DNA QUALITY

The Hidden Benefits of Real-Time PCR: Assessing and Addressing Qualitative Challenges

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INTRODUCTION

Over the past year many scientists in the forensic DNA community have implemented real-time PCR into their laboratories to improve the ability to quantify DNA extracts. Historically, the primary means of quantification was a hybridization assay such as the Quantiblot® system (Applied Biosystems) in which the intensity of sample dots was compared to the intensity of standard dots to estimate a concentration. While this approach works, there are several negative aspects associated with it, such as extensive hands-on time, limited dynamic range and subjective interpretation. Many scientists wanted a method that would minimize these limitations and got it with the emergence of real-time PCR. While some scientists in the field may have been hesitant to change to real-time PCR, the decision to make the switch became easier when Applied Biosystems announced the discontinuation of Quantiblot®.

Real-time PCR uses the same principles as Quantiblot®, but the difference is that quantification is achieved by measuring the amount of fluorescence emitted from a sample as it proceeds through a PCR. There are two different approaches to real-time PCR. The first is one in which the amount of fluorescence increases as the PCR proceeds. For example, with the TaqMan® system (Applied Biosystems) PCR primers bind to sites adjacent to the region to be amplified and a probe binds to the region amplified. The probe is labeled with both a fluorescent reporter molecule and a fluorescence-quenching molecule. When the reporter and quencher molecules are both bound to the probe, fluorescence is quenched. However, when the primers and probe anneal and the primers are subsequently extended, the reporter molecule is cleaved from the probe, and fluorescence is released. Another approach to real-time PCR is one in which the amount of fluorescence decreases as the PCR proceeds. An example of this approach is the Plexor™ HY System (Promega). In a Plexor™ reaction, one of the primers contains a modified iso-dC nucleotide that is linked to a fluorescent tag. Therefore, prior to the start of the PCR, maximum fluorescence is being emitted. The fluorescent primers are gradually incorporated into PCR products. As PCR progresses, quencher molecules that are linked to iso-dG nucleotides are incorporated opposite the fluorescent-labeled iso-dC nucleotides in the primer, resulting in the quenching of fluorescence. While commercially available real-time PCR kits are attractive, to get the most useful information possible from your real-time PCR system, some degree of optimization may be necessary.

USING REAL-TIME PCR TO ASSESS DNA QUALITY

Real-time PCR has had an obvious impact on the ability of forensic DNA laboratories to quantify DNA more efficiently and more accurately. However, real-time PCR also offers qualitative information about DNA extracts that was never previously available to forensic scientists: specifically, the abilities to predict the presence of degraded DNA and PCR inhibitors in evidentiary DNA extracts prior to amplification and analysis. While assessments regarding degradation and inhibition could be made in the past, they could only be performed post-analysis after significant amounts of extract and time had already been wasted.

DNA QUALITY

The Delaware Office of the Chief Medical Examiner (OCME) DNA Unit wanted a real-time PCR system that not only would provide accurate quantification of amplifiable DNA but would also predict degradation and inhibition. Therefore, we decided to validate a two-assay system that combined a homemade kit with a commercial kit. The first assay is a nuclear DNA quantification method that was developed by the California Department of Justice (DOJ) and uses an amplicon size (~170 base pairs) that is comparable to the size of STR loci (1). The second assay is the commercially available Quantifiler® Y kit from Applied Biosystems that provides a male DNA concentration.

The decision to use the California DOJ assay for nuclear DNA quantification rather than one of the commercially available real-time PCR kits was made to ensure that the amount of DNA being quantified truly reflected the amount of DNA that would amplify in an STR amplification. Some of the commercially available kits such as Quantifiler® use amplicons for quantification that are significantly smaller in size than the amplicons in the STR kits. This is problematic in that real-time PCR kits are able to successfully amplify DNA that STR kits cannot amplify. The result is an overestimation of amplifiable DNA; therefore not enough DNA is added to the STR amplification. Such a problem can be overcome by simply customizing the assay to use a larger amplicon.

We decided to use the Quantifiler® Y kit for male DNA quantification because the assay contains an Internal Positive Control (IPC), which will fail if the extract contains an inhibitor and thus can be used for predicting inhibition. It should be noted that, in addition to the presence of an inhibitor, the IPC may also fail if there are extremely high concentrations of DNA present or if the assay was not set up correctly. However, if those factors can be discounted, then the IPC can act as a strong predictor of inhibition.

The specific combination of assays chosen also allows us to predict degradation since the Quantifiler® Y assay uses an amplicon size (~62 base pairs) that is nearly three times smaller than that of the California DOJ assay and, therefore, is less susceptible to degradation. If the template DNA is degraded, then the concentration of DNA detected by the two assays will differ. While a ratio that indicates more nuclear DNA than male DNA suggests the presence of female DNA, observing a ratio in which there is more male DNA than nuclear DNA suggests that the smaller amplicon is amplifying more than the larger amplicon and therefore the DNA template is degraded.

Being able to assess DNA degradation at the quantification stage is essential now that methods for addressing degraded samples, such as mini-STRs, are becoming more prevalent. In addition, the Delaware OCME DNA Unit felt it was necessary to research methods to better address inhibitors, since the ability to assess inhibition has improved. The most common approach used today is to dilute the template DNA during PCR setup. While this approach does dilute out the inhibitor, it also results in a less-than-optimal amount of template in the reaction. Another potential avenue is to alter the PCR by adding reagents such as bovine serum albumin to prevent the inhibitors from interacting with the *Taq* DNA polymerase or to add extra *Taq* DNA polymerase to overpower the negative effects of the inhibitors. However, the most attractive tactic is to simply remove the inhibitor. This can be done using DNA extraction kits that minimize the co-extraction of inhibitors with DNA or purification methods that remove inhibitors from DNA extracts. Since we can only reliably predict the presence of inhibitors after the DNA has been extracted, the latter would be a better course of action.

METHODS TO REMOVE PCR INHIBITORS

Two methods for purifying DNA extracts were found to be reliable and effective means of addressing inhibitors. The first involves the use of the QIAamp® kit from Qiagen, and the second involves the PowerClean™ DNA clean-up kit from MO BIO Laboratories, Inc. The Qiagen protocol called "Cleanup of Genomic DNA" (2) uses the wash buffers and spin columns associated with a standard QIAamp® kit to remove inhibitors from DNA extracts. The PowerClean™ DNA clean-up kit protocol (3) is a more aggressive procedure that first precipitates and pellets the majority of inhibitors, then subjects the supernatant to washes on a spin column to further purify the sample. Both methods were tested with the known inhibitors hematin,

Table 1. Comparison of QIAamp® and PowerClean™ Purification Methods for Inhibitor Removal.

Inhibitor	Amount That Causes Complete Inhibition	Purification Method	Maximum Amount of Inhibitor Removed	Percentage of DNA Recovery
Hematin	10ng/µl of PCR	QIAamp® kit	64µg	33.33%
		PowerClean™ kit	64µg	10.60%
Humic Acid	2.5ng/µl of PCR	QIAamp® kit	1µg	31.14%
		PowerClean™ kit	100µg*	12.71%
Indigo Dye	500ng/µl of PCR	QIAamp® kit	200µg	26.08%
		PowerClean™ kit	20mg	8.73%

*Full STR profile was obtained, but contamination was present.

DNA QUALITY

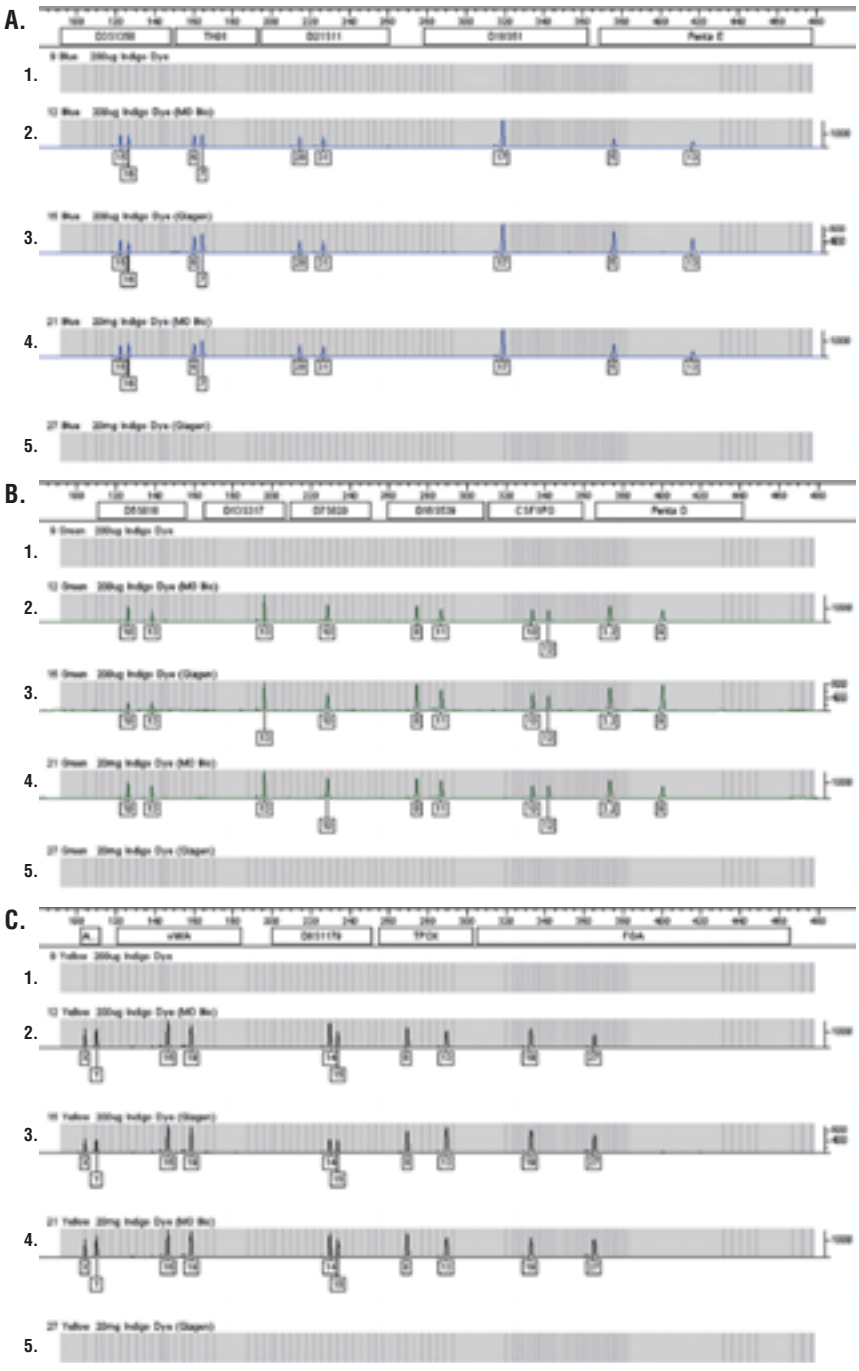

Figure 1. Removal of PCR inhibitors. DNA samples containing the indicated amount of indigo dye were left untreated or were cleaned up using the QIAamp® or PowerClean™ kit. Equal amounts of DNA were amplified using the PowerPlex® 16 System[d–g]. **Panel A.** The fluorescein-labeled PowerPlex® 16 loci. **Panel B.** The JOE-labeled loci. **Panel C.** The TMR-labeled loci. **Row 1.** 200µg indigo dye—Untreated. **Row 2.** 200µg indigo dye—PowerClean™ kit. **Row 3.** 200µg indigo dye—QIAamp® kit. **Row 4.** 20mg indigo dye—PowerClean™ kit. **Row 5.** 20mg indigo dye—QIAamp® kit.

humic acid and indigo dye. Both methods successfully removed significant amounts of inhibitor, but the PowerClean™ method, with its additional pelleting step, removed higher levels of inhibitor than the QIAamp® method (Figure 1). However, because some DNA is lost during that additional pelleting step, there is a trade-off with respect to the amount of inhibitor that can be removed and the amount of original DNA that can be recovered (Table 1). Therefore, the type of sample and the potential amount of DNA within the sample must be considered on a case-by-case basis to determine which method should be used to remove inhibitors.

CONCLUSION

The preceding discussion is a perfect example of why it is necessary to continually pursue technological advances in our laboratories. An improvement in one aspect of the testing process naturally leads to improvements in other aspects of the process. While the implementation of real-time PCR promised an improved quantification method, in reality the impact that it will have on our field is much more extensive. Not only are there additional benefits of being able to predict degradation and inhibition, but those abilities then lead to the implementation of additional technologies such as mini-STRs and inhibitor-removal methods. The key is to take advantage of the technology.

REFERENCES

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