Application Note

The effectiveness of STR Quality Sensors to inform rework strategies and improve STR success of challenging samples

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Introduction

The Investigator® 24plex GO! and Investigator 24plex QS kits are 6-dye multiplex assays for the identification of human reference and casework samples, respectively. In addition to the expanded CODIS core and three additional autosomal loci (SE33, D2S1338 and D19S433), these kits also include two quality sensor targets (QS1 and QS2) that serve as internal PCR controls. The presence, absence or relative amplification of these quality sensors can assist the interpretation of STR profiles and direct analysts toward more effective rework strategies (1).

The use of direct PCR for database and reference samples has greatly increased laboratory throughput by employing automated solutions and bypassing the time-consuming and costly DNA extraction and quantification processes. Due to the predictable nature of databasing samples (buccal swabs and FTA® cards), full STR profiles can be generated from a majority of them. However, lack of purification can leave some samples vulnerable to PCR inhibition, while other complicating factors, such as DNA degradation and low amounts of DNA (LT-DNA), can also affect downstream STR success (1–5). Sources of inhibition can originate from the collection/preservation method used or the sample itself. The main sources of PCR inhibitors in reference blood samples include endogenous hematin and EDTA from blood collection tubes and FTA cards (6). DNA degradation may occur in samples stored improperly or in a facility with limited climate control. For buccal swabs, poor collection and storage techniques as well as exogenous inhibitors from food and beverages can produce low template and/or inhibited profiles (7).

In this study we selected a set of challenging samples (N=53) that generated less than 90% reported alleles in the first amplification round. To assess the values of the QS markers included in ▷

the STR kits, the samples were then reworked based on the quality of the electropherogram (EPG) with the QS markers redacted (Figure 1A) and in conjunction with the QS markers (Figure 1B). Results from each of the reworks were analyzed to determine which strategy, if either, improved the profile quality and the number of STR alleles reported.

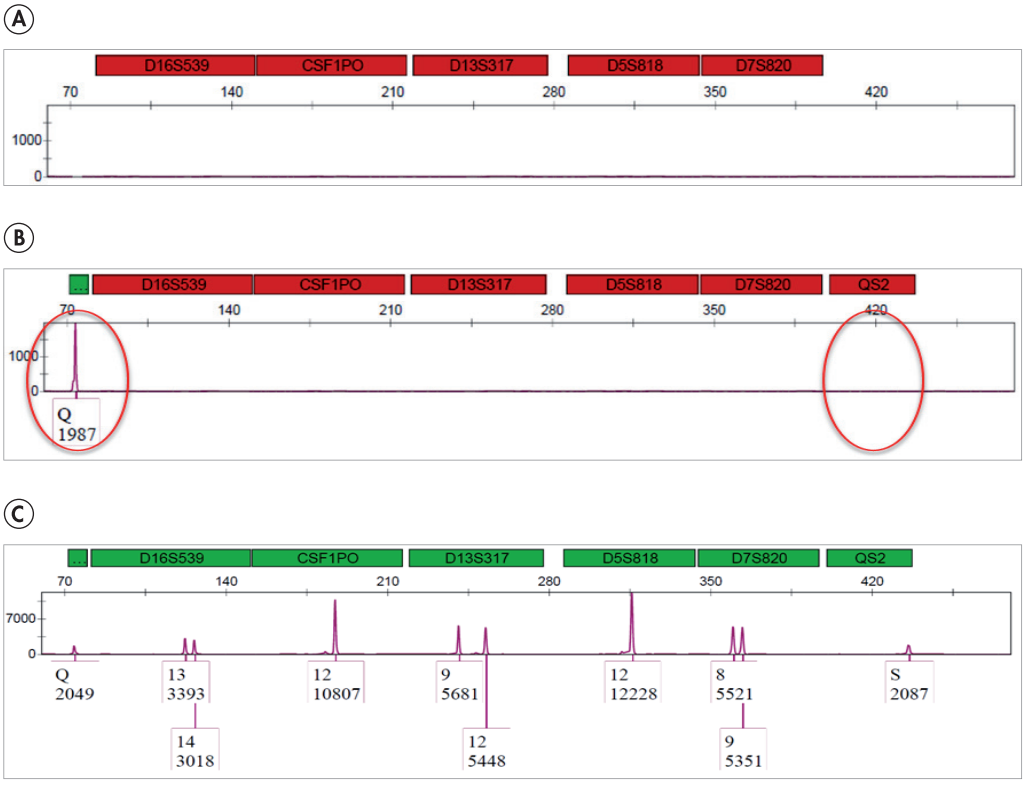

Figure 1. Example EPG (purple channel only) for a low blood volume FTA sample. The sample was analyzed after: **A** first-pass direct amplification (shown with QS markers redacted), **B** secondary direct amplification with new punch (S marker failed), and **C** lysis of new punch with the Investigator 24plex GO! lysis buffer before secondary direct amplification (rework determined by failed S marker in B).

Methods

Sample collection and preparation

Databasing samples

Reference DNA samples were collected from informed and consenting participants pursuant to IRB 2018-05-40949 approved by Sam Houston State University. A subset of samples (N=53) from a larger sample pool were chosen for this study based on STR profile completeness. Blood samples were collected via venipuncture and spotted on Whatman® FTA cards (GE Healthcare). Saliva samples were collected using sterile cotton tipped applicators (Puritan Medical Products Company), the Bode Buccal DNA Collector® system (Bode Technology) or Whatman FTA cards

with Easicollect™ devices (GE Healthcare) using manufacturers' recommended protocols (hereafter referred to as cotton swabs, Bode swabs and saliva FTA samples, respectively).

With the exception of room temperature controls, databasing samples were subjected to a variety of simulated environmental conditions, including incubation in a hot and humid environment for up to 27 weeks, UV exposure for between 1 minute and 24 hours, and poor collection methods, such as single cheek swipes for buccal swabs. Low blood volume samples were simulated using purple-topped blood collection tubes with less than 0.75 ml of blood. Blood from the collection tubes was then deposited onto FTA cards.

Casework-like samples

DNA extracts previously identified as inhibited, low-template or degraded were used in this study. Inhibited and degraded samples were sourced from cadaver muscle biopsies stored in a liquid preservative and unpreserved nylon swabs. In addition, samples spiked with inhibitors (hematin, melanin, humic acid and ethanol) were prepared using neat inhibitors and control DNA.

DNA quantification and amplification

Setup for all quantification and amplification reactions was performed using a QIAGEN QIAgility® liquid handling platform. All mock casework samples were quantified using the Investigator Quantiplex® Pro RGQ kit on a QIAGEN Rotor-Gene® Q and results were analyzed with the QIAGEN Data Handling Tool.

Databasing samples were amplified using the QIAGEN Investigator 24plex GO! chemistry. DNA in both cotton and Bode swabs was amplified according to the manufacturer's guidelines using 26 PCR cycles. For both saliva and blood FTA cards, single 1.2 mm punches were manually deposited into the wells of a 0.2 ml 96-well plate and 20 µl of STR GO! lysis buffer was directly added to the punches. The plate was then centrifuged and incubated at 95°C for 5 minutes before 2 µl of the crude lysate was added to 20 µl GO! Master Mix. Additionally, a subset of blood FTA samples were directly amplified without use of the GO! lysis buffer. DNA in FTA blood and saliva samples was amplified for 27 PCR cycles.

All sample dilutions for mock casework samples targeted a final DNA input per reaction of 0.8 ng. Samples were amplified with the QIAGEN Investigator 24plex QS Kit according to the manufacturer's protocol on either a ProFlex™ (Applied Biosystems) or Veriti™ (Applied Biosystems) thermal cycler.

Capillary electrophoresis and data analysis

Amplified fragments were separated and detected on an Applied Biosystems® 3500 Genetic Analyzer on a 36 cm capillary array using handbook-defined settings. Data analysis was completed using GeneMapper IDX v1.4 with tertiary analysis being accomplished with in-house Excel® (Microsoft Corp.) workbooks. Stochastic and analytical thresholds were set at 200 RFU and 100 RFU, respectively.

Sample reworks

Strategy determination

To assess the benefits of the QS markers present in STR profiles, strategies for reworking samples were determined by analyzing STR profiles with and without the QS marker information visible. To avoid bias, a forensic DNA analyst from an external crime laboratory was asked to interpret EPGs with the QS markers redacted. For databasing samples, the analyst was provided with the sample type (blood vs buccal) and substrate (FTA card vs Bode/cotton swab) and asked to indicate their rework strategy. Quantification information was also provided to the analyst during STR profile evaluation for casework samples, as these data would normally be available regardless of the STR chemistry used. The analyst used their experience and their laboratory's standard operating procedures (SOPs) to assess the profile quality and determine the appropriate rework strategy (if any).

In addition to the rework approach indicated by the external analyst (blinded to QS markers), samples were also examined by a different analyst who indicated their rework strategy based on the performance of the QS markers. With the QS marker information, inhibition was suspected when the Quality Sensor S/Q allele ratio was below 70% and confirmed when one or both QS markers dropped out. Samples were classified as low template and/or degraded when the QS markers were balanced and low RFUs were observed consistently throughout the EPG (roughly average peak height of <750 RFUs). The absence of DNA template in the PCR was determined by the presence of balanced QS markers and no other alleles called. In general, rework strategies were determined as follows: adding more template to the PCR amplification (for suspected low or degraded template), diluting the lysate/extract before re-amplifying (for suspected PCR inhibition), or processing a new punch from the same sample (for suspected failed amplification due to no template).

Strategy implementation

Rework strategies for each sample were designated, executed and compared. If the strategies with and without the QS markers were the same, the rework was only performed once. Databasing samples requiring an increased template included either an additional microliter of lysate (3 µl total) if available or two 1.2 mm punches added to the PCR. For samples identified as inhibited, either a 1:3 dilution or a punch wash with GO! lysis buffer was performed before re-amplification. Inhibited casework samples were diluted as much as 1:15 and re-quantified prior to re-amplification. Finally, samples categorized as having no DNA template in the PCR were reprocessed either as a new punch or from the original lysate/extract when applicable. All PCR cycling conditions and CE parameters remained the same as for the initial amplification.

Results and Discussion

As expected, reworking challenging samples resulted in more complete STR profiles compared to the original amplifications (Figure 2). Compared to the inhibited samples, the degraded/low template samples produced more complete STR profiles but also yielded wider variations in first-pass amplification success rates. Overall, the increase in allele recovery after applying both rework strategies was comparable for degraded/low template samples but differed for those that were inhibited (Figure 2). The greatest improvement in STR success was achieved when inhibited samples were reworked based on information provided by the QS markers (29/29 samples improved) rather than relying on the quality of the EPG alone (20/29 samples improved; Figure 2). Although a trained analyst may be able to identify signs of PCR inhibition within the profile, this study found that inhibition was more reliably and accurately detected based on the behavior of the QS markers.

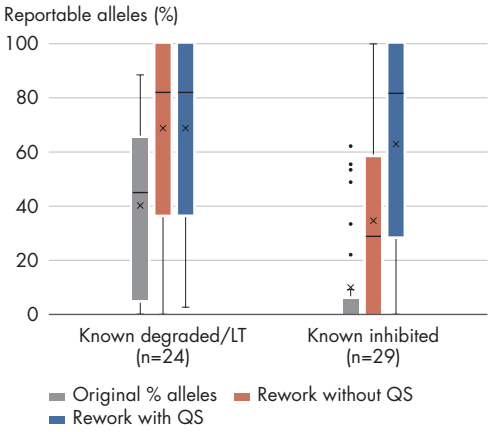

Figure 2. Comparison of STR results before and after reworks with and without QS marker information for challenging samples. Outliers are shown and sample means are represented by the 'x' symbols.

Interestingly, direct amplification of several low blood volume FTA cards resulted in complete amplification failure. We suspect that the accumulation of EDTA in these samples from both the blood tubes and the unwashed FTA cards resulted in high levels of PCR inhibition. Without the QS marker information, the external analyst assumed the punch contained no DNA template and the rework strategy was to process a new punch, which yielded the same results as the initial assays (Figures 1A and 1B). However, with the QS marker information available, inhibition was indicated due to one or both QS markers failing to amplify, and the appropriate rework strategy was identified. In these cases, a wash with GO! lysis buffer was performed and full STR profiles were recovered (Figure 1C).

For samples that had concordant rework strategies regardless of the QS markers, an increase in correct alleles called was achieved 94% of the time. It is important to note that although the rework strategies were identical, the QS markers did provide the analyst with high confidence regarding the level of DNA degradation in the samples. One sample that showed no improvement after rework was highly degraded/low template, and therefore the maximum amount of template was ▷

already being amplified. Another sample still showed signs of extreme inhibition after several dilution attempts, which was confirmed by the QS marker information.

Of the 53 samples reworked, 20 were identified as having different rework strategies based on the characteristics of the DNA profile provided to the analysts, either with or without the QS markers being masked. This demonstrates that although experienced analysts are frequently able to correctly identify the likely cause of a loss of alleles or overall poor profile quality, there are still circumstances where the true issue may be more ambiguous. When the QS markers were used to assess the likely cause of a poor STR result, the reason for PCR failure could be readily identified and the correct rework strategy employed (Figure 3). The QS markers were most beneficial in resolving failed amplifications and highly inhibited samples (Figure 4). The rework strategy based on the QS markers resulted in all but two of those 20 samples having full or nearly full STR profiles (>97%), whereas rework strategies without the QS markers were frequently less successful.

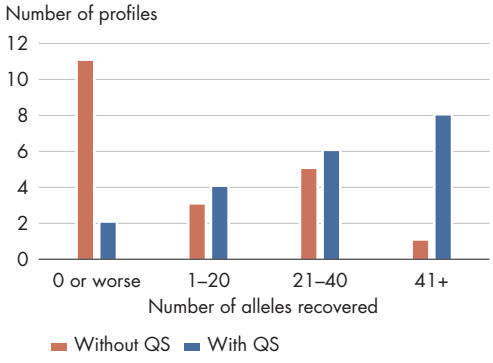

Figure 3. Improvement in profile completeness based on rework strategy. Distribution of the number of alleles recovered (compared to the original amplification) for 20 samples that had different rework strategies based on analysis of the DNA profile with (blue) and without (orange) QS marker information provided.

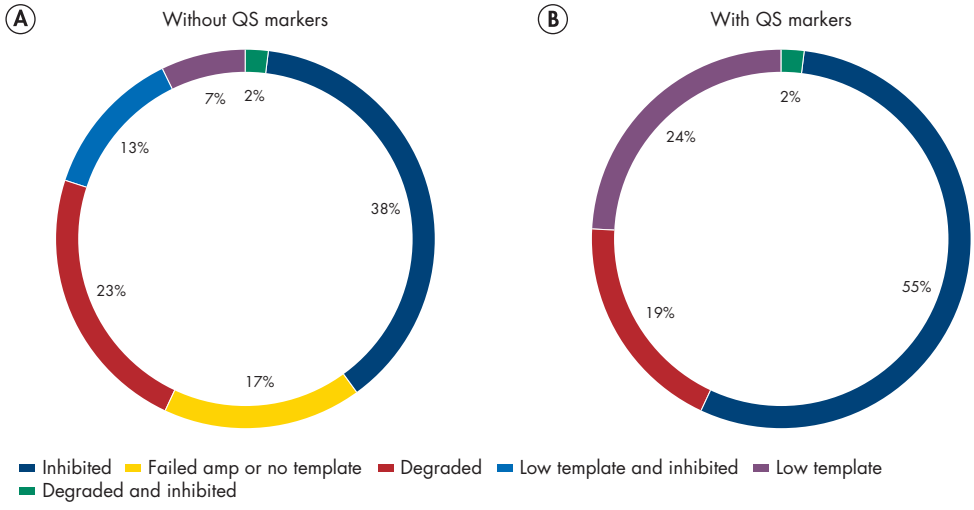

Figure 4. Assignment of the suspected cause of suboptimal DNA profiles. Electropherograms were interrogated **A** without the QS markers visible and **B** With the QS markers visible (N=53).

In addition to recovering more loci, rework strategies based on QS marker information also resulted in improvements in general profile quality. Profile quality of the first amplification was poor, and only 3.2% of samples showed an average peak height ratio (APHR) above 60% and only 13.2% of samples had an average peak height (APH) above 200 RFUs (stochastic threshold). In comparison, 32% of reworks without the QS markers and 47% samples with QS markers showed an APHR above 60%. Also, 47% of samples without QS markers and 55% of samples with QS markers had an APH above 200 RFUs (Figure 5). Overall, when QS markers were used to determine the rework strategy, consistently more alleles were recovered and a greater number of samples with balanced, reportable profiles were obtained.

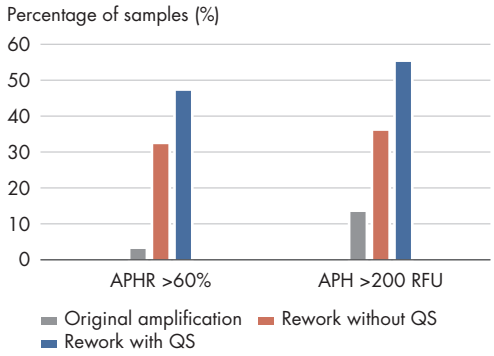

Figure 5. Quality metrics of STR profiles. The number of samples with reasonably balanced and quality STR profiles (APHR >60%, and APH >200 RFUs) before (grey) and after reworks with (blue) and without (orange) QS marker information for challenging samples was determined (N=53).

Conclusion

Most forensic DNA analysts are able to distinguish between degraded, low template and inhibited STR profiles after adequate training and experience. However, there are many instances when the best approach for how to rework a particular sample to improve results may not be apparent. This study demonstrates that QS markers can be used as a straightforward, consistent and valuable tool to assist in the interpretation of STR profiles from challenging samples. The ability to distinguish between highly inhibited samples, severely degraded DNA and failed amplification informs the analyst on the most appropriate rework strategy to eliminate or minimize the number of reworks performed, saving both time and resources, which ultimately improves overall efficiency.

References

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Ordering Information

Product	Contents	Cat. no.
Investigator 24plex QS Kit (100)	Primer Mix, Fast Reaction Mix including Taq DNA Polymerase, Control DNA, allelic ladder 24plex, DNA size standard 24plex (BTO), and nuclease-free water	382415
Investigator 24plex QS Kit (400)	Primer Mix, Fast Reaction Mix including Taq DNA Polymerase, Control DNA, allelic ladder 24plex, DNA size standard 24plex (BTO), and nuclease-free water	382417
Investigator 24plex GO! Kit (200)	Primer Mix, Fast Reaction Mix 2.0 including Taq DNA polymerase, Control DNA, allelic ladder 24plex, DNA size standard 24plex (BTO)	382426
Investigator 24plex GO! Kit (1000)	Primer Mix, Fast Reaction Mix 2.0 including Taq DNA polymerase, Control DNA, allelic ladder 24plex, DNA size standard 24plex (BTO)	382428
Investigator ESSplex SE QS Kit (100)	Primer Mix, Fast Reaction Mix including Taq DNA Polymerase, Control DNA, Allelic Ladder ESSplex SE QS, DNA size standard 550 (BTO) and Nuclease-Free Water	381575
Investigator ESSplex SE QS Kit (400)	Primer Mix, Fast Reaction Mix including Taq DNA Polymerase, Control DNA, Allelic Ladder ESSplex SE QS, DNA size standard 550 (BTO) and Nuclease-Free Water	381577

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