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A Study of PCR Inhibition Mechanisms Using Real Time PCR*,†

ABSTRACT: In this project, real time polymerase chain reaction (PCR) was utilized to study the mechanism of PCR inhibition through examination of the effect of amplicon length, melting temperature, and sequence. Specifically designed primers with three different amplicon lengths and three different melting temperatures were used to target a single homozygous allele in the HUMTH01 locus. The effect on amplification efficiency for each primer pair was determined by adding different concentrations of various PCR inhibitors to the reaction mixture. The results show that a variety of inhibition mechanisms can occur during the PCR process depending on the type of co-extracted inhibitor. These include Taq inhibition, DNA template binding, and effects on reaction efficiency. In addition, some inhibitors appear to affect the reaction in more than one manner. Overall we find that amplicon size and melting temperature are important in some inhibition mechanisms and not in others and the key issue in understanding PCR inhibition is determining the identity of the interfering substance.

KEYWORDS: forensic science, inhibition, DNA typing, real time polymerase chain reaction, HUMTH01, humic acid, hematin, melanin, calcium

Degraded and environmentally challenged samples can produce numerous problems in forensic DNA typing including loss of signal, peak imbalance, and allele dropout. However, DNA degradation is not the only issue encountered when analyzing challenging samples. Many such samples contain substances which are co-extracted with the DNA and inhibit the polymerase chain reaction (PCR). While the effect of the presence of inhibitors is well known, the mechanism for PCR inhibition is often unclear. A better understanding of these processes should help the analyst recognize and troubleshoot problematic samples. This paper describes the utilization of real time PCR to study the mechanism of various PCR inhibitors and examines the effect of amplicon length, sequence, and melting temperature on the process.

While a number of methods have been developed to improve PCR amplification in the presence of inhibition (1–3), little is known of the underlying causes of inhibition in PCR. Three potential mechanisms include: (i) binding of the inhibitor to the polymerase (4,5); (ii) interaction of the inhibitor with the DNA; and (iii) interaction with the polymerase during primer extension.

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In previous work (6) we have determined that certain primers with a higher melting temperature are less affected by inhibition (Fig. 1), and that not all inhibitors have the same effect on different STR loci. This suggests that the sequence of the amplicon or primer may have an affect on PCR inhibition. Primers with higher melting temperatures are more strongly bound to the DNA and may possibly prevent the inhibitor from binding to the DNA. Alternatively, the inhibitor may bind to the DNA and block or interfere with primer extension. This could provide one explanation as to why shorter amplicons improve PCR sensitivity.

Inhibitors can also affect PCR efficiency through binding to the polymerase and/or blocking necessary reagents. The purpose of this research is to examine inhibited PCR reactions in an attempt to better understand the general mechanisms of these interactions. If inhibitors bind to the polymerase and deactivate it, template size, melting temperature, and sequence should not affect results and all amplicons should be inhibited at roughly the same rate. If the inhibitors bind to the DNA and are influenced by primer or sequence, sequences with different melting temperatures should be inhibited at different rates and the total amount of template available to the polymerase at that locus may be reduced. If the inhibitor interacts with the polymerase or template during primer extension, longer amplicons should be inhibited at lower inhibitor concentrations than shorter amplicons for the same locus.

Real time PCR (qPCR) was selected as a means of testing inhibition for several reasons. First, since it is a PCR process, inhibition can be detected due to changes in either the efficiency of the reaction (7) or by changes in the threshold cycle (C_t), which indicates that lower concentrations of DNA are being amplified (8). Second, analysis of the PCR product is possible through a measurement of the melt characteristics of the amplicon (9). A change in the melt curve demonstrates modification of the PCR product, presumably due to inhibitor binding. Third, a variety of inhibitor treatments may be directly compared by examining the relative amounts of PCR product produced by different levels of inhibition. Examination of

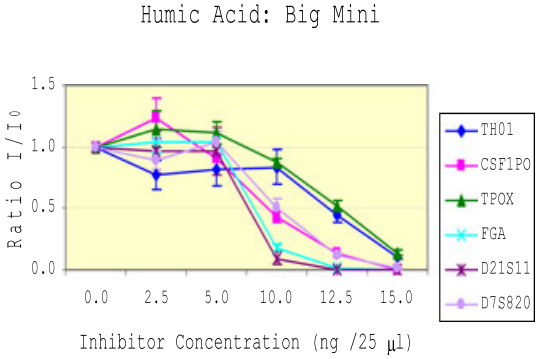

FIG. 1—*Inhibition by humic acid in amplification of various STR loci in the Big Miniplex STR kit. DNA samples were spiked with different concentrations of humic acid ranging from 0 to 15 ng/25 µL. I/I_0 is the ratio of signal with inhibitor in the sample to the signal without inhibitor in the sample. TH01 and TPOX are inhibited at a higher concentration than the other four loci. These two loci have the highest primer melting temperatures of the set. This suggests that the inhibitor is binding to the DNA and is displaced by the primers due to higher bond strength of the primers (6).*

these criteria should provide important information on how various types of inhibitors affect the amplification of DNA template during PCR and aid the analyst in identifying the particular class of inhibitor that is interfering with sample analysis.

Materials and Methods

DNA Standards

DNA standard K562 was used for primer optimization. For the inhibition tests, a standard solution of genomic DNA (TH01 9.3 homozygous genotype) was collected via multiple buccal swabs. The swabs were extracted by organic separation (phenol/chloroform/isoamyl alcohol [Sigma Aldrich, St. Louis, MO]) using a previously published protocol (10). The extracts were combined into one stock solution, quantified using the Alu qPCR protocol published by Nicklas et al. (11), and diluted to approximately 2 ng/µL concentration.

Primer Design

Primers for the HUMTH01 locus were designed using the GenBank sequence accession number D00269 and the online primer design program Primer3 (12). The default settings available were used for all parameters except product size, primer length, and primer melting temperature. A primer length of 20 bp was used as a default unless it was necessary to increase the length to improve specificity. Target amplicon size ranges were: 100–150, 200–300, and 300–400 bp; and target melting temperatures were: 58, 60, and 62°C. Nine sets of primers were designed to produce three amplicons (100, 200, and 300 bp) at each of the three melting temperatures. The oligonucleotide primers were manufactured by Integrated DNA Technologies (Coralville, IA) and were purified by standard de-salting by the manufacturer. In order to confirm the specificity of the amplification, amplification of the K562 standard DNA was performed for each of the nine primer sets using the Miniplex PCR protocol described previously (13) with 5 ng of template DNA. The products were separated and analyzed on the Agilent 2100 Bioanalyzer (Waldbronn, Germany) using the DNA 1000 Assay kit according to manufacturer's protocols (14).

TABLE 1—*Final inhibitor concentrations for the 20 µL reaction mix.*

Inhibitor	Units	1	2	3	4	5	6	7
Calcium	µM	0.1	0.2	0.3	0.4	0.5	0.6	0.7
Humic acid	ng/µL	0.5	1	1.5	2	2.5	3	3.5
Collagen	ng/µL	16	20	24	28	32	36	40
Melanin	ng/µL	1	1.5	2	2.5	3	3.5	4
Hematin	µM	1.5	1.75	2	2.25	2.5	2.75	3
Indigo	µM	100	150	200	250	300	350	400
Tannic acid	ng/µL	1.5	2	2.5	3	3.5	4	4.5

TABLE 2—*Size (approximate), melting temperature, and sequences for the final five TH01 primers.*

Size/T_m	Primer	Sequence
100 bp (T_m 60)	Forward	5′-AAATAGGGGGCAAAATTCAAAG-3′
	Reverse	5′-CACAGGGAACACAGACTCCAT-3′
200 bp (T_m 60)	Forward	5′-ATTGGCCTGTTCCTCCCTTA-3′
	Reverse	5′-CAAGGTCCATAAATAAAAACCCATT-3′
300 bp (T_m 60)	Forward	5′-GCAAAATTCAAAGGGTATCTGG-3′
	Reverse	5′-GGAAATGACACTGCTACAACTCAC-3′
300 bp (T_m 58)	Forward	5′-ATAGGGGGCAAAATTCAAAG-3′
	Reverse	5′-CCTGTGTCCCTGAGAAGGTA-3′
300 bp (T_m 62)	Forward	5′-AAATTCAAAGGGTATCTGGGCTCT-3′
	Reverse	5′-ACCTGGAAATGACACTGCTACAAC-3′

Real Time PCR Analysis

Real time PCR was performed on the Corbett Rotorgene 6000 (Corbett Robotics, Sydney, Australia), with SYBRGreenI (Invitrogen, Carlsbad, CA) intercalating dye. The reaction components were based on a previously published protocol (11), with three modifications. To enhance the effect of the various inhibitors, BSA was not added, the amount of Taq polymerase was reduced by half to 0.02 U/µL, and the primer concentrations were reduced by an order of magnitude to 0.21 µM. Additionally, Ramp Taq® polymerase (Denville Scientific, Metuchen, NJ) was used instead of AmpliTaq® Gold. A genomic DNA standard (homozygous 9.3 HUMTH01 STR allele) was added to the reaction mixture for a final concentration of 2 ng/µL. The inhibitor was added last to reach a final reaction volume of 20 µL. Control (noninhibitor) samples were performed using the same protocol, with an equivalent volume of ddH_2O used in place of the inhibitor.

Cycling conditions for the reaction were as follows: an initial hold for 10 min at 95°C; then cycling for 20 sec at 95°C to denature, 20 sec at an annealing temperature of 53, 55, or 58°C, depending on the melting temperature of the primer, and a 20 sec extension at 72°C. The melt cycle involved a 90 sec pre-melt at a temperature of 72°C followed by a temperature ramp from 72 to 95°C, with a 5 sec hold at each 1°C step of the ramp.

Inhibitor Preparation

The inhibitor stock solutions were prepared as follows: hematin (ICN Biomedicals, Aurora, OH), 100 mM in 0.1 N sodium hydroxide (Fisher Scientific, Waltham, MA); calcium hydrogen phosphate (Aldrich, Milwaukee, WI), 100 mM in 0.5 N hydrochloric acid (Fisher Scientific); indigo (Tokyo Kasei Kogo Co., Ltd, Tokyo, Japan), 100mM in 2% Triton X (Sigma); indigo carmine (MP Biomedicals, Aurora, OH), 100 mM in water; melanin (ICN Biomedicals), 1 mg/mL in 0.5 N ammonium hydroxide (Fisher Scientific); collagen (from calf skin) (Sigma), 1 mg/mL in 0.1 N acetic acid (Fisher); humic acid (Alfa Aesar, Ward Hill, MA), 1 mg/mL in water; and tannic acid (Sigma), 1 mg/mL in water. All subsequent dilutions were prepared in water.

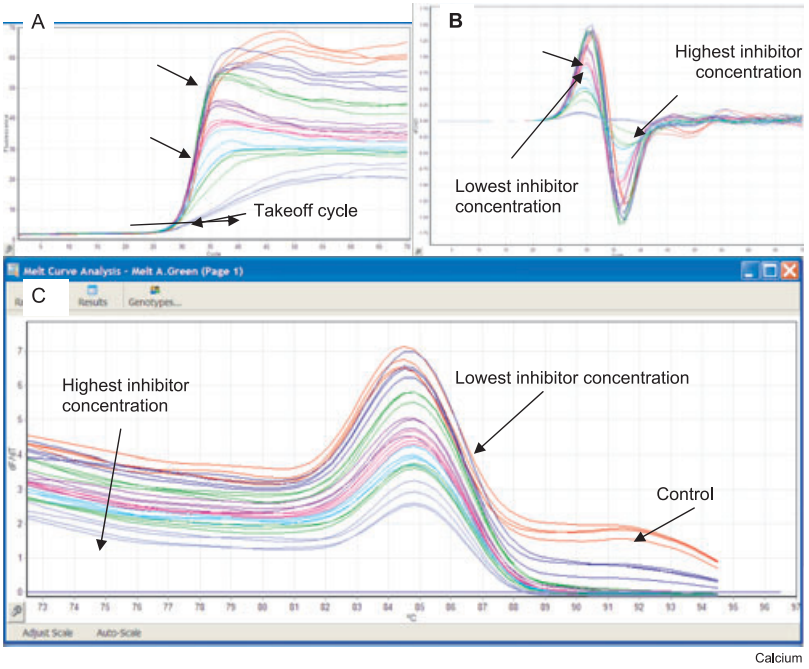

FIG. 2—*Real time data showing the effect of varying levels of calcium added to the 100 bp primer set (see Table 1 for concentrations). (A) Real time amplification curve, (B) comparative quantitation (first derivative of A), and (C) product melting temperature analysis. As seen in plot A, there is little effect on the take-off cycle (C_t), however the efficiency of reaction (slope of exponential amplification curve) changes greatly as does the final product concentration. In (C), the DNA melt curve shows little if any effect with added calcium. These results are consistent with calcium's role as a Taq inhibitor.*

Inhibitor Concentrations

A range of concentrations was tested to determine the concentration of inhibitor that would produce a change in the signal output. The starting concentrations were based on previous work with these inhibitors, where the concentration required for allele dropout with the miniSTR primer sets was determined (6). These qPCR tests were conducted using a primer set producing a 200 bp amplicon with a T_m of 60°C (Primer set 2). The final range of concentrations for each inhibitor is presented in Table 1.

Polymerase and Magnesium Tests

The maximum concentration of each inhibitor was used to test the effects of increased Taq polymerase and Magnesium. Three concentrations of Taq were tested: 1X, 1.5X, and 2X of the standard concentration (0.02 U/μL); and three concentrations of Magnesium were tested: 1X, 2X, and 3X of the standard concentration (62.5 mM). Additionally, a range of Taq concentrations from 1/4X to 2X were tested on noninhibited DNA to determine the effect of lower Taq concentrations on amplification with the TH01 primers.

Data Analysis

In examining the mechanism of PCR inhibition on amplification by real time PCR, four effects were examined, amplification efficiency, product quantity, take-off cycle, and melt curve. The first effect, differences in relative amplification efficiency, was evidenced by changes in the slope of the exponential amplification curve compared to the noninhibited control sample. The second effect was determined by the relative quantity of product. When the intensity of the qPCR amplification curve levels off at a lower relative fluorescence than the control, there is evidence of a limiting effect produced by a reduction in the availability of one or more of the components of the PCR reaction mixture (primers, Taq, magnesium, dye, or dNTPs). The third effect, a change in C_t value or "take-off cycle," indicates a relative decrease in the amount of DNA template available for amplification. The fourth effect is the melt curve for the PCR products produced following the qPCR. A lower melt temperature for the amplified products indicates that the strength of the hydrogen bonding of the product has decreased. Melt curve analysis is generally used to determine a change in the sequence of the PCR product. In these studies, the DNA sequence was held constant while the inhibitor concentration was varied. Thus a change in the melt curve indicates the presence of inhibitor binding to the DNA.

A comparison between amplicons of different lengths (with the same melting temperature) and primer sets with different melting temperatures (with the same amplicon length) was made to determine the effect of size and primer melting temperature on PCR inhibition. A ratio of the C_t cycle between the inhibited sample and the uninhibited sample (I_0/I) was calculated for each inhibitor concentration to determine the effect of the range of concentrations on the various primer sets.

Results and Discussion

The experimental design for this study utilized a series of primer sets to compare the effect of amplicon length and primer melting temperature (T_m). Three primer sets with the same melting temperature of 60°C producing amplicon lengths of 100, 200, and 300 bp were used to determine the effect of length on PCR inhibition. In addition, a second set of primers producing an amplicon length of 300 bp but with melting temperatures of 58, 60, and 62°C were used to determine the effect of melting temperature. Other primers producing 100 and 200 bp amplicons were not used as they were less efficient or did not produce clean amplification products. Overall, five primer pairs were selected (Table 2). Seven inhibitors were examined and their effects on PCR amplification were determined using the real time system.

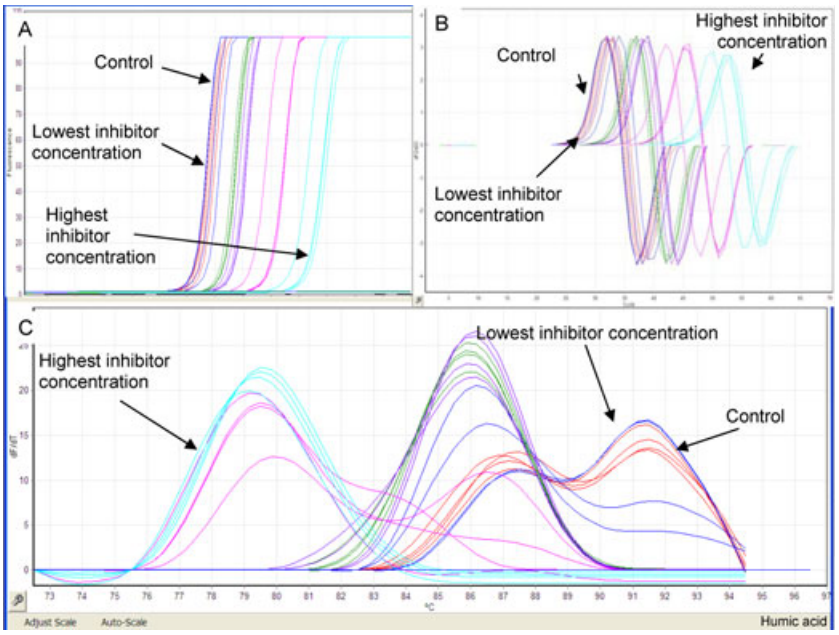

FIG. 3—*Real time data showing the effect of varying levels of humic acid added to the 300 bp primer set (Set 3) (see Table 1 for concentrations). (A) Real time amplification curve, (B) comparative quantitation (first derivative of A), and (C) product melting temperature analysis. As seen in plot A, there is a shift in the take-off cycle (C_t), however the efficiency of reaction (slope of exponential amplification curve) does not change, nor is there any major loss in product. In Fig. 2C, the DNA melt curve shows extensive changes with inhibitor concentration. These results are consistent with humic acid inhibiting the PCR through binding the DNA and reducing the amount of available template.*

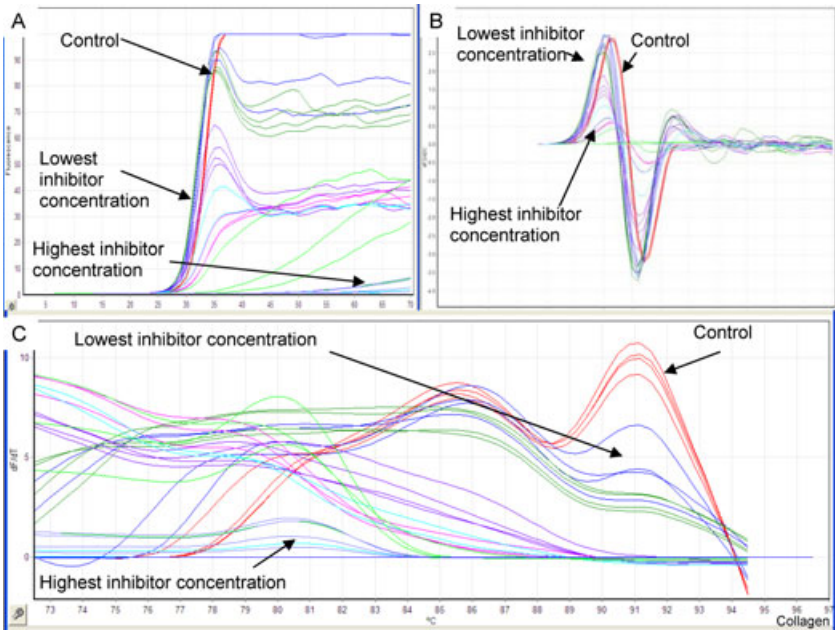

FIG. 4—*Real time data showing the effect of varying levels of collagen added to the 300 bp primer set (Set 3) (see Table 1 for concentrations). (A) Real time amplification curve, (B) comparative quantitation (first derivative of A), and (C) product melting temperature analysis. As seen in plot A, there is little effect on the take-off cycle (C_t), however the efficiency of reaction (slope of exponential amplification curve) changes greatly as does the final product concentration. In addition, a drop off in fluorescence occurs over time. In Fig. 2C, the DNA melt curve changes at higher levels of inhibitor. These results are consistent with Taq inhibition, but unlike calcium, there is also some binding to the DNA template at later stages of the reaction and higher inhibitor concentrations.*

Calcium

Calcium, a major inorganic component of bone (5) was the first inhibitor examined. Inhibition by calcium reduced the efficiency of the amplification, showed evidence of limiting reagents, and produced no change in the melt curve for all primer sets (Fig. 2). Addition of magnesium and Taq polymerase up to three times the normal concentration produced a minor increase in the amplification efficiency. There was no difference in C_t for the different size amplicons or the primer sets with different melting temperatures.

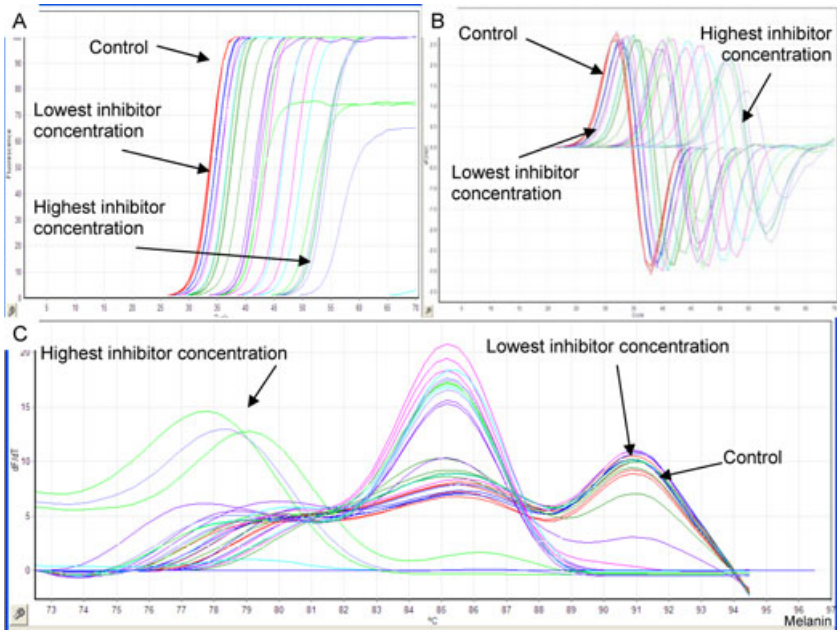

FIG. 5—*Real time data showing the effect of varying levels of melanin added to the 300 bp primer set (Set 3) (see Table 1 for concentrations). (A) Real time amplification curve, (B) comparative quantitation (first derivative of A), and (C) product melting temperature analysis. As seen in plot A, there is a strong effect on the take-off cycle* (C_t)*, however the efficiency of reaction (slope of exponential amplification curve) undergoes little change with [inhibitor]. In Fig. 2C, the DNA melt curve shows three transitions as the [inhibitor] increases. These results are consistent with melanin inhibiting the PCR through binding the DNA and reducing the amount of available template.*

These results were consistent with our expectation that calcium is a Taq inhibitor, competing with magnesium and reducing the reaction efficiency and total amount of product.

Humic Acid

Humic acid is a component in soils (15), and may be encountered in samples that have been buried, particularly in skeletal remains. Inhibition by humic acid did not reduce the efficiency of the amplification or show evidence of limiting reagents (Fig. 3). However, a change in the melt curve was observed for the two larger amplicons and for all primer sets there was an increase in the C_t cycle as the concentration of inhibitor rose. The smallest amplicon dropped out at the lowest inhibitor concentration and additional Taq or magnesium did not relieve inhibition. These results indicate that humic acid inhibits the PCR reaction through sequence specific binding to DNA, limiting the amount of available template.

Collagen

Collagen is a component in connective tissue and bone (16), and may be encountered in DNA extracts from skeletal samples. Inhibition by collagen reduced the amplification efficiency, and produced a change in the melt curve for all primer sets. There was slight reduction in C_t with inhibitor concentration for all amplicons, although the larger amplicons required higher inhibitor concentrations for the C_t to increase. Interestingly, for the larger amplicons, a loss of signal was observed during later cycles, presumably due to fluorescent quenching (Fig. 4). Additional Taq and magnesium did not appear to improve amplification of inhibited samples. Collagen, different from humic acid, appears to bind DNA but does not alter the availability of DNA template. Instead the binding appears to affect Taq processivity.

Melanin

Melanin is a pigment found in hair and skin, and is a possible inhibitor present in telogen hair samples (17). No change in efficiency, melt curve, or C_t cycle was observed for the smallest amplicon with the addition of melanin to the reaction mix. For all other amplicons, a loss of signal occurred at the highest inhibitor concentrations, an increase in the C_t cycle with inhibitor concentration was observed, and melt curve effects were observed (Fig. 5). The 100 bp amplicon was less affected by inhibition than the larger two amplicons, and the 60 T_m amplicon required a higher inhibitor concentration to produce a change in the C_t cycle. Additional Taq and magnesium did not improve amplification for inhibited samples. Thus melanin, like humic acid inhibits the PCR reaction through sequence specific binding to DNA, limiting the amount of available template. Smaller amplicons appear to be less inhibited by this material presumably due to fewer binding sites.

Hematin

Hematin is a metal chelating molecule found in red blood cells (18), and may be encountered in dried blood stains. Inhibition by hematin produced a reduction in final product formation (limiting effect) for all amplicons. A shift in the C_t cycle at high inhibitor concentrations was observed for all but the smallest amplicon, and minor changes were observed in melt curves for the larger amplicons. The larger amplicons were also affected by inhibitor concentration sooner than the small amplicons, and the amplicon with the lowest T_m appeared to be the least affected by inhibition (Fig. 6). Additional Taq did not reduce inhibition by hematin, but additional magnesium increased the effects of inhibition in samples with hematin. Based on the fact that there is minimal shift in the template melt curve we believe hematin to be a Taq inhibitor.

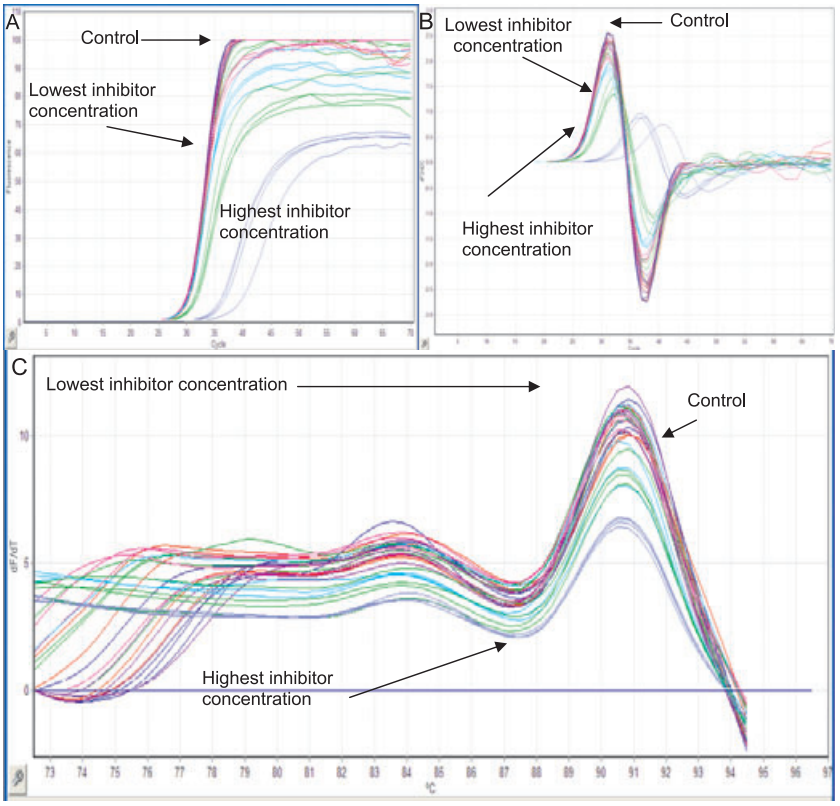

FIG. 6—*Real time data showing the effect of varying levels of hematin added to the 200 bp primer set (Set 2) (see Table 1 for concentrations). (A) Real time amplification curve, (B) comparative quantitation (first derivative of A), and (C) product melting temperature analysis. As seen in plot A, there is an effect on the take-off cycle at high inhibitor concentrations (C_t), as well as effects on the efficiency of reaction (slope of exponential amplification curve) and the production of PCR product. In Fig. 2C, the DNA melt curve shows minimal effects with increasing [inhibitor]. These results are consistent with hematin as a Taq inhibitor and also show its ability to reduce PCR product formation.*

Tannic Acid

Tannic acid is an agent found in leather, as well as in some types of plant material (19). It may also be encountered in samples which have been exposed to leaf litter. No change in the melt curve was observed for samples inhibited with tannic acid for any of the primer sets (Fig. 7). The smallest amplicon and lowest melting temperature primer set did not produce a C_t shift in the presence of tannic acid, however a C_t shift was observed for the larger amplicons. Some loss of product through limiting effects was observed for all primer sets but there was no significant change in reaction efficiency. Additional Taq and additional magnesium did relieve inhibition by tannic acid. Tannic acid thus appears to be a Taq inhibitor that also affects availability of the DNA template.

Indigo

Indigo is a dye used in certain types of fabrics, and this inhibitor may be encountered in DNA extracted from stains on denim or other dyed fabrics (20). Analysis of this inhibitor by qPCR proved to be problematic. Amplification could not be detected by the instrument due to interference by the dark blue color of the reaction mixture. It was decided that this was not a realistic representation of an inhibited sample, and the real time results indicated a loss of efficiency that was possibly related to the quenching of the dye.

Overall Results

The results of these experiments indicate that there are major differences in the mechanism by which different inhibitors affect the PCR reaction (Table 3). Some of the inhibitors, such as calcium and tannic acid, appear to be interacting with the polymerase. This is evidenced by the improvement in amplification with additional Taq enzyme, indicating a competitive inhibition reaction. Calcium, a divalent cation, is likely acting as a competitive inhibitor to magnesium, a cofactor for the polymerase enzyme. However, the addition of increased levels of magnesium to the reaction mixture does not relieve the inhibition. Tannic acid inhibition is reduced with both the addition of Taq and magnesium. Tannic acid contains a large number of electronegative groups, and could be chelating the magnesium which would render the Taq inoperable. The improvement of the reaction with an excess of magnesium supports this hypothesis. Humic acid produces both a shift in the C_t cycle and a melt curve change. For this substance both amplicon size and primer melting temperature affect the level of inhibition. This inhibitor is binding to the DNA and the effect is related to sequence and the strength of the hydrogen bonds in the amplicon.

Other inhibitors, such as hematin and melanin, appear to affect the processivity (rate of extension) of the DNA polymerase during primer extension. For these compounds, the larger size amplicons are more sensitive to inhibition than smaller ones, indicating that the polymerase is being affected during primer

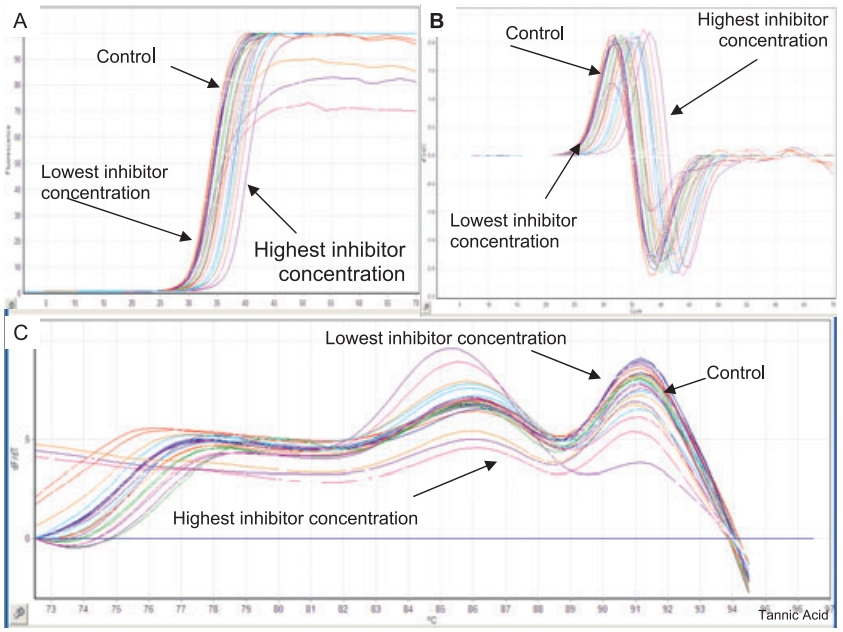

FIG. 7—*Real time data showing the effect of varying levels of tannic acid added to the 300 bp primer set (Set 3) (see Table 1 for concentrations). (A) Real time amplification curve, (B) comparative quantitation (first derivative of A), and (C) product melting temperature analysis. As seen in plot A, there is an effect on the take-off cycle (C_t), however the efficiency of reaction (slope of exponential amplification curve) does not change. In Fig. 2C, there are very minor DNA melt curve effects with added calcium. These results are consistent with tannic acid affecting the quantity of available DNA template.*

TABLE 3—*Summary of effects on qPCR for the five primer sets and seven inhibitors.*

Inhibitor	Melt	Efficiency	Limiting	C_t Shift	Other
Calcium		All	All		
Hematin	6	1,2,6,9	All	2,3,6,9	
Melanin	3,6		6	2,3,9	
Humic acid	2,3,6,9		6	All	
Collagen	2,3,6,9	1,2,3	1,6	1,3,6,9	3,9†
Tannic acid				2,3,9	
Indigo*					2*

Melt effects indicate changes in qPCR melt curves, efficiency effects indicate a reduction of slope of qPCR response, limiting effects indicate a loss in final product intensity and C_t shifts indicate an increase in cycle threshold with increasing inhibitor concentration. Primer sets: 1—100 bp T_m 60; 2—200 bp T_m 60; 3—300 bp T_m 60; 6—300 bp T_m 58; 9—300 bp T_m 62.

*Only one primer kit tested with indigo due to dye effect.

†Loss of intensity in later cycles.

extension. Since a change in the melt curve is also observed for these inhibitors, it is probable that the inhibitor is binding to the DNA rather than the polymerase. While tannic acid also produces a C_t shift (and loss of available DNA template) for the larger amplicons, it does not affect the melting temperature (Fig. 7). This indicates that the inhibitor is binding Taq instead of the DNA.

Collagen appears to be binding to the DNA due to a melt curve shift, but the larger amplicons are less affected. In addition, the signal from the amplified samples decreases with the number of cycles, which indicates some sort of effect (quenching) of the reaction. A possible explanation for this is that the collagen is overwhelming the DNA and reducing the signal obtained from the intercalating dye. The smaller amplicons would be more likely to be overwhelmed due to the size of the collagen molecule in comparison to the size of the amplified DNA of the smaller amplicon.

Hematin and indigo, as well as the highest concentrations of tannic acid and melanin, had melt curves where incomplete melting was present (the signal never reaches baseline at low temperatures). This same phenomenon, as well as the lower maximum level of amplification associated with limiting effects, was observed for lower concentrations of SYBR Green in uninhibited samples (Fig. 8). This suggests that these inhibitors function in such a way to limit the incorporation of the dye in the DNA strand, or have a quenching effect on the dye itself.

A summary of all results and effects is listed in Table 4.

Conclusions

A variety of inhibition mechanisms have been observed in the analysis of the inhibition of PCR by a variety of known inhibitors, and some inhibitors, such as tannic acid, appear to affect the reaction in more than one manner. While smaller amplicon size does appear to be advantageous in the propensity of inhibition for some compounds, this is not a consistent rule for all inhibitors. Thus the hypothesis that reduced sized amplicons are more efficient in amplifying samples that are inhibited is not always correct.

For those amplicons with higher primer melting temperatures, the sequence of the amplicon as well as the primer is likely to determine the level of inhibition for those inhibitors which bind (intercalate) with the DNA. For those inhibitors which are interfering with the Taq, the addition of PCR components such as Taq or magnesium may alleviate the problem, but the extent to which this will help may vary. While an understanding of the mechanism of these inhibitors can help the analyst in attempts to alleviate the problem, an identification of the inhibitors present and their relative concentrations are necessary to effectively address the problem. Identification of possible inhibition cannot always be made by

TABLE 4—*Summary of results of inhibition studies. Responses to increasing levels of inhibition are listed along with postulated mechanisms. Also listed are the effect of various additives (Taq, Mg, BSA) used to reduce inhibitory effects. Results with individual primer sets are listed in Table 3.*

Inhibitor	Effect on Melt Curve	Loss of Efficiency	Loss of Product Intensity	Reduction in C_t	Mechanism of Inhibition	Effect of Added Taq/Mg	Effect of Added BSA(6)
Calcium		Yes	Yes		Taq inhibitor	Minor improvement	No effect
Humic acid	Yes		Limited	Yes	DNA template binding	No effect	Reduced inhibition
Collagen	Yes	Some	Some	Yes	Taq inhibitor and DNA template binding	No effect	No effect
Melanin	Some		Limited	Some	DNA template binding/amplicon size effect	No effect	Reduced inhibition
Hematin		Yes	Yes	Yes	Taq inhibitor/amplicon size effect	Increased inhibition w/Mg	Reduced inhibition
Tannic acid				Some	Taq inhibitor but no loss of efficiency	Reduced inhibition	Reduced inhibition
Indigo*					n/a	n/a	n/a

Note: Response was listed as yes if at least four of the five primer sets exhibited an effect. If two–three of five primer sets exhibited an effect the response was listed as some. The response was listed as limited if only one primer set exhibited an effect.

*Indigo interfered with real time fluorescence and could not properly be tested.

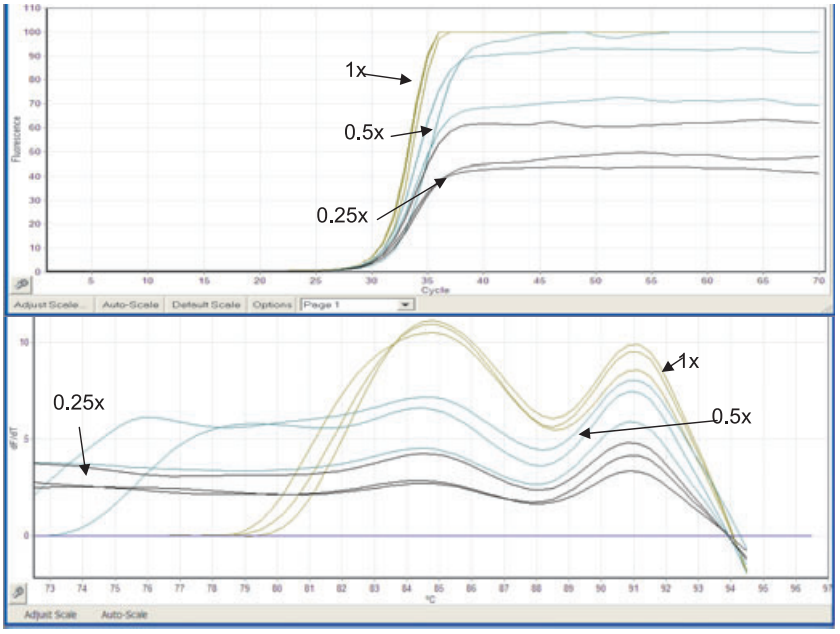

FIG. 8—*Real time data showing the effect of varying levels of SYBR Green added to the primer set 2 (200 bp). (A) Real time amplification curve, (B) comparative quantitation (first derivative of A), and (C) product melting temperature analysis. As seen in plot A, there is little effect on the take-off cycle (C_t), however the efficiency of reaction (slope of exponential amplification curve) changes as does the apparent final product concentration. In Fig. 2C, the DNA melt curve shows minor effects as the [SYBR Green] is dropped. As SYBR Green is the visualizing agent for all reactions, these data indicate a potential effect that could occur if inhibitors block the interaction of SYBR Green with product.*

visual inspection, but the qPCR data can indicate the presence of these inhibitors.

With the exception of calcium and collagen, additional BSA can often relieve inhibition when added to the PCR reaction (6). Sample dilution is also a useful technique but will further reduce template concentration. Other treatments, such as rinsing the sample with NaOH (1) or purification with silica based spin columns (2) or agarose (6) result in a loss of DNA template (21).

Overall, knowledge of the type of inhibitor present, especially melt curve data from SYBR Green based qPCR data should help the analyst select the best method to effectively remove inhibitors without compromising the amount of DNA or further compromising the PCR reaction. This knowledge will also help the analyst determine the type of STR analysis to use, and if reduced sized amplicons will improve their results.

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