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The effect of internal control sequence and length on the response to PCR inhibition in real-time PCR quantitation

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ABSTRACT

PCR inhibitors can originate from a variety of sources and can co-extract with the DNA template, resulting in reduced amplification and/or dropped alleles. Currently real time PCR is used to provide a check for the presence of PCR inhibition by monitoring the quality of amplification of an internal control. In this paper we examine the effect of internal control length and sequences on its sensitivity to PCR inhibition by varying concentrations of commonly encountered PCR inhibitors. Data from both amplification and melt curves were evaluated. The results show that while amplicon sequence has minor effects on amplification efficiency and melt curves, amplicon length has a more dramatic effect, regardless of inhibitor type. Given the increasing variety of STR typing kits and their documented differences in performance with respect to inhibition, the data obtained in this study can be used to assist designers of real time PCR kits to adjust their internal PCR controls (IPC) to permit a more targeted estimation of inhibition.

1. Introduction

A key step in producing a DNA profile is to determine the concentration of DNA present in an extracted sample. Once this is known, an optimal volume of sample can then be added to the subsequent STR amplification. This is commonly performed by real time PCR, which can be a sensitive and human specific DNA-quantification technique when used with appropriate primer sets. However, DNA samples of forensic interest do not always come from the most pristine conditions and often contain co-extractable PCR inhibitors. These inhibitors can be detected in a DNA sample using real time PCR by including a non-human target in each reaction tube and examining the respective cycle threshold (C_t) values for this amplicon [1]. The internal control sequence is added at a set concentration, and should therefore produce a constant C_t value for each reaction. However, if an inhibitory substance is present in a particular sample, the normally constant C_t value for the internal control sequence may be increased as the inhibitor can block access to the DNA template. In addition, co-extracted inhibitors can alter amplification efficiency and affect the melt temperature (T_m) of the amplified DNA [2–4]. Increases in the C_t value of the real time PCR amplification internal control generally indicate the presence of interfering substances which affect the ability of the polymerase to amplify the DNA. It is expected that such inhibitors would affect the unknown quantity of DNA template and the known amount of internal control DNA in a similar fashion. This increase in C_t is likely the result of the inhibitor binding to the DNA or sterically hindering it, thereby reducing the total amount of accessable DNA template [1]. When such a process occurs, it is reasonable to expect changes in the melt curves due to increasing interactions with the double stranded DNA. Of course it is also possible for the inhibitor to affect the *Taq* polymerase by partially disabling it resulting in a reduction in the processivity of the enzyme. Inhibitors that alter *Taq* processivity tend to produce changes in amplification efficiency, altering the shape and slope of the real time amplification curve. For example, inhibitors such as EDTA or Ca^{2+}, chelate or otherwise prevent the Mg^{2+} ions from interacting with the enzyme, which results in sub-optimal performance or deactivation of the *Taq* [1].

Ultimately, PCR inhibition is manifested by a reduction in intensity or a complete loss of alleles following the multiplex PCR amplification of STRs. The inhibition can be generic, resulting in the loss of larger alleles; or it can be more specific, affecting certain alleles more than others, regardless of size. One method of alleviating these inhibitory effects is by diluting the DNA sample [5]; however, dilution decreases the concentration of the DNA as well as the inhibitor. Better understanding of the mechanism of inhibition would permit the user to develop specific mitigation techniques such as spin filtration, the addition of BSA, or the use of smaller amplicons, based upon the real time response [6]. Another challenge is the choice of internal control length and sequence. A variety of real time PCR kits exist, each with different control

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sequence lengths and chemistries. Anecdotal evidence suggests that the internal control sequences in these kits vary in response to different inhibitors.

Thus, in this study we examine the effects of commonly encountered PCR inhibitors on internal controls of varying amplicon length and sequence structure, hypothesizing that the sequences with greater guanine and cytosine content and smaller length would be the least prone to inhibitory interactions. With this information, it should be possible to improve the response of real time PCR methods by developing more responsive IPC probes, and improving the ability of laboratories to understand and detect PCR inhibition.

2. Materials and methods

2.1. IPC DNA and primer sequences

Three 400 bp DNA sequences were randomly generated to create IPCs with 53%, 44% and 32% GC content based upon the range seen in typical forensic STRs. The sequences were run against the nucleotide BLAST database [7], and only those with no match to any known sequences were chosen. These were ordered from Integrated DNA Technologies (IDT) as a custom gene sequence [8]. Forward and reverse primers were designed using Primer3 [9] and unlabeled primers were synthesized by IDT [9]. The reverse primer targets were placed within the main sequence to create a total amplicon length of 80 bp, 160 bp and 230 bp. The forward IPC primer was labeled at the 5′-end with an Iso-C base and Cal-Fluo-Red 610 fluorescent tag (BioSearch Technologies [10]) for use with the Plexor® HY System (Promega Corporation, Madison, WI).

2.2. Inhibitors

Inhibitor stocks of tannic acid, bile salts, collagen, guanidinium isothyanate, hematin, humic acid, melanin and urea were made according to Opel et al. [1] and subsequent dilutions were made with water. In previous research [13], we found tannic acid to be a *Taq* polymerase inhibitor, while bile salts, humic acid, hematin, and melanin were DNA binding inhibitors. Lastly guanidinium, urea and collagen acted as mixed mode inhibitors, affecting both the polymerase and the DNA template [13]. Thus these inhibitors provided a wide range of differing effects to test our hypothesis.

2.3. PCR reactions

Volumes of master mix, primer mix and DNA were prepared per reaction according to manufacturer's specifications [11], where up to 5 μL of the 7 μL of water was replaced with inhibitor to get the final concentrations listed in Table 1. Each of the nine individual IPC amplicon sequences was performed in triplicate at each inhibitor concentration and the average change in C_t, efficiency and T_m was determined for the uninhibited control and for the concentration where 50% inhibition occurred. The sequences that produced the greatest change in each of the 3 criteria were tallied to identify which amplicon was the most impacted by inhibition.

Table 1
List of inhibitors, their mode of inhibition and common sources, along with each reaction's final inhibitor concentration.

Inhibitor	Type	Sources	Final reaction concentrations
Bile salts	DNA	Feces	0, 0.5, 1.0, 1.5 μg/μL
Collagen	Mixed	Bone, tissue	0, 25, 50, 75, 87.5, 100, 112.5 ng/μL
Guanidinium	DNA	Guanidinium salts	0, 1.5, 4.5, 7.5 ng/μL
Hematin	DNA	Blood	0, 15, 30, 45, 60, 75, 90μM
Humic acid	DNA	Soils	0, 10, 20, 30, 40, 60 ng/μL
Melanin	DNA	Hair, tissue	0, 20, 40, 60 ng/μL
Tannic acid	Taq	Leather, plant material	0, 7.5, 11.25, 18.5 ng/μL
Urea	Mixed	Urine	0, 100, 300, 500 mM

2.4. Sample analysis

Cycle threshold values and melt temperatures were obtained using the Plexor Analysis Software version 1.5.4.18 [12]. The real time amplification curves were simulated using the Weibull Model, as by Thompson [13] and the reaction efficiencies were estimated using the slope value of the Weibull function given as $y = a - b^{(-cx\wedge d)}$ in CurveExpert Professional version 1.6.0 [14]. This sigmoidal regression uses all of the points in the amplification to generate an equation to the PCR amplification curve. The Weibull *d* variable can be used as an estimate of the linear slope of the amplification the curve; this value can then be used to estimate reaction efficiency when R^2 values are 0.99 or greater [15]. Control PCR amplifications with no inhibitor present were assumed to amplify with the greatest efficiency, and all subsequent inhibited amplification efficiencies are represented as a percentage thereof.

Pairwise comparisons were performed on the average values of the control to the average values of the second inhibitor concentration (Table 1) for each amplicon of each inhibitor to determine the significance of changes seen in cycle threshold, melt temperature and efficiency values using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corporation, Armonk, NY).

3. Results and discussion

The goal of these experiments was to examine the overall ability of different internal control sequences to predict the

Table 2
The average difference in C_t between the control and the inhibited sample +/− the standard error. All boldface samples indicate a significant change from the control where $p < 0.05$. Italicized samples indicate the amplicon most affected by the presence of the inhibitor.

ΔC_t	Bile salts 1.0 ng/μL	Collagen 100 ng/μL	Guanidinium 4.5 ng/μL	Hematin 60 uM	Humic acid 40 ng/μL	Melanin 16 ng/μL	Tannic acid 11.25 ng/μL	Urea 300 mM
Autosomal	1.6 ± 0.1	**3.8 ± 0.2**	0.2 ± 0.1	**2.0 ± 0.2**	**2.5 ± 0.2**	**2.3 ± 0.2**	**0.6 ± 0.1**	**1.9 ± 0.2**
Y	**1.4 ± 0.1**	**5.2 ± 0.1**	**1.9 ± 0.1**	**4.3 ± 0.1**	**2.4 ± 0.2**	**1.4 ± 0.1**	**1.3 ± 0.1**	**1.4 ± 0.3**
53% GC 80bp	**1.1 ± 0.1**	**2.3 ± 0.1**	0.1 ± 0.1	**1.6 ± 0.1**	**1.1 ± 0.1**	**1.4 ± 0.1**	−0.3 ± 0.1	**0.9 ± 0.1**
53% GC 160bp	**2.6 ± 0.1**	***9.8 ± 0.8***	**1.3 ± 0.1**	**0.4 ± 0.1**	**1.3 ± 0.1**	**1.3 ± 0.1**	**1.3 ± 0.1**	**1.0 ± 0.1**
53% GC 230bp	***14.8 ± 0.9***	**8.6 ± 1.2**	**0.6 ± 0.1**	**2.3 ± 0.2**	**1.7 ± 0.1**	**3.1 ± 0.1**	**2.1 ± 0.1**	**2.1 ± 0.1**
44% GC 80bp	**1.5 ± 0.1**	**1.8 ± 0.1**	**−0.3 ± 0.1**	**1.3 ± 0.1**	**2.3 ± 0.4**	**2.0 ± 0.1**	**1.7 ± 0.1**	**1.2 ± 0.1**
44% GC 160bp	**3.9 ± 0.1**	**4.9 ± 0.1**	**1.1 ± 0.1**	**1.7 ± 0.1**	**1.7 ± 0.1**	**1.2 ± 0.1**	**0.5 ± 0.1**	**2.7 ± 0.1**
44% GC 230bp	**4.7 ± 0.5**	**5.4 ± 0.2**	***2.5 ± 0.1***	**2.9 ± 0.2**	**1.1 ± 0.1**	***4.1 ± 0.3***	**2.2 ± 0.1**	**2.7 ± 0.1**
32% GC 80bp	**0.9 ± 0.1**	**1.6 ± 0.1**	**−0.6 ± 0.1**	**1.7 ± 0.1**	**1.9 ± 0.1**	**2.7 ± 0.1**	**0.3 ± 0.1**	**1.0 ± 0.1**
32% GC 160bp	**2.1 ± 0.1**	**5.9 ± 0.1**	0.1 ± 0.1	**2.9 ± 0.1**	**2.7 ± 0.1**	**1.7 ± 0.1**	***2.5 ± 0.1***	**2.1 ± 0.1**
32% GC 230bp	**11.2 ± 0.9**	**5.9 ± 0.1**	**2.0 ± 0.1**	***3.7 ± 0.1***	***4.1 ± 0.1***	**2.8 ± 0.1**	**1.8 ± 0.1**	***4.1 ± 0.1***