Application Note

Comparison of four commercial qPCR kits for analyzing inhibited and degraded forensic samples

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Introduction

Forensic STR analyses rely on accurate DNA quantification and input of optimal DNA amounts for success and are often complicated by casework samples that are degraded, only available in low amounts or contain inhibitors. Accurately predicting the DNA quality prior to analysis can reduce costs and time for STR amplification (1). Several quantitative real-time PCR (qPCR) kits are commercially available for assessing DNA for degradation and presence of inhibitors, enabling prediction of STR typing success and providing a streamlined and efficient forensic analysis workflow.

These qPCR kits are based on a four-target system: small and large human autosomal targets, male targets and an internal PCR control (IPC) for inhibition. The test sample is amplified, and then a degradation index (DI) is calculated from the resulting small and large target DNA concentrations (2–6). The IPC enables detection of PCR inhibitors that may have been co-extracted with the nucleic acids, for example, hematin from blood, humic acid from soil, calcium from bone, melanin from hair or salt in aqueous solutions (7–9). Although presence of these inhibitors can cause STR results to mimic those from assays with degraded DNA or too little DNA (7–8), adjusting subsequent assays to improve the results requires a different approach. Assessing the quality of forensic samples with respect to concentration, degradation or presence of inhibitors before STR amplification increases first-pass success rates and simplifies assay troubleshooting.

In this study, we tested the performance of four different qPCR kits for analyzing high molecular weight DNA, low template samples, degraded samples and DNA spiked with various inhibitors. The following kits were assessed (table 1): Investigator® Quantiplex® Pro Kit (QIAGEN), Quantifiler® Trio DNA Quantification Kit (Thermo Fisher Scientific), PowerQuant® System (Promega Corporation) and InnoQuant® HY (InnoGenomics Technologies). Of these kits, the Investigator Quantiplex Pro Kit contains the longest large target amplicon (353 bp), which more closely reflects the upper range of loci in STR amplification kits. The Investigator Quantiplex Pro Kit also contains a large IPC amplicon (434 bp), which reflects the Quality Sensor™ in its corresponding STR analysis kits (Investigator 24plex QS and Investigator 24plex GO! Kits) (1).

Table 1. Study design metrics and description of targets for the various kits.

	Large Target	Small Target	Male Target	Internal PCR Control (IPC)	Degradation Index (DI)	Degradation Flags	IPC ΔC_T	Inhibition Flags
Investigator Quantiplex Pro Kit	353 bp*,†	91 bp*	81 bp*	434 bp*	DNA concentration of small target divided by DNA concentration of large target†,‡,§,¶,**	>10*,†	Average of standards IPC C_T minus sample IPC C_T†	>1*,†
Quantifiler Trio DNA Quantification Kit	214 bp‡,§	80 bp‡,§	75 bp‡,§	130 bp‡,§		1–10, >10§	Sample IPC C_T minus average of standards (1–5) IPC C_T‡,§	>2‡,§
PowerQuant System	294 bp§§,¶	84 bp¶	81 and 136 bp¶	435 bp¶		>2¶	A sample's IPC is compared to a standard's C_T (based on nearest quant)¶	>0.3¶
InnoQuant HY	207 bp**	80 bp**	79 bp**	172 bp**		2.5–20, >20††	Sample IPC C_T minus average of standards (1–5) IPC C_T**	>2**

* As described in the *Investigator Quantiplex Pro Kit Handbook* and in the † QIAGEN Quantification Assay Data Handling and STR Setup Tool v2.01.

‡ As described in Holt et al. (5) and in the § Quantifiler HP and Trio DNA Quantification Kits User Guide, 2017.

¶ As described in Ewing et al. (4) and in the PowerQuant System Technical Manual.

** As described in Loftus et al. (3) and in the InnoQuant HY User Guide and in †† van den Berge et al. (10).

‡‡ Different region from the same locus as the small target; targets are partially overlapping.

§§ Different region from the same locus as the small target.

Materials and methods

DNA standards and samples

Two human male DNA standards (NIST SRM 2372 Component A and TaqMan® Control Genomic DNA (Thermo Fisher Scientific)) were spiked with defined amounts of inhibitors: hematin, humic acid, calcium, melanin or salt (see Figure 1). Each DNA standard was diluted to 1 ng/µl, followed by a 1:2 dilution into various concentrations of inhibitors, resulting in a final DNA concentration of 0.5 ng/µl. Inhibitor-free samples containing 0.5 ng/µl of DNA were also included as controls. All samples were quantified in duplicate.

The degraded samples included DNA from bones (n=5), decomposed tissues (n=5) and formalin-damaged samples from embalmed tissues (n=5). These human remain samples were obtained from the Applied Anatomical Research Center (AARC) in the Center for Biological Field Studies at Sam Houston State University, Huntsville, Texas, USA. DNA from the bone samples was extracted using the QIAamp® DNA Investigator Kit (QIAGEN) or the PrepFiler® BTA Forensic DNA Extraction Kit (Thermo Fisher Scientific). DNA from decomposed tissues was extracted using the QIAamp DNA Investigator Kit on the QIAcube® (QIAGEN) and the forensic casework samples protocol. DNA from embalmed samples was extracted using the QIAamp DNA FFPE Tissue Kit (QIAGEN).

DNA quantification and assessment

DNA from all samples was quantified using the Investigator Quantiplex Pro Kit, the Quantifiler Trio DNA Quantification Kit, the PowerQuant System and the InnoQuant HY Kit according to the manufacturers' instructions. DNA quantification was carried out on a 7500 Real-Time PCR System (Thermo Fisher Scientific) using each kit's respective template. The sample DI and inhibition index (IPC ΔC_T value) were determined for each sample.

STR analysis of degraded samples

To assess the correlation between the sample DI and the success of downstream STR analysis, STR amplification and analysis were performed on all degraded samples (n=15), pairing each commercial qPCR kit with its corresponding STR amplification kit: Investigator 24plex QS Kit (QIAGEN), GlobalFiler® PCR Amplification Kit (Thermo Fisher Scientific) and PowerPlex® Fusion 6C System (Promega Corporation), following manufacturers' instructions. STR amplification was not performed for the InnoQuant HY Kit analysis, due to the limited amount of DNA available.

When possible, 0.8 ng DNA was included in each STR reaction, according to the concentration determined from the small target measurement. For samples containing <0.053 ng/µl of DNA, 15 µl of DNA was added to each reaction. PCR amplification was performed using a ProFlex™ PCR System (Thermo Fisher Scientific) with each STR kit's recommended cycling parameters. A 3500 Genetic Analyzer (Thermo Fisher Scientific) with a 36 cm capillary array and POP4 polymer platform was used for separation and detection of the PCR products. Data analysis was performed with the GeneMapper ID-X Software v1.4 (Thermo Fisher Scientific). The following thresholds were used for data interpretation: analytical threshold (AT) of 100 relative fluorescent units (RFUs) and stochastic threshold (ST) of 200 RFUs for Investigator 24plex QS, AT of 150 RFUs and ST of 600 RFUs for GlobalFiler and AT of 175 RFUs and ST of 400 RFUs for PowerPlex Fusion 6C. Single factor ANOVA analysis in Microsoft® Excel® was used for assessing statistical significance of the data, with $P<0.05$ accepted as the level of significance.

Results and discussion

Inhibition

All four qPCR kits provided similar inhibition indices at lower inhibitor concentrations, but the DNA quantification results varied between kits and across the inhibitors tested (Figure 1A–E). For samples spiked with hematin, a statistically significant difference between kits was determined by one-way ANOVA ($F_{3,36}$ = 6.4779, p = 1.28E-03). The inhibition indices for hematin were similar for all kits until the PowerQuant System began flagging inhibition at 500 µM, the Quantifiler Trio at 600 µM and the InnoQuant HY at 900 µM. The Investigator Quantiplex Pro Kit provided more accurate quantification results (average of 0.4359 ng/µl when compared to 0.5 ng/µl and a variance of 0.00451) (Figure 1A). ▷

Inhibition flagging for the humic acid-spiked samples was not observed until 200 ng/µL with the PowerQuant System and the InnoQuant HY kit, 250 ng/µl with the Investigator Quantiplex Pro Kit and 400 ng/µl with Quantifiler Trio. The difference in DNA concentrations obtained with the different kits was statistically significant ($F_{3,36}$ = 14.2939, p = 2.7E-06). In the presence of humic acid, the Investigator Quantiplex Pro Kit provided more accurate DNA concentrations than the other kits, and the small target was more tolerant (average 0.5075 ng/µl with a variance of 0.00176). The InnoQuant HY kit appeared to be most susceptible to high levels of humic acid out of the kits tested (Figure 1B).

Inhibition flagging for the calcium-spiked samples was observed at 15 mM with PowerQuant and InnoQuant HY, at 25 mM with Quantifiler Trio and at 30 mM with the Investigator Quantiplex Pro Kit. The difference in DNA concentrations obtained with the different kits was statistically significant ($F_{3,36}$ = 15.2388, p = 1.5E-06). In the presence of calcium, the Investigator Quantiplex Pro Kit provided more accurate DNA concentrations than the other kits, and the small target was more tolerant (average 0.4038 and smallest variance of 0.00144) (Figure 1C). These results suggest that the Investigator Quantiplex Pro Kit is highly suitable for analyzing skeletal samples, which often contain calcium from the bone and humic acid from the soil where bones are retrieved.

Inhibition flagging for the melanin-spiked samples was observed at 75 ng/µl with the Investigator Quantiplex Pro and InnoQuant HY kits and at 175 ng/µl with the PowerQuant System. Again, the difference in DNA concentrations obtained with the four kits was statistically significant ($F_{3,36}$ = 11.4574, p = 2E-05). However, a separate one-way ANOVA that excluded the InnoQuant HY kit revealed that the difference between the three remaining kits was not statistically significant. This indicates that the InnoQuant HY was more susceptible to melanin inhibition than the other kits, which showed no significant difference in performance. In addition, only the Investigator Quantiplex Pro and PowerQuant systems flagged inhibition in these samples (Figure 2). Furthermore, only the Investigator 24plex STR Kit was able to demonstrate whether inhibition impacts STR amplification due to its inclusion of the Quality Sensor peaks (Figure 2).

Inhibition flagging for the salt-spiked sample was observed at 200 mM with the Investigator Quantiplex Pro Kit and the PowerQuant System and at 400 mM for the InnoQuant HY. The Quantifiler Trio indicated no inhibition until the IPC target failed to amplify at a salt concentration of 800 mM. The difference in DNA concentrations obtained with the four kits was statistically significant ($F_{3,36}$ = 6.8143, p = 9.4E-04). However, at salt concentrations above 500 mM, only the Quantifiler Trio provided DNA quantification results (Figure 1E). The Quantifiler Trio showed the highest tolerance to these salt concentrations (average = 0.3142 ng/µL, variance = 0.01264).

Our results demonstrate that some quantification kits have higher tolerance to certain inhibitors than others. In particular, the Investigator Quantiplex Pro Kit was more tolerant than the other kits to the presence of various inhibitors, except for salt. In addition, in cases where the small target DNA concentration was decreased by 50% or more (<0.25 ng/µl) due to severe inhibition, the IPC target still indicated the presence of inhibitors for all four kits. These results support the importance of consulting the IPC data before increasing the sample analysis volume, as inadvertently increasing the amount of inhibitor in the reaction will reduce STR success or lead to complete failure of the PCR.

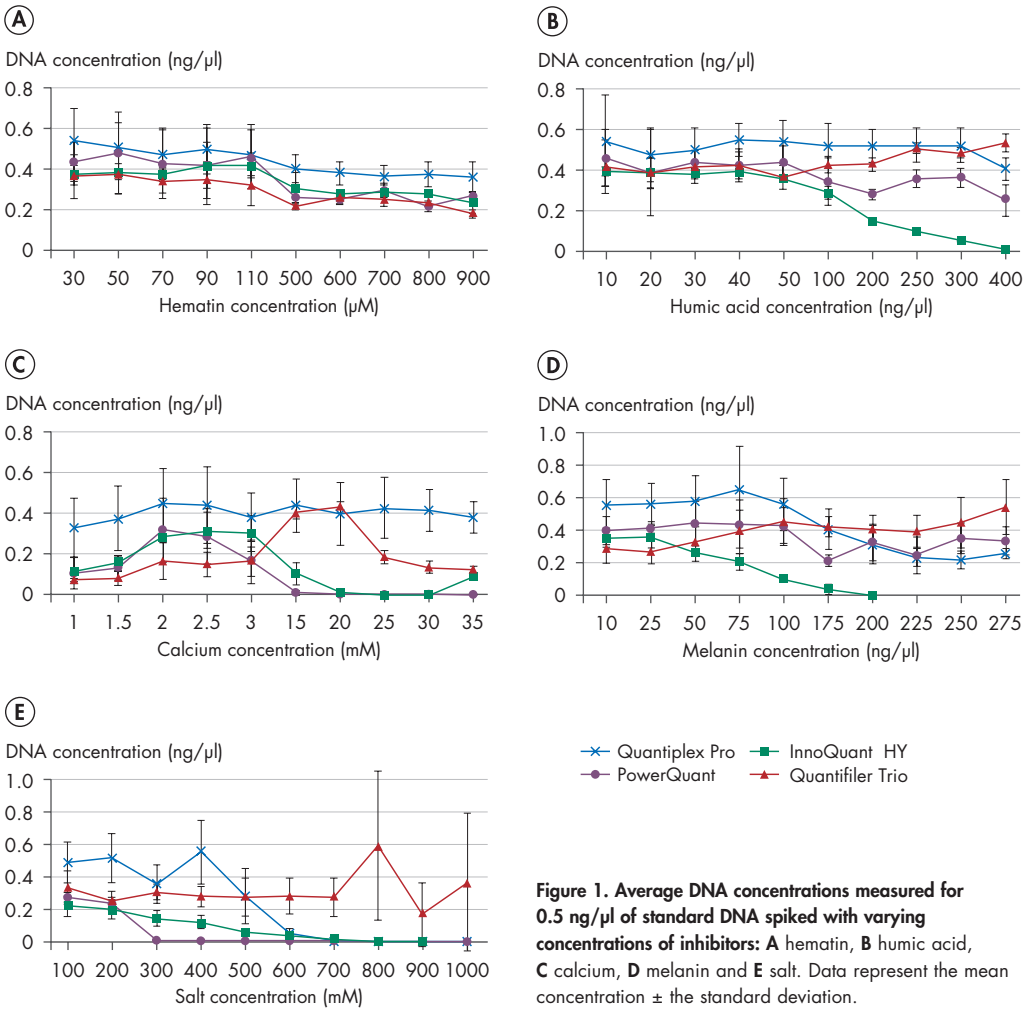

Figure 1. Average DNA concentrations measured for 0.5 ng/µl of standard DNA spiked with varying concentrations of inhibitors: A hematin, **B** humic acid, **C** calcium, **D** melanin and **E** salt. Data represent the mean concentration ± the standard deviation.

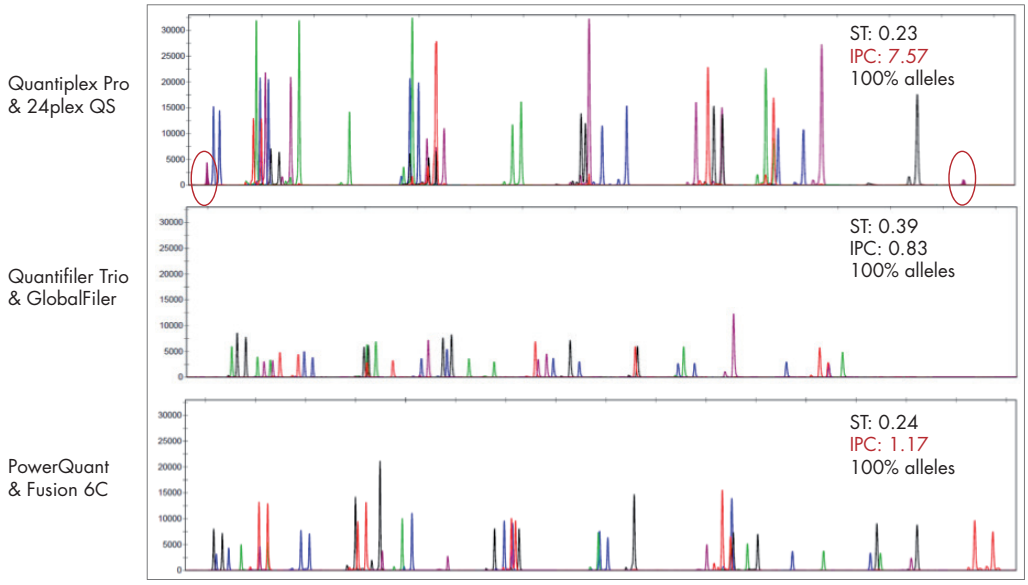

Figure 2. Electropherograms of DNA spiked with 225 ng/µl melanin. The sample was quantified using each qPCR kit and then amplified with the corresponding STR kit based on the small amplicon quantity. **ST:** small target, **IPC:** Internal PCR Control (ΔC_T value). IPC values in red indicate that the qPCR kit flagged possible PCR inhibition. Red circles indicate the presence of both Quality Sensors in the Investigator 24plex QS Kit, indicating PCR inhibition.

Degradation

DNA samples from bone (n=5), decomposed tissue (n=5) and formalin-damaged samples from embalmed tissue (n=5) were quantified with each of the four qPCR kits. For all of the degraded samples, the resulting average DNA concentrations based on the small target were similar for each kit tested, but the Quantifiler Trio kit consistently provided the highest large target concentration (Figure 3A). As a result, analysis using the Quantifiler Trio kit resulted in the smallest DIs for each set of degraded samples (Figure 3B). In terms of degradation flags, all samples had a DI >1 and <10 according to the Quantifiler Trio kit, indicating moderate degradation. The highest DIs were observed with the Investigator Quantiplex Pro Kit, with a DI >1 for all samples; only 60% of the samples had a DI >10, indicating degradation (Figure 3B). The difference in results from these two kits is primarily related to the size difference between the two large targets (~140 bp). The PowerQuant System correctly indicated degradation in all of the degraded samples (DI >2), while the InnoQuant HY indicated moderate degradation in 73% of samples (DI >2.5 and <20) and severe degradation in 27% (DI >20).

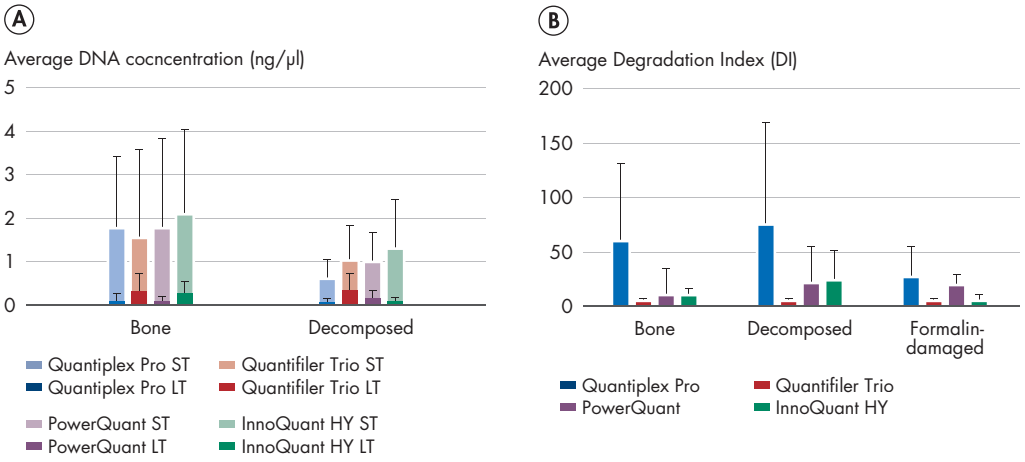

Figure 3. Average DNA quantification results for degraded human samples. A Average DNA concentrations obtained from each qPCR kit. **ST:** small target, **LT:** large target. **B** Average degradation index calculated from each qPCR kit analysis. Data represented as mean ± standard deviation.

STR analysis

For STR analysis, each of the qPCR kits in this study except for the InnoQuant HY was tested with its respective STR amplification kit. Based on the small target, the most successful STR analyses were observed with the Investigator Quantiplex Pro plus Investigator 24plex QS Kits and the Quantifiler Trio plus GlobalFiler kits; however, there was a large variation in the average DIs obtained with these qPCR kits (Figure 4). While both qPCR kits indicated degradation in the test samples, full profiles were still produced in 53% of the samples with the Investigator 24plex QS Kit and in 47% of the samples with the GlobalFiler Kit. The PowerQuant System similarly indicated degradation in all samples, but the PowerPlex Fusion 6C resulted in the highest incidence of allele dropout based on the small target DNA concentration (Figure 4). Figure 5 shows a representative example of the difference in STR profile quality when the same sample was quantified and amplified with each qPCR and STR kit combination.

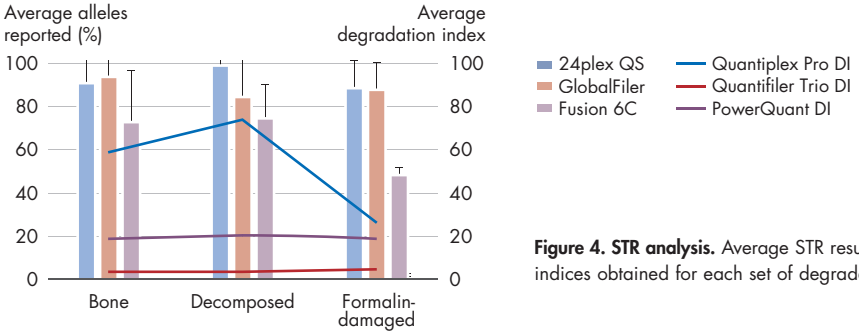

Figure 4. STR analysis. Average STR results and degradation indices obtained for each set of degraded samples.

Similar to other studies (3–5), we observed that high inhibitor concentrations often preferentially influence amplification of the large target, indirectly affecting the resulting DI. This can lead to incorrect flagging of these samples as severely degraded, so it is important to consider both the DI and IPC data together when assessing sample integrity prior to STR analysis.

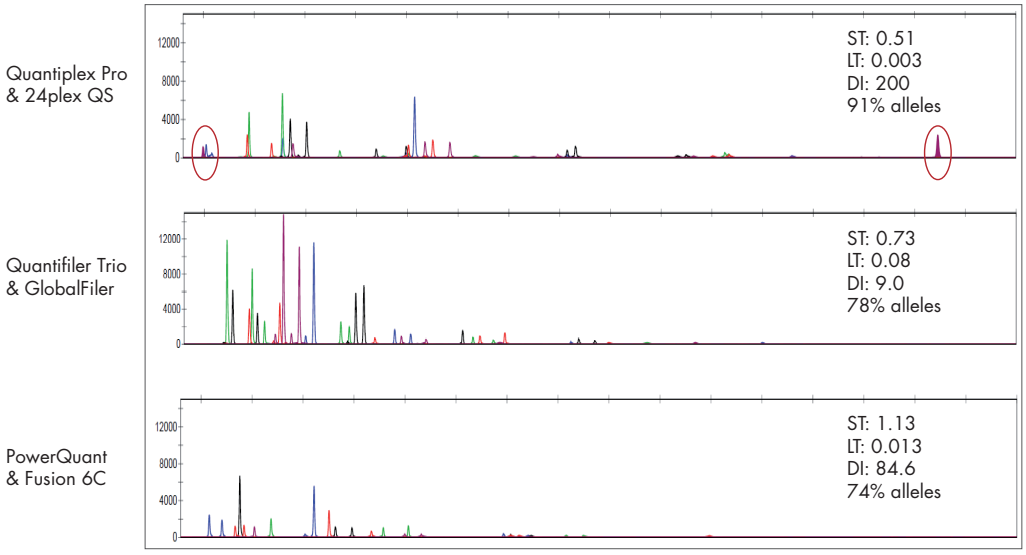

Figure 5. Electropherograms of DNA extracted from decomposed muscle tissue. The sample was quantified using each qPCR kit and then amplified with the corresponding STR kit based on the small amplicon quantity. **ST:** small target, **LT:** large target, **DI:** degradation index. Red circles indicate the presence of both Quality Sensors in the Investigator 24plex QS Kit, indicating no PCR inhibition.

Conclusion

All of the kits included in this study performed similarly for high-quality DNA samples containing low levels of inhibitors. However, in samples containing higher levels of inhibitors, the Investigator Quantiplex Pro Kit was most tolerant, providing more accurate quantification results in all spiked samples, except for those with high salt concentrations. All of the kits provided comparable small target DNA concentrations but varied regarding DNA degradation prediction. The STR typing results varied between the different kits, but the Investigator 24plex QS and GlobalFiler kits provided a higher number of complete profiles than the PowerPlex Fusion 6C.

While increasing the amount of input DNA for samples predicted to be degraded can increase first-pass STR typing success, it can also introduce unwanted experimental artifacts. Our results show that considering both sample degradation and inhibition together is important to assay success. Using commercially available qPCR kits with laboratory-defined DI and inhibition flags can improve prediction of sample integrity, streamlining the forensic workflow and allowing analysts to triage evidence before conducting expensive and time-consuming genetic analyses.

In addition, in the case of the Investigator 24plex QS Kit, the presence of a Quality Sensor in the STR kit provides a further quality control check on the performance of the PCR. The Quality Sensor data was shown to correlate with the information provided by the IPC in the Investigator Quantiplex Pro Kit. This additional feedback has the potential to further streamline DNA processing when determining the appropriate re-work strategy for failed samples.

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