheshire, UK) [20,21]. DNA extractions on nine samples were performed in dedicated ancient DNA (aDNA) facilities at LJMU following previously published protocols [22,23]. The cementum-rich root tip of the teeth was sampled as it has been shown to preserve DNA better than most types of bone [24–26]. Teeth were first cleaned with 1% sodium hypochlorite and ddH_2O, before using a multi-tool drill at the lowest possible rpm (ca 100 rpm) to obtain ~75 mg tooth powder/sample. The work surface (a dead-air fume cabinet) was thoroughly cleaned after preparing each sample with one extraction blank included per seven

human samples.

2.4. Quantification and STR analysis setup

All thermal cycling was carried out using a Rotor Gene Q 5Plex HRM Instrument running Q-Rex Software v1.0 at 20 μL standard reaction size. Reaction mixtures for both Quantiplex® Pro RGQ and PowerQuant® kits were assembled according to manufacturer's specifications and contained 2 μL of either control DNA standard, sample DNA, or amplification grade water as a negative control. Samples processed with the Quantiplex® Pro RGQ kit used the manufacturers recommended cycling and fluorescence acquisition settings as specified in the technical manual and analysed according to the manufacturers guidelines [27]. The PowerQuant® system was run using a custom template file provided by the manufacturer (Promga Pers. Comm.) with post-qPCR, raw sample data analysed using the Q-Rex Absolute Quantification HID plug-in. PCR threshold values and threshold start cycles were set according to manufacturer's specifications. After analysis, all kit data were exported from the Q-Rex software into Microsoft Excel to prepare graphs and calculate mixture/degradation ratios.

STR profiling was performed using the Investigator® 24plex QS kit [17] at 10 μL total PCR reaction volume. DNA from representative samples of each study were added to each PCR reaction at the concentrations specified in the methods above. Thermal cycling was performed according to the manufactures guidelines on a T100™ Thermal Cycler (BIO-RAD). Capillary electrophoresis was performed using a SeqStudio Genetic Analyser (Applied Biosystems). PCR samples were prepared for analysis by mixing 1 μL of amplified DNA (diluted 1:10 with DNA-grade water) with 12 μL of a formamide and 0.5 μL DNA size standard 24plex (BTO) mixture. Default instrument and quality control protocol parameters for fragment analysis were used as recommended by the Investigator® 24plex QS kit handbook [28] with a 7 s injection time. Results were analysed in GeneMapper 6 software [29] with allele calls generated automatically by a pre-optimised Investigator 24plex QS allele bin panel. After data review, some samples were re-run with a 30 s injection time. Peak height data and allele calls for each locus was tabulated and exported for analysis in Microsoft Excel.

3. Results & discussion

3.1. Quantification accuracy results

3.1.1. Study one

All standard curve data passed the acceptance criteria set by the manufacturers [27] (Table 1). The Quantiplex® Pro RGQ and PowerQuant® kits recommends the use of four DNA standards run in duplicate to generate a standard curve for quantification. This is fewer than that recommended by other kits [30,31] and may have a negative impact on the linearity of the standard curve if one of the standards is incorrectly prepared, causing a high leverage data point. Regression analysis using Minitab 19.2 [32] showed no significant difference in the y-intercept for the autosomal marker ($P = 0.949$), the male marker ($P = 0.744$), and the autosomal degradation marker ($P = 0.999$) between each kit when seven standards were used, suggesting that both kits are capable of accurate quantification of both autosomal and male components. Importantly, there were no significant differences in the y-intercept for the autosomal marker ($P = 0.961$), the male marker ($P = 0.983$), the autosomal degradation marker ($P = 0.824$) and the male degradation marker ($P = 0.9049$) for the Quantiplex® Pro RGQ kit when either four or seven standards were used, suggesting that four standards can be used to generate a standard curve without reducing data quality.

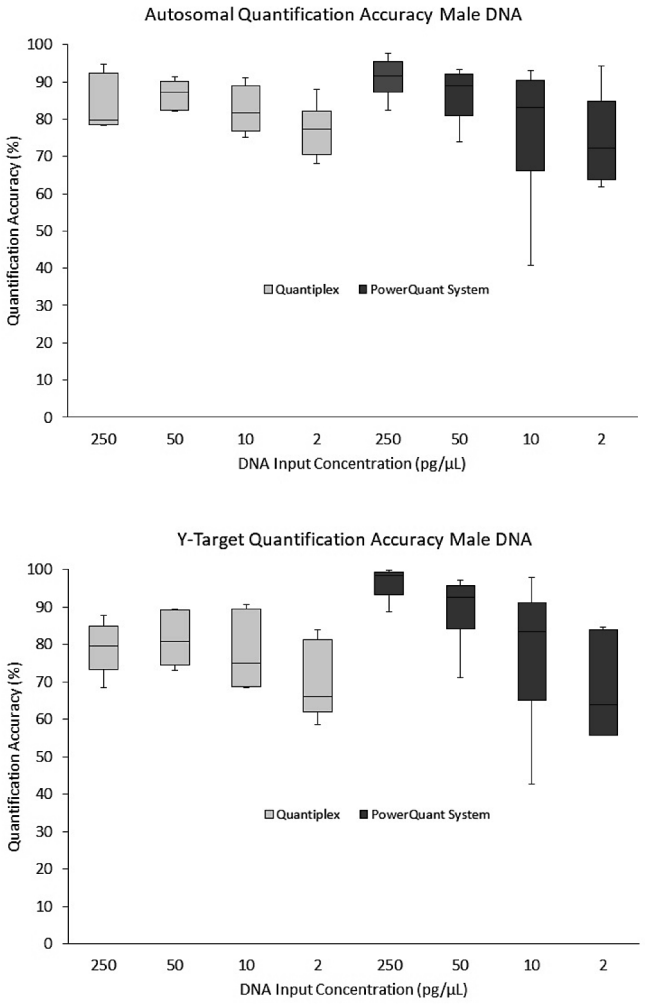

Fig. 1. A comparison of the accuracy of DNA quantification values against decreasing DNA input amounts between the QIAGEN Quanitplex® Pro RGQ and the PowerQuant® kits for autosomal DNA and male marker DNA. n = six for each concentration.

Table 1
Amplicon size and standard curve metrics for Quantiplex® Pro RGQ and PowerQuant® kits.

Quantification Kit	Marker and Amplicon Size (bp)	Repeatability with Four Standards (Mean ± 1SD)			Repeatability with Seven Standards (Mean ± 1SD)		
		Slope	Intercept	R^2	Slope	Intercept	R^2
Qiagen Quantiplex Pro RGQ	Autosomal (91 bp)	−3.25 ± 0.05	22.37 ± 0.20	0.99 ± 0.00	−3.32 ± 0.07	22.90 ± 0.07	0.99 ± 0.00
	Male (81 bp)	−3.28 ± 0.09	22.06 ± 0.32	0.99 ± 0.00	−3.32 ± 0.04	22.30 ± 0.10	0.99 ± 0.00
	Auto Deg. (353 bp)	−3.30 ± -0.17	21.65 ± 0.39	0.99 ± 0.00	−3.24 ± 0.13	22.97 ± 0.34	0.99 ± 0.00
	Male Deg. (359 bp)	−3.37 ± 0.07	21.17 ± 0.67	0.99 ± 0.00	−3.42 ± 0.13	22.05 ± 0.16	0.99 ± 0.00
Promega PowerQuant System	Autosomal (84 bp)	−3.31 ± 0.09	21.88 ± 0.03	0.99 ± 0.00	−3.31 ± 0.06	21.87 ± 0.06	0.99 ± 0.00
	Male (81 & 136 bp)	−3.03 ± 0.10	22.75 ± 0.08	0.99 ± 0.00	−3.06 ± 0.01	22.80 ± 0.06	0.99 ± 0.00
	Auto Deg. (294 bp)	−3.25 ± 0.07	22.74 + 0.22	0.99 ± 0.00	−3.26 ± 0.01	22.50 ± 0.34	0.99 ± 0.00

3.1.2. Study two

Quantification accuracy was assessed by calculating the difference between the observed and expected quantification values across four DNA input levels (Fig. 1). Both the PowerQuant® and Quantiplex® Pro RGQ kits provide autosomal quantification values close to the expected concentration of the standard. Compared to the PowerQuant® System, the Quantiplex® Pro RGQ kit showed no significant difference in its precision in quantifying both autosomal and male DNA at all but one of the DNA input concentrations tested (**F-Test** autosomal DNA; 250 pg/μL $P = 0.2278$, 50 pg/μL $P = 0.1044$, 10 pg/μL $P = 0.0144$, 2 pg/μL $P = 0.1021$. **F-Test** male DNA; 250 pg/μL $P = 0.1382$, 50 pg/μL $P = 0.2367$, 10 pg/μL $P = 0.0822$, 2 pg/μL $P = 0.2657$). The significant difference in precision between kits observed when quantifying 10 pg/μL autosomal DNA is attributed to a single outlying data point with the PowerQuant® system that underestimated DNA concentration. As such, our data suggests that both kits perform broadly similar in their ability to accurately quantify a given concentration of DNA template across the ranges tested in this study. Sensitivity data from the Quantiplex® Pro RGQ kit has been reported as low as 0.016 pg input DNA [33], below the reported recommended dynamic range of 50 ng–0.5 pg total input DNA. While the data presented here cannot confirm these detection limits, the data does demonstrate that the Quantiplex® Pro RGQ kit is able to accurately quantify DNA within the reported dynamic range, and performs similarly to the PowerQuant® system as a commercial equivalent qPCR kit. Quantification results were supported by STR data generated from both male and female samples which show a trend for decreasing allele peak heights with DNA input (Fig. 2). All alleles were observed in the 250 pg and 50 pg input samples with instances of allelic dropout observed in 19 of the 24 loci at the 10 pg input level (Amelogenin, D3S1358, D21S11, CSF1PO amplified) and 22 of the 24 loci at the 4 pg input level (Amelogenin amplified). The failure to amplify alleles at such low input concentrations are consistent with the manufacturer's STR validation data [34] again supporting the quantification results of the samples used in this study.

3.2. Laboratory controlled mixture and degradation results

3.2.1. Study three

By quantifying autosomal and sex-linked PCR targets, it is possible to infer the biological sex of whomever deposited a particular forensic sample [3]. It can also support the detection of mixed DNA samples where male DNA may be present in extremely small quantities against a background of female-source DNA. Such samples may be encountered in sexual assault casework where male seminal material may be collected alongside female epithelial cells during examination. Such samples are usually subject to differential extraction to separate the male and female fractions [35] or the entire sample is subject to Y-STR typing [36]. Consequently, the ability to detect and quantify minor male components in a larger female fraction is important to inform further processing. Both kits were able to quantify autosomal and male DNA at all mixtures tested and the autosomal:male (A:Y) ratios were calculated (Fig. 3). All samples returned A:Y ratios within error of the expected ratios. Statistical analysis shows no significant difference in the A:Y ratios observed between kits at any mixture ratio (**T-Test** Male single-source $P = 0.19$, Male:Female 1:1 $P = 0.27$, Male:Female 1:25 $P = 0.29$, Male:Female 1:50 $P = 0.21$), suggesting the kits are roughly equal in performance when detecting mixtures.

STR analysis of laboratory mixed DNA samples show that major:major mixtures were observed across all loci in male:female DNA samples mixed at a 1:1 ratio concurring with the mixture detection result from the quantification kit (Fig. 3). Furthermore, the allele balance between male and female mixtures suggests that the single source samples were mixed in equal ratios prior to STR analysis. Major:Minor mixtures were also observed at the 1:25 ratio, again supporting the mixture detection result provided by the quantification with the minor male contributor easily visible in loci with no neighbouring alleles. Where loci had neighbouring alleles the minor male contributor alleles were indistinguishable from stutter (Fig. 4). No mixed profiles were observed at the 1:50 male:female mixture ratio despite the quantification assay flagging the sample. The results suggest that there is a range in which the Quantiplex Pro RGQ kit can predict the presence of mixed STR profiles but past a certain ratio (1:25 observed in this study) there will be preferential amplification of the major contributor leading to a full, single source STR profile. However, as the mixture ratio reported by the quantification kit is relatively accurate, careful interpretation of the result should allow the forensic analyst to correctly predict the occurrence of mixtures when moving on to STR amplification.

3.2.2. Study four

Several commercial qPCR kits contain multiple primer sets that co-amplify both small and large DNA fragments to reflect the degradation observed in the autosomal target [10–12]. Long DNA fragments are known to be more susceptible to degradation than short fragments and by calculating the ratio of long and short, a degradation index (DI) can be generated that provides a measure of the integrity of DNA. A unique

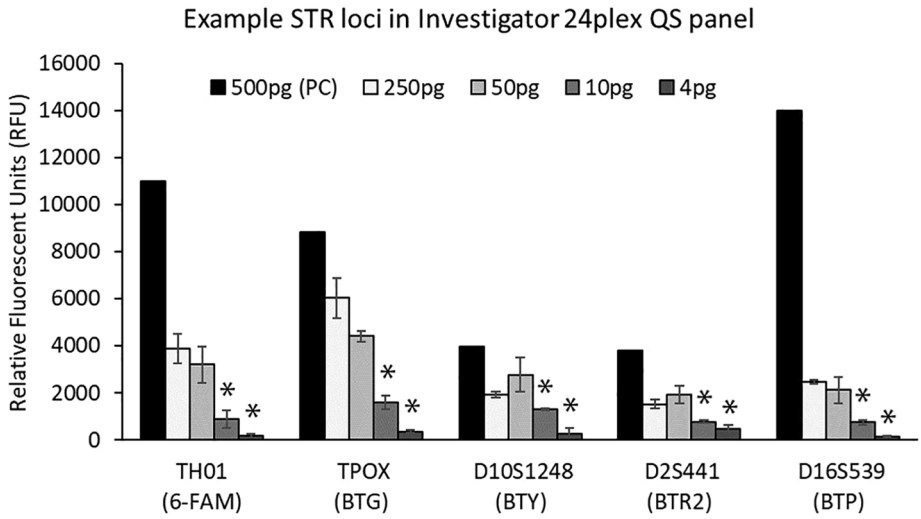

Fig. 2. Allele peak heights for the smallest size range loci for each dye set observed across a DNA dilution series after amplification using Investigator 24plex QS Kit. * Denotes instances of observed allelic dropout.

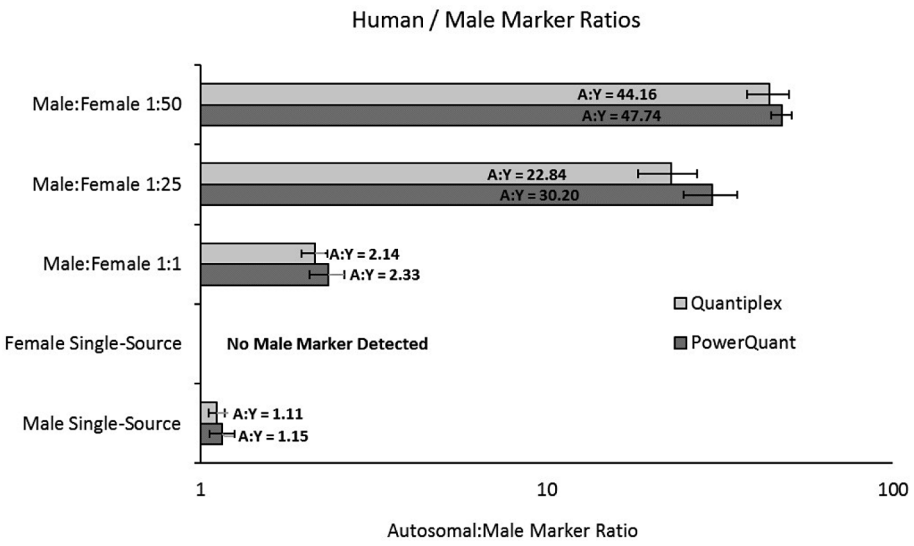

Fig. 3. A comparison of the expected (Y-axis) and observed (X-axis) quantification (ng/μL) ratios of male:female DNA mixtures obtained when using the Quantiplex® Pro RGQ and PowerQuant® kits. n = three at each mixture ratio. Male DNA was not quantified in the female single-source samples. Error bars represent one standard deviation.

feature of the Quantiplex® Pro RGQ kit is the inclusion of multiple primer sets to identify degradation in both autosomal and Y targets. As such, the kit may be of utility in laboratories that routinely analyse degraded DNA samples. Under default analysis settings by proprietary data analysis software, a Degradation Index (DI) value ≥ 10 (Quantiplex Pro RGQ) or ≥ 2 (PowerQuant®) is automatically identified as possibly degraded. The data generated from the laboratory-controlled degradation study for both Quantiplex® Pro RGQ and PowerQuant® kits show that degradation indices increase with the duration of sonication (Fig. 5). The sonicated samples displayed consistently higher DI values for the male target using the Quantiplex® Pro RGQ kit, which would infer that the male target is likely to be detected by the software as being 'degraded' before the autosomal marker. One explanation for the observed differences in detection is the relative size differences of the autosomal and male targets (Table 1) which show an amplified fragment length of 353 bp for the autosomal degradation marker and 359 bp for the male degradation marker. Size variation has been cited as a possible reason for observed differences in degradation indices

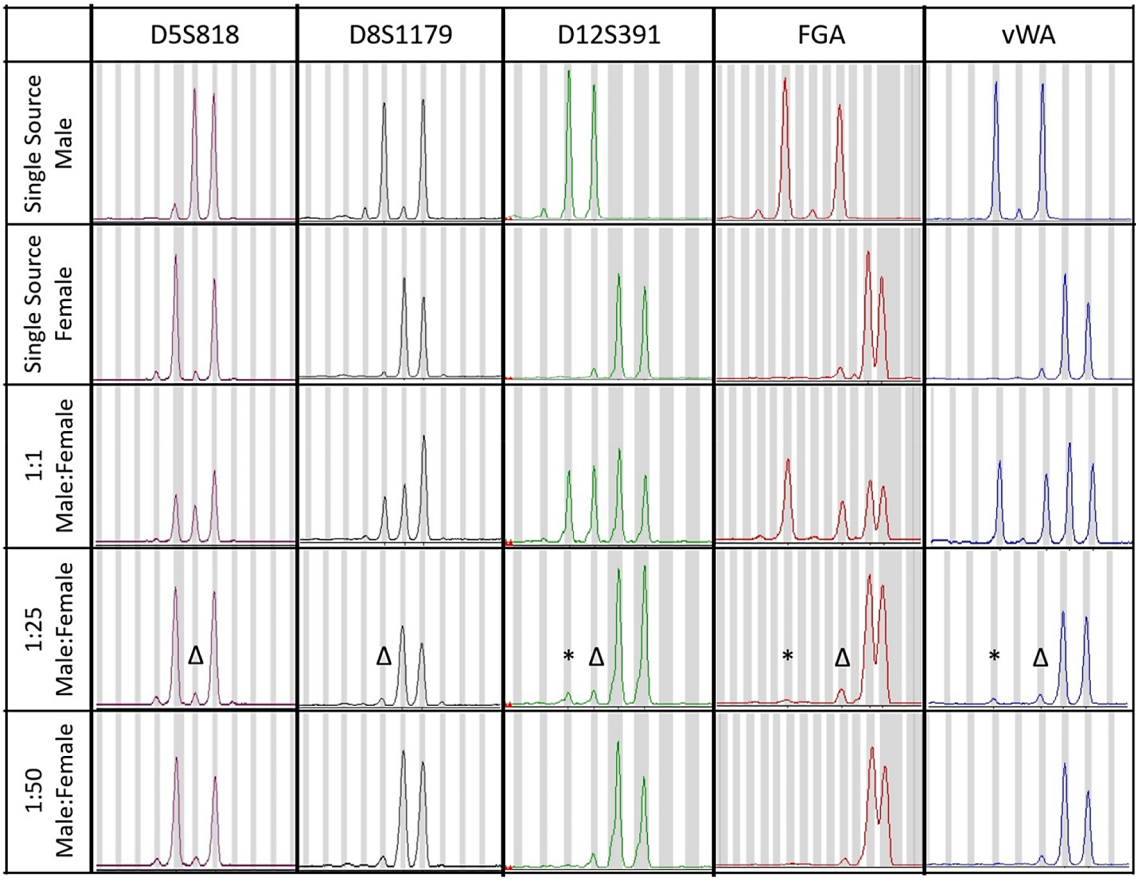

Fig. 4. Electropherogram of five representative loci amplified from various mixed DNA samples using the Investigator 24plex QS Kit. * Denotes instances of minor contributor alleles in 1:25 mixtures; Δ denotes instances where minor contributor alleles cannot be differentiated from allelic stutter.

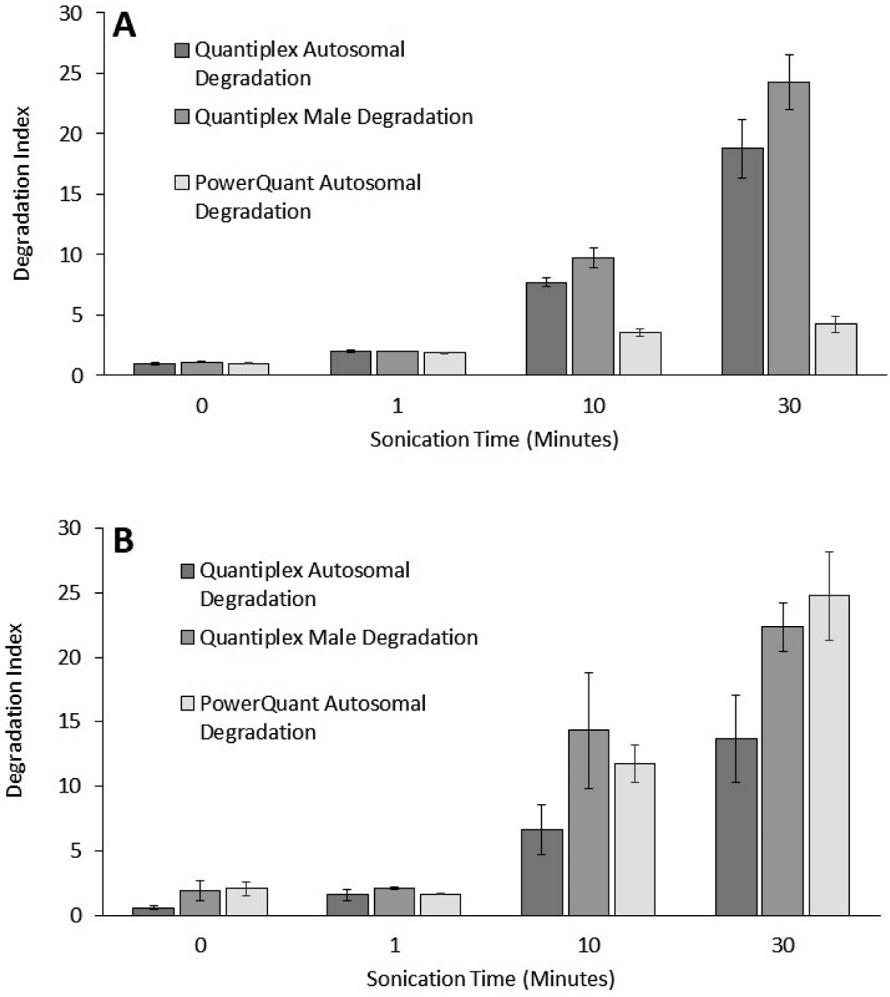

Fig. 5. Degradation indices obtained from a time-course of sonicated male DNA run with QIAGEN Quantiplex® Pro RGQ Kit and PowerQuant® System. A = Degradation indices of sonicated male DNA quantified using both kits at 250 pg/μL input. B = Degradation indices of sonicated male DNA quantified using both kits at 25 pg/μL input. n = six at each time point. Error bars represent one standard deviation.

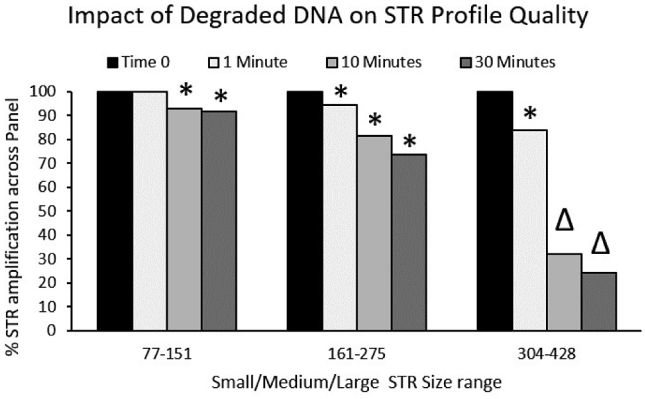

Fig. 6. Amplification success (%) for sonicated DNA at various time points. * indicates instances where allelic dropout was observed; Δ indicates where locus dropout was observed.

between kits [13] so may also explain the slight differences observed here, although the 6 bp size difference seems unlikely to account for such large differences in DI. Another possible explanation may be that the primer used to amplify the male degradation marker may be less efficient at amplification in the assay, consistently leading to fewer copies of the large target being amplified and a bias in the relative ratios. However, without the primer sequences being published this cannot be verified.

At both 250 pg/μL and 25 pg/μL of DNA template, 10 min of sonication was enough to flag the male marker as potentially degraded with the Quantiplex® Pro RGQ kit (Average DIs = 9.75 and 14.32 respectively) whereas the autosomal markers remained below the degradation detection threshold. All samples quantified by the Quantiplex® Pro RGQ were flagged by the QIAGEN Data Handling Tool as possibly degraded following 30 min of sonication. The PowerQuant® technical manual recommends flagging [Auto]/[Degradation] ratios of ≥ 2:1 as degraded, which is lower than the 10:1 ratio used by the QIAGEN Data Handling Tool. At 250 pg/μL DNA input, autosomal DNA was flagged as degraded using PowerQuant® settings at 10 and 30 min of sonication, but would not have been flagged by the QIAGEN Data Handling Tool (Average DIs = 3.53 and 4.23 respectively). This discrepancy requires careful consideration by the forensic analyst using these kits to avoid false positive and negative flagging of samples for further processing and may require in-house optimisation based on equipment and sample type. When using 25 pg/μL of template DNA, degradation indices using the PowerQuant® kit were significantly greater at 10 and 30 min compared to using 250 pg/μL input (**T-Test** $P = 0.006$ and $P = 0.03$ respectively). This suggests that smaller quantities of template are more prone to degradation, and that this information will be captured during analysis. The range of autosomal DI values reported in Fig. 3 are markedly lower than the average

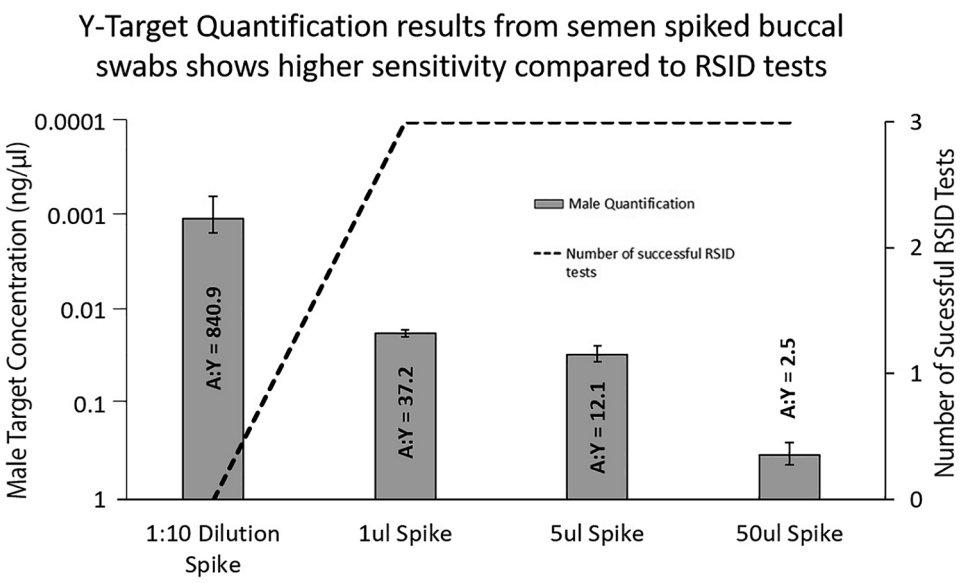

Fig. 7. Detection of male material from spiked swabs at four different semen volumes. Black dashed line represents number of positive RSID tests detecting semenogelin (right axis). Grey bars represent concentration (ng/µL) of male DNA obtained presented as log scale (left axis). A:Y = average ratio of Autosomal to Y target calculated after DNA quantification. Data suggest that the qPCR approach is more sensitive than the RSID test. n = three at each semen volume. Error bars represent one standard deviation.

Table 2
Sample information relating to the sources of ancient DNA processed with the Quantiplex® Pro RGQ kit.

Sample ID	Skeleton ID	Type	Year Excavated	Sex Based on Osteological Identification	Sex Based on DNA Quantification	Sex Based on STR Profiling	
						Amelogenin	DYS391
ASM8	847	Pre-molar	2016	Female	Male	Male (XY)	Male
ASM10	854	Molar	2016	Female	Female	No Call	No Call
ASM11	823	Incisor	2015	Not defined	No DNA	No Call	No Call
ASM12	856	Pre-molar	2016	Not defined	Female	Female (X Call)	No Call
ASM14	865	Canine	2016	Male	Male	Male (XY)	Male
ASM17	873	Canine	2016	Not defined	Female	Female (X call)	No Call
ASM18	797	Pre-molar	2015	Not defined	Male	Male (Y Allele)	No Call

autosomal DI values reported from previous research [13]. In the study by Holmes, average DI values of ~58, ~75, and ~25 were reported for bone, decomposed tissue and formalin-damaged tissue respectively when using the alternative QIAGEN quantification kit, Quantiplex® Pro. This suggests that the sonication approach used in our study could have been performed over a longer time period to mimic the DI values representative of bone and degraded tissue. This said, the STR profiles obtained from the laboratory degraded samples shows a decay curve with the percentage of amplified loci decreasing with length of sonication and, importantly, size of DNA fragment (Fig. 6). All alleles were observed in un-sonicated samples across all fragment sizes, while sample degraded for 1 min began to show allelic dropout for medium (161–275 base pair) and large (304–428 base pair) fragment sizes. Allelic dropout was also observed in the samples sonicated for 10 min and 30 min with whole loci (SE33, D21S11, D7S820) failing to amplify in the large fragment size range (Fig. 6).

3.3. Mock casework mixture and degradation studies

3.3.1. Study five

The use of immunoassay tests to detect male seminal material is common when processing sexual assault samples that may contain both male and female biological fractions. Data from the male:female spiked swabs show that the RSID test can detect the presence of seminal material when spiked at 50 µL, 5 µL and 1 µL volumes but not at the 0.1 µL (100 nL) volume. This is much less sensitive than previously reported for the RSID test which shows seminal material being detected at the 2.5 nL volume [37]. The difference in reported limit of detection is likely due to experimental differences between this study and others, which often measure sensitivity based on a dilution series derived from a single homogenised solution at a high starting concentration [37,38]. The method employed in the current study is considered a more realistic approach to describe the sensitivity as the amount of seminal material on the swab was varied *before* recovery. After the RSID test, the remaining buffer solution underwent DNA extraction and quantification where the average autosomal:male marker ratios were never less that 2:1 (Fig. 7) meaning the QIAGEN Assay Data Handling Tool flagged all mixtures.

The amplification of the male DNA target at the 0.1 µL semen spike by the Quantiplex® Pro RGQ highlights an instance of non-concordance between the two approaches and suggests that there may be instances where male DNA from semen can be recovered after a negative RSID result (Fig. 7). The amount of male DNA recovered from the 0.1 µL semen spike was quantified at 1.1 pg/µL which is below the limit of detection reported by many Y-STR kits [39,40] so it is debatable as to whether the sample would yield an STR profile. Differences in detection limits between immunoassay and PCR based approaches are common and do not invalidate the use of such tests for sample prioritisation and/or body fluid identification, and further testing on a larger number of samples may be required to understand the extent to which non-concordant results occur.

A mixed STR profile was observed after amplification using the Investigator 24plex QS Kit at the 50 µL semen spike but not at the other spike volumes (data nor shown) despite the quantification software flagging all as possible mixtures. The results are broadly consistent with those observed in the laboratory mixed samples in that over-dilution of the male contributor will lead to non-amplification of male DNA. The lack of a mixed STR profile in the 5 µL spike despite having an A:Y ratio of 12.1 also highlights the stochastic nature of amplification at low male contributor levels since only ~20 pg of male DNA was present in

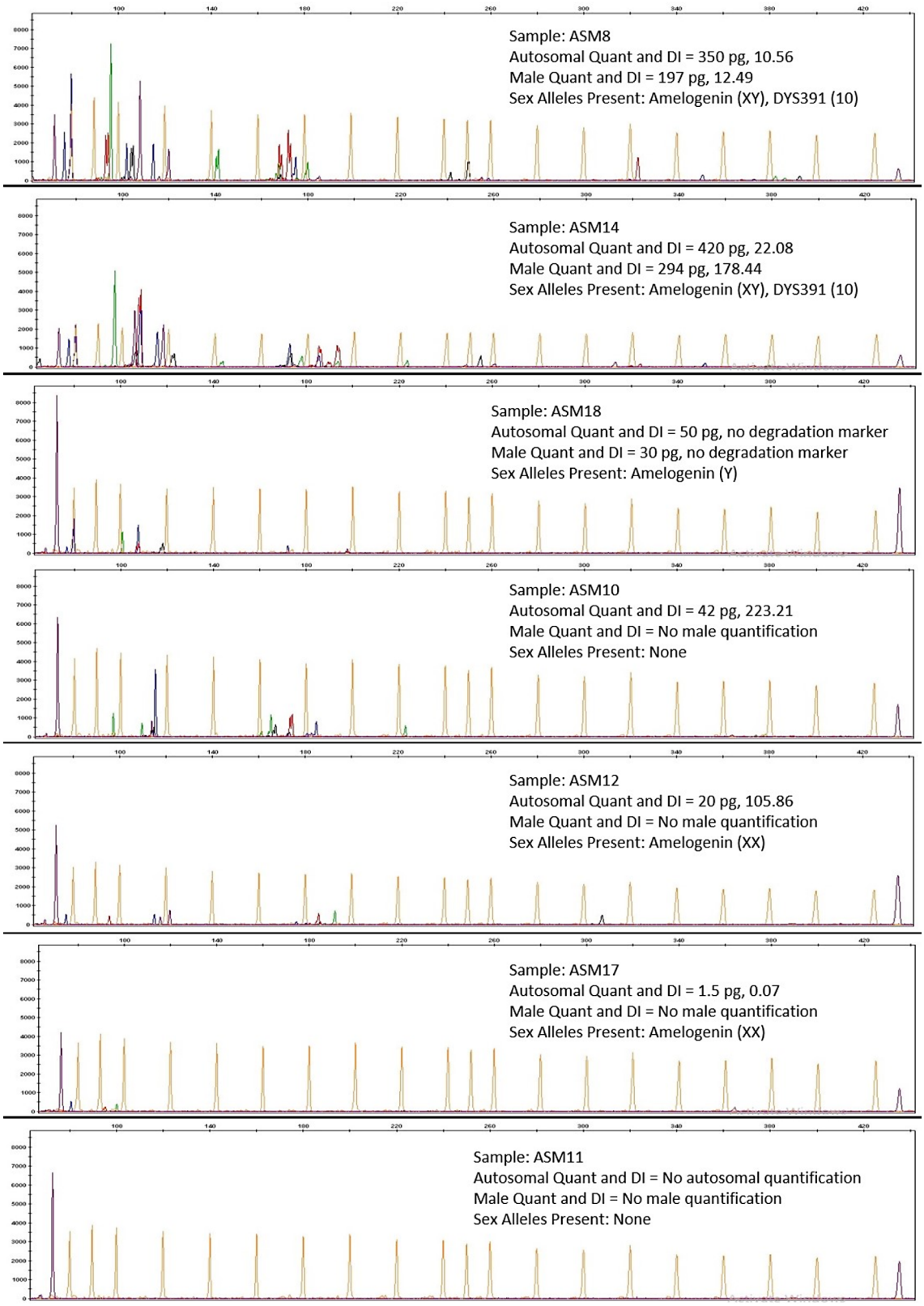

Fig. 8. STR profiles obtained from ancient DNA samples post-PCR. Each profile contains sample information regarding autosomal and male DNA quantification, degradation indices, and presence of sex alleles.

this sample, whereas a major:minor mixture profile was observed in the laboratory controlled samples at a higher mixture ratio 1:25 which also included 20 pg male DNA. Together, the data from the SARC samples suggest that the Quantiplex® Pro RGQ kit is capable of detecting mixed samples across a range of ratios, although the extent to which this is observed in the resulting STR profile will vary as a function of mixture ratio and the amount of male contributor. Consequently, using the mixture metric to accurately predict a mixture is likely possible with

each laboratory needing to set their own thresholds for interpretation using laboratory controlled samples.

3.3.2. Study six

Prior to quantification of ancient DNA samples sourced from teeth, osteological identification of skeletons was performed. The skeleton number, year of excavation, and ostelogical sexing information for each sample is displayed in Table 2. Of the nine ancient DNA samples, DNA was quantified in six of them (no quantification values obtained for ASM7, ASM11, and ASM15). For samples that amplified both autosomal markers, higher autosomal DNA quantities were generally recorded for samples with a lower autosomal DI (ASM8 and ASM14), while samples with low autosomal DNA quantities showed a higher DI (ASM10 and ASM12). According to the analysis software, the DI values for ASM15 and ASM18 were 'not applicable' due to the non-amplification of the autosomal degradation marker, while no DI was given for ASM7 and ASM11 due to the non-amplification of the autosomal marker. The trend for high autosomal DNA concentrations returning low autosomal DI scores was not observed in relation to the male markers, where the sample ASM14 returned both a high male DNA concentration and a high DI score. This result was due to very poor amplification of the male degradation marker (calculated at 0.002 ng/μL). It is considered unlikely that this result is due to contamination during sample processing by staff as any contamination with recently shed, un-degraded DNA would likely amplify both male and male-degradation markers equally. It is considered more likely due to amplification imbalance between the male marker and male degradation marker, either through different amplification efficiencies of primers or stochastic DNA amplification. Indeed, the results from sonicated male DNA samples at 500 pg and 50 pg show that the male DI is often much higher than the autosomal DI, which is exacerbated in low template, degraded samples. The result suggests that it may be common to observe single source male samples, which have a low autosomal DI and a high male DI. Internal control C_t values for all samples were far below the inhibition threshold value, suggesting the results were not due to the presence of PCR inhibitors and none of the samples were flagged as possible mixtures. Results from the QIAGEN Data Handling Tool supports the osteological identification of ASM14 as male as a relatively high quantification of ~300 pg/μL male DNA was obtained. Additionally, no male DNA was detected in sample ASM10, supporting the osteological identification of this sample as female. However, male DNA quantities of ~200 pg/μL were observed in sample ASM8, which had been osteologically identified as female. With regards to samples with unknown osteological data, low (< 30 pg/μL) male DNA was quantified in samples ASM7, ASM11, ASM17, and ASM18. Lastly, no autosomal or male DNA was amplified in sample ASM15, so no information about the sex of this individual was obtained from this experiment. Although still widely used, osteological methods for sex identification are noted for their unreliability, even with specialised computer software analysis tools [41]. Together, the data suggest that the Quantiplex® Pro RGQ kit may be a useful tool to support existing methods for determining sex in ancient remains and for prioritising the best samples for further analysis, although it is considered likely that a consensus result from multiple sample observations may be needed.

All samples underwent STR profiling (Fig. 8). STR amplification was observed across a number of alleles with no evidence of contamination from technical staff or mixtures observed between samples. Decay curves were observed in the two samples that contained the most DNA (ASM8, ASM14) with a reduction in relative peak height of the large STR fragments. Some samples showed the complete non-amplification of large STR fragments (ASM18, ASM10, ASM12) which correlated with a higher DI or non-amplification of the DI marker during quantification. Another sample (ASM17) showed a very low DI value and also a low quantification value with stochastic amplification observed at a few loci across the size range. Sample ASM11 did not provide any quantification data (amount or DI) and also failed to provide an STR profile. Across all samples, sex allele calls agreed with sample sexing based on quantification results, in that Y-specific alleles were only present in samples that quantified male DNA at any level. Together these results show good evidence that the quantification results (DNA concentration and DI) correlate well with the proceeding STR results suggesting that the Quantiplex® Pro RGQ kit can aid in the prioritisation of samples for further processing.

4. Summary

The data obtained from this series of experiments show that the Quantiplex® Pro RGQ kit and PowerQuant® HY kit are similar in terms of quantification accuracy and sensitivity against both autosomal and male DNA targets. The detection of male:female mixtures from both controlled DNA and mock samples suggest that the described kit may be well suited to sexual assault casework where it is necessary to detect low level male DNA in female samples. In addition, the DNA degradation data appear to determine DNA integrity in a reproducible manner while data from ancient DNA samples provides supporting evidence that the Quantiplex® Pro RGQ kit can aid identify samples for further processing. In all instances, STR data was able to corroborate quantification results, although in the case of mixture detection the Quantiplex® Pro RGQ kit provided evidence of minor:major male:female DNA mixtures where STR profiles returned a single-source female DNA profile. This suggests that laboratories may need to validate their own interpretation guidelines to fully utilise the information provided by the quality metrics.

5. Disclaimer

The views expressed in the submitted article are those of the authors listed and not an official position of the institution, funder or collaborative commercial company.

6. Sources of support

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Conflict of interest declaration

The work was performed as part of a Beta Testing trial involving the QIAGEN Quantiplex Pro RGQ kit. Part of this work was presented in part at the 8th Qiagen Investigaor Conference in Prague, 2018.

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