Int J Legal Med (2012) 126:961–968
DOI 10.1007/s00414-011-0616-z

TECHNICAL NOTE

Effects of humic acid on DNA quantification with Quantifiler® Human DNA Quantification kit and short tandem repeat amplification efficiency

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Received: 21 April 2011 / Accepted: 25 August 2011 / Published online: 13 September 2011

Abstract Correct DNA quantification is an essential part to obtain reliable STR typing results. Forensic DNA analysts often use commercial kits for DNA quantification; among them, real-time-based DNA quantification kits are most frequently used. Incorrect DNA quantification due to the presence of PCR inhibitors may affect experiment results. In this study, we examined the alteration degree of DNA quantification results estimated in DNA samples containing a PCR inhibitor by using a Quantifiler® Human DNA Quantification kit. For experiments, we prepared approximately 0.25 ng/μl DNA samples containing various concentrations of humic acid (HA). The quantification results were 0.194–0.303 ng/μl at 0–1.6 ng/μl HA (final concentration in the Quantifiler reaction) and 0.003–0.168 ng/μl at 2.4–4.0 ng/μl HA. Most DNA quantity was undetermined when HA concentration was higher than 4.8 ng/μl HA. The C_T values of an internal PCR control (IPC) were 28.0–31.0, 36.5–37.1, and undetermined at 0–1.6, 2.4, and 3.2 ng/μl HA. These results indicate that underestimated DNA quantification results may be obtained in the DNA sample with high C_T values of IPC. Thus, researchers should carefully interpret the DNA quantification results. We additionally examined the effects of HA on the STR amplification by using an Identifiler® kit and a MiniFiler™ kit. Based on the results of this study, it is thought that a better understanding of various effects of HA would help researchers recognize and manipulate samples containing HA.

Keywords DNA quantification · Real-time PCR · Short tandem repeat · Humic acid · PCR inhibitor

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Introduction

Short tandem repeat (STR) typing is a useful technology for identifying individuals in forensics. Commercially available STR kits such as the AmpFlSTR® Identifiler® PCR Amplification kit (Applied Biosystems, Warrington, UK) and PowerPlex® 16 System (Promega, Madison, WI, USA) have been extensively used for STR typing. For obtaining reliable STR typing results by using the kits, it is necessary to use the proper amount of DNA template recommended by the commercial STR kit manufacturer [1, 2]. Typically commercial STR kits work optimally with an input DNA template of approximately 1 ng. If the incorrect amount of the template is added to the PCR, several phenomena such as heterozygote peak imbalance, allele dropout, split peak, and off-scale peak can occur [3–5]. In addition, because

forensic evidence can contain minute amounts of DNA, it is necessary that limited material be utilized optimally. For these reasons, researchers often quantify amounts of DNA template prior to STR amplification. There are various methods for DNA quantification such as UV spectrophotometry, fluorimetric assay, and real-time PCR assay [6, 7]. Among these methods, DNA quantification kits based on real-time PCR assays are mainly used because they have the advantage of reflecting both the quality and quantity of the DNA template [8–12].

DNA extracted from forensic samples may contain PCR inhibitors which can prevent the amplification process. DNA extraction from remains buried in soil may result in the co-extraction of soil components, mainly humic acid (HA) or other humic substances [8]. Humic substances represent a mixture of partially characterized polyphenols that are produced from chemical and/or biological decomposition of organic matter [13]. These compounds readily co-purify with DNA and may inhibit PCR amplification and cause false negative results [14, 15].

Some studies have reported that HA interferes with DNA quantification by UV spectrophotometry and fluorimetric assay [16, 17]. However, no research on the relative suppression of quantitative PCR results as a function of HA content, with respect to DNA quantity, has yet been conducted. Although the fact that DNA quantification is unstable when the PCR inhibitor concentration increases can be deduced from the kit manual [18], it is difficult to derive any further explanation for the effects of PCR inhibitors on DNA quantification results, because the company's validation study on a DNA quantification kit focused on a correlation of the internal PCR control (IPC) and PCR inhibition levels. An alternative correlation analysis of DNA quantification results and the presence of PCR inhibitors in a real-time PCR assay would be more relevant to DNA analysis using STR amplification. We attempted to examine the degree of alteration of DNA quantification results estimated in DNA samples containing HA by using a Quantifiler® Human DNA Quantification kit (Applied Biosystems).

It is known that HA negatively affects STR amplification with commercial STR kits [19, 20]. STR kits optimized for the genotyping of degraded and/or inhibited DNA samples are used for challenged samples [21, 22]. However, the efficiency of these kits in overcoming PCR inhibition by HA is not calculated as a numerical value. Therefore, we attempted to amplify samples containing various HA concentrations by using an AmpFℓSTR® Identifiler® PCR Amplification kit and an AmpFℓSTR® MiniFiler™ PCR Amplification kit (Applied Biosystems) which is optimized for challenged samples. We analyzed the phenomena of PCR inhibition obtained from the two STR kit reactions.

Material and methods

Sample preparation

Three human DNA samples, K562 DNA (Promega), Raji DNA (Applied Biosystems), and G147A DNA (Promega) were prepared. The initial concentration of these commercially available DNA samples was 852, 200, and 210 ng/μl, respectively. The DNA samples were diluted to a concentration of 0.5 ng/μl in TE buffer (10 mM Tris, 0.1 mM EDTA (TE-4), pH 8.0). HA (Fluka, Buchs, Switzerland) was prepared at 1,000 ng/μl in 10 mM NaOH, and the HA solution was diluted to concentrations ranging from 20 to 400 ng/μl in distilled water. Equal DNA sample and HA solution volumes were mixed, producing 0.25 ng/μl DNA samples containing various HA concentrations in the 10–200 ng/μl range.

Measurement of DNA quantification value and level of PCR inhibition

Three DNA samples containing 0, 10, 20, 30, 40, 50, 60, and 70 ng/μl of HA were used for Quantifiler® Human DNA Quantification kit (Applied Biosystems) reactions. The reaction was conducted twice in a total volume of 25 μl containing 2 μl of DNA sample containing HA, 10.5 μl of Quantifiler human primer mix, and 12.5 μl of Quantifiler PCR mix according to the manufacturer's protocols [18] with the ABI Prism® 7,000 Sequence Detection System (Applied Biosystems). When 2 μl of samples were added to the 25 μl Quantifiler reaction, the final concentrations of HA were 0, 0.8, 1.6, 2.4, 3.2, 4.0, 4.8, and 5.6 ng/μl. Data analysis was performed by using 7,000 SDS software v1.1 (Applied Biosystems). According to the Quantifiler manual [18], the presence of PCR inhibitors was estimated by the C_T value of IPC. A C_T value of over 30 cycles may indicate PCR inhibition in the DNA sample.

DNA concentration is often estimated by using a UV spectrophotometer. We attempted to determine the effects of HA on DNA quantification values by using a NanoDrop® ND-1,000 (NanoDrop Technologies, Wilmington, DE, USA) as a UV spectrophotometer and to compare the values with those from a Quantifiler kit. The quantification by the NanoDrop was conducted in duplicate with the same samples which were used for Quantifiler reactions.

STR amplification and data analysis

Of the three DNA samples, K562 and Raji DNA were used for STR amplification. Because G147A DNA was made by a mixture of various human DNAs, STR results were difficult to represent and thus, G147A DNA was excluded

from STR amplification. STR amplification was conducted twice by using both the AmpFℓSTR® Identifiler® and AmpFℓSTR® MiniFiler™ PCR Amplification kits (Applied Biosystems). PCR amplification by using the Identifiler kit was performed in a reaction volume of 10 μl containing 4 μl of PCR Mix, 2.0 μl of Primer Set, 1 U AmpliTaq Gold® DNA Polymerase, and 3.8 μl of DNA sample containing HA. PCR amplification by using the MiniFiler kit was performed in a reaction volume of 10 μl containing 4 μl of Master Mix, 2.0 μl of Primer Set, 0.2 μl of distilled water, and 3.8 μl of DNA sample containing HA. When 3.8 μl of DNA samples were added to the 10 μl STR amplification, the final concentrations of HA were from 0 to 76 ng/μl. Thermal cycling was performed by using the GeneAmp® PCR system 9700 (Applied Biosystems) under each kit's recommendation condition [1, 23]. Typing of PCR products was carried out on an ABI 3,130 Genetic Analyzer (Applied Biosystems), and the data were analyzed by using Genemapper ID v3.2 (Applied Biosystems). The data from the Identifiler and MiniFiler were analyzed with peak thresholds of 50 and 100 relative fluorescence units (RFU), respectively.

Results and discussion

DNA quantification values by using a Quantifiler kit

The mean DNA quantification values of the three DNA samples with non-HA were 0.234–0.275 ng/μl (Table 1). The quantification values were 0.228–0.303 ng/μl at 0.8 ng/μl HA (final concentration in Quantifiler reactions), 0.194–0.279 ng/μl at 1.6 ng/μl HA, 0.122–0.168 ng/μl at 2.4 ng/μl HA, 0.045–0.066 ng/μl at 3.2 ng/μl HA, 0.003–0.014 ng/μl at 4.0 ng/μl HA, and almost undetermined at ≥4.8 ng/μl HA. At ≤1.6 ng/μl HA, the constant patterns of the changes in quantification values were not identified, showing that the values were increased or decreased by the DNA samples tested when compared as Quantifiler reaction results with non-HA. However, the quantification values were decreased by 38.5–51.4% in the reactions with all of the three samples at 2.4 ng/μl HA and decreased by ≥71.8% in the reactions at ≥3.2 ng/μl HA. These results indicate that HA concentrations exceeding a certain value may interfere with DNA quantification by using the Quantifiler kit and lead to underestimated quantification or false negative results.

The presence of PCR inhibitors was estimated by the C_T value of IPC. The mean C_T values of the IPC of all DNA samples with non-HA were 28.0 (Table 1). The C_T values of IPC ranged between 28.4 and 31.0 when Quantifiler reactions were conducted with 0.8–1.6 ng/μl HA. These results indicate that HA concentrations in this range do not significantly affect PCR amplification. However, the C_T values of IPC were 36.5–37.1 at 2.4 ng/μl HA and undetermined at ≥3.2 ng/μl HA. These results indicate that HA concentration in this range may affect PCR amplification.

The reason for unreliable DNA quantification results obtained from samples containing high level of HA may be that a Quantifiler kit is manufactured based on PCR amplification. The Quantifiler kit is designed that DNA concentration and PCR inhibition level can be detected by different fluorescent dyes, such as FAM™ and VIC®, respectively. However, because the PCR inhibitor spiked with the DNA sample when concentrations of HA were increased and resulted in PCR inhibition, DNA quantification was adversely affected. Therefore, it is necessary for researchers to know the point that underestimated DNA quantification results appear. This result was commonly observed at an HA concentration of 2.4 ng/μl, showing 38.5–51.4% decreases in quantification values compared to those of non-HA. In addition, the C_T values of IPC were 36.9, 37.1, and 36.5 at K562, Raji, and G147A DNA samples, respectively. The similar phenomena were observed when the HA concentration is 2.4 ng/μl in Quantifiler reaction with 0.1 ng/μl DNA, showing a mean of 34.7% decreased quantification values compared to those from non-HA; the C_T values of IPC were 32.0, 33.0, and 31.7 at K265, Raji, and G147A DNA samples, respectively (data not shown). These results indicate that DNA quantification value can be underestimated when the C_T value of IPC is around 32. Therefore, researchers should carefully interpret DNA quantification results regarding C_T values of IPC because a high C_T value of IPC can show underestimated DNA quantification results.

If researchers input the DNA template into the STR amplification by the quantification value obtained from the Quantifiler kit without considering the C_T value of IPC, which is high, despite following DNA quantity by the manufacturer's recommendation, unstable STR typing results can be obtained. Because the amount of template DNA containing a PCR inhibitor is larger in STR amplification reactions, the following results can be obtained: STR amplification is inhibited because the PCR inhibitor increases in STR reactions with excessive input of DNA template containing PCR inhibitors. In contrast, off-scale or split peaks may occur by the use of STR kits that are designed to overcome high levels of PCR inhibitors, such as the MiniFiler kit. We must note that incorrect DNA quantification results by the presence of PCR inhibitors can cause unstable STR results. Therefore, if researchers understand the alteration in DNA quantification values of samples containing PCR inhibitor using the Quantifiler kit, they can easily deal with STR typing problems occurred by incorrect quantification results as aforementioned. Taken together, our study may help researchers to reduce the

Table 1 DNA quantification values by the Quantifiler kit and the NanoDrop ND-1,000 and C_T values of IPC by the Quantifiler kit

HA (ng/μl)	DNA quantification value (ng/μl)			C_T value of IPC
	Quantifiler kit		NanoDrop	Quantifiler kit
	Mean	Alteration rate of mean DNA quantification value (%)	Mean	Mean
K562				
0	0.251±0.04[a]	–	1.1±0.85[a]	28.0±0.09[a]
0.8	0.228±0.00	−9.2[b]	9.4±0.78	28.9±0.00
1.6	0.194±0.04	−22.7	14.1±2.40	31.0±0.45
2.4	0.122±0.03	−51.4	23.4±1.48	36.9±0.01
3.2	0.045±0.02	−82.1	31.3±0.07	Und.
4.0	0.003±0.00	−98.8	41.0±0.42	Und.
4.8	Und.	Und.	48.3±1.06	Und.
5.6	Und.	Und.	56.9±0.14	Und.
Raji				
0	0.275±0.02	–	0.9±0.42	28.0±0.07
0.8	0.303±0.01	+10.2	13.2±0.21	28.4±0.02
1.6	0.279±0.04	+1.5	19.5±0.85	30.7±0.06
2.4	0.168±0.01	−38.9	28.3±1.48	37.1±1.33
3.2	0.061±0.03	−77.8	34.5±0.57	Und.
4.0	0.008±0.00	−97.1	43.1±0.71	Und.
4.8	0.000/Und.	Und.	51.1±0.71	Und.
5.6	Und.	Und.	59.9±1.13	Und.
G147A				
0	0.234±0.00	–	1.4±0.71	28.0±0.06
0.8	0.263±0.02	+12.4	10.8±0.28	28.4±0.02
1.6	0.215±0.02	−8.1	17.9±0.07	29.9±0.31
2.4	0.144±0.01	−38.5	26.0±0.85	36.5±0.43
3.2	0.066±0.01	−71.8	33.5±0.99	Und.
4.0	0.014±0.00	−94.0	43.2±1.34	Und.
4.8	0.001±0.00	−99.6	48.8±0.28	Und.
5.6	Und.	Und.	57.9±1.48	Und.

C_T threshold cycle for log phase amplification of IPC, *Und.* undetermined

[a]Values of DNA quantification and C_T of IPC are the means of results obtained from duplicate experiments

[b]Values are the alteration ratios of DNA quantification values based on Quantifiler reaction results with no HA

unnecessary use of DNA template in STR amplification, for which only a limited amount of DNA could be available.

Comparison of DNA quantification results by using Quantifiler kit and NanoDrop

DNA quantification values by using the NanoDrop showed extreme differences from the Quantifiler kit results (Table 1). The mean quantification value was 0.9–1.4 when 0.25 ng/μl DNA samples containing no HA were tested. Quantification values were 9.4–13.2 and 56.9–59.9 ng/μl at DNA samples containing 10 and 70 ng/μl HA, respectively, which corresponded to 0.8 and 5.6 ng/μl HA in the Quantifiler reactions, respectively. The results demonstrate that DNA quantification values by using a NanoDrop are overestimated, while those by using a Quantifiler kit are underestimated due to an increase in the HA concentration.

In the NanoDrop results, HA concentrations can be reflected in the DNA quantification values. Earlier studies have explained the reason by the fact that since HA possesses high absorption coefficients in the UV spectral range, it considerably impairs nucleic acid quantification by using a UV spectrophotometer [16]. It is difficult to find out previous studies on the difference in DNA quantification values estimated by using a Quantifiler and a NanoDrop in DNA samples containing PCR inhibitors. In our study, we observed different patterns of response to HA by each method (Fig. 1).

STR typing results of samples containing HA

In the Identifiler results, all of the 16 STR loci were amplified at 0–3.8 ng/μl HA (final concentration in the Identifiler) (Table 2). At 7.6 ng/μl HA, 15–16 loci were

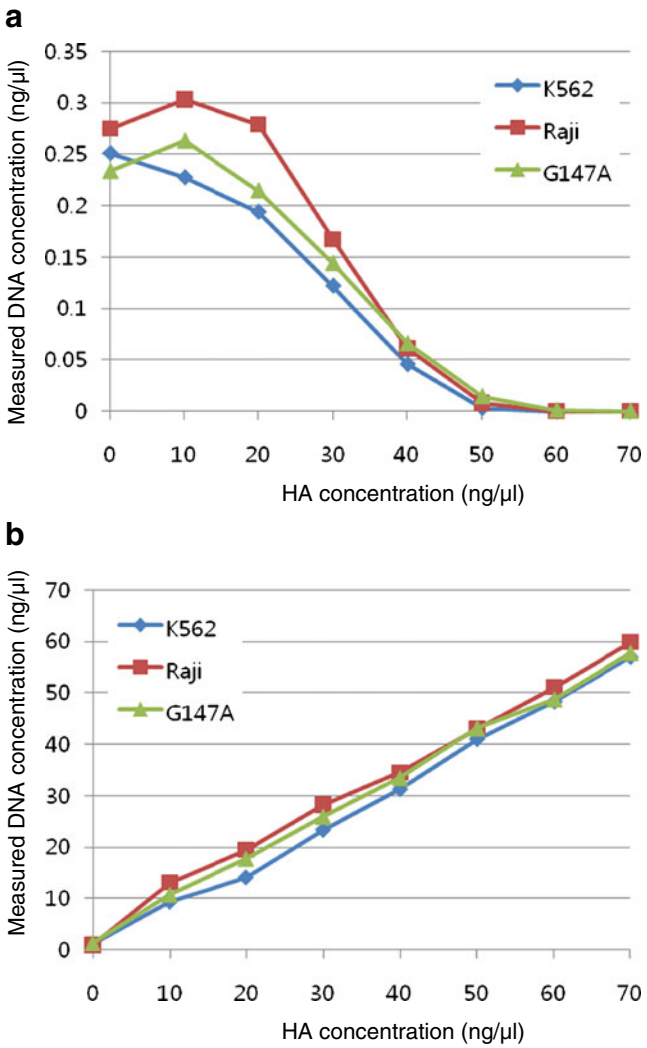

Fig. 1 Comparison of DNA quantification results by using the Quantifiler kit (**a**) and the NanoDrop (**b**). The values of the *X*-axis mean HA concentrations in DNA samples. HA concentrations ranging from 10 to 70 ng/μl in (**a**) corresponded to those ranging from 0.8 to 5.6 ng/μl in Quantifiler reactions

amplified. These results show that the HA concentration range does not significantly affect the STR amplification efficiency. However, the number of STR loci amplified was significantly decreased at ≥11.4 ng/μl HA, only seven to eight loci of the 16 STR loci were amplified. At ≥15.2 ng/μl HA, the amplification was almost inhibited. In the MiniFiler results, all of the nine STR loci were amplified at 0–34.2 ng/μl HA (Table 3). At 38 ng/μl HA, eight to nine loci were amplified. The PCR inhibition was relatively evident at ≥45.6 ng/μl HA, where 5–7 loci of the nine STR loci were amplified. The number of loci amplified was continuously decreased and almost all loci were not amplified at ≥68.4 ng/μl HA. From the results obtained from the two STR kits, we know that the MiniFiler kit can overcome inhibitory effects of HA five times better than the Identifiler kit based on STR amplification (7.6 ng/μl HA in the Identifiler and 38 ng/μl HA in the MiniFiler).

Based on the relationship between the Identifiler results and the C_T values of the IPC obtained from the same DNA samples, it is conceivable the C_T values may significantly reflect the levels of PCR inhibition. The number of amplified STR loci was decreased by an increase in the C_T value of over 30 cycles. When the C_T values were 28.0–31.0, 36.9–37.1, and undetermined, 15–16, 7–8, and 0–2 STR loci were amplified in the Identifiler reactions. In the MiniFiler results, all of the STR loci were amplified even at 34.2 ng/μl HA, although the C_T values of the IPC were undetermined at this HA concentration. It is probable that negative results are obtainable with the Identifiler kit in samples with undetermined C_T values which are frequently observed in forensic practice. The MiniFiler kit can produce STR loci even in similar situations. The use of the MiniFiler kit rather than the Identifiler kit would save DNA template, experiment materials, and time in problematic samples. If the sample with an increased C_T value of the IPC shows negative results even by using the MiniFiler kit, various methods to overcome such problems should be applied to the sample considering the amount of DNA or PCR inhibitors. A previous study using dilution method reported that when both DNA quantity and the C_T value of the IPC were undetermined in DNA extracted from bloodstains on cotton, an adequate amount of DNA without inhibiting STR amplification was obtainable after a ten-fold dilution of the sample [24]. However, if there is a suspicion of a small amount of DNA and a large amount of inhibitors in samples, the dilution method would be ineffective for positive STR results. In this case, a direct approach for removing inhibitors by using an additional washing method or an alternative DNA extraction would be more effective.

Overall, relatively long amplicons were inhibited early in both kits due to an increase in HA concentration. However, amplicon size was not the only factor for the PCR inhibition. The PCR inhibition level by HA was different between individual STR loci. In the Identifiler results of K562 DNA samples containing 11.4 ng/μl HA, TPOX (alleles 8, 9; 229.60, 233.54 bp) locus was amplified, but D21S11 (alleles 29, 30, 31; 203.98, 207.91, 211.86 bp) locus was not amplified (Fig. 2a, b). In the MiniFiler results of Raji DNA samples containing 53.2 ng/μl HA, D2S1338 (alleles 22, 22; 147.80 bp) and D18S51 (alleles 17, 17; 164.08 bp) loci were amplified, but D16S539 (alleles 8, 11; 87.38, 99.69 bp) locus, which is the shortest amplicon, was not amplified (Fig. 2c–e). Similar results were observed in K562 samples with the MiniFiler reactions. Also, it was found that D2S1338 and CSF1PO loci were more amplified

Table 2 Identifiler results with DNA samples containing various HA concentrations

Locus	HA concentration in Identifiler reactions (ng/μl)									
	0–3.8		7.6		11.4		15.2		19	
	K562	Raji	K562	Raji	K562	Raji	K562	Raji	K562	Raji
C_T value of IPC[a]	28.0–28.9	28.0–28.4	31.0	30.7	36.9	37.1	Und.	Und.	Und.	Und.
D19S433	14,14.2[b]	14,14.2[b]	+	+	+	+	−	+	−	−
Amelogenin	*X*	*X,Y*	+	+	+	+	−	+	−	−
D3S1358	16	15,16	+	+	+	+	−	−	−	−
D8S1179	12	14,15	+	+	+	+	−	−	−	−
D5S818	11,12	10,13	+	+	−	+	−	−	−	−
vWA	16	16,19	+	+	+	+	−	−	−	−
TH01	9.3	6,7	+	+	+	+	−	−	−	−
D21S11	29,30,31	28,31	+	+	−	−	−	−	−	−
FGA	21,24	19,27	+	+	−	−	−	−	−	−
D13S317	8	13	+	+	−	−	−	−	−	−
TPOX	8,9	8,13	+	+	+	+	−	−	−	−
D16S539	11,12	8,11	+	+	−	−	−	−	−	−
D7S820	9,11	10	+	+	−	−	−	−	−	−
D18S51	15,16	17	−	+	−	−	−	−	−	−
CSF1PO	9,10	10,12	+	+	−	−	−	−	−	−
D2S1338	17	22	+	+	−	−	−	−	−	−
Numbers of amplified loci	16	16	15	16	7	8	0	2	0	0

+ when all alleles on each STR locus are repeatedly amplified in duplicated amplification,− when all alleles on each STR locus are not repeatedly amplified

[a] The C_T values of the IPC are the same as those which are shown in Table 1. HA concentrations ranging from 0.8 to 4.0 ng/μl in Quantifiler reactions corresponded to those ranging from 3.8 to 19.0 ng/μl in Identifiler reactions because DNA samples used in the Identifiler reactions were the same as those used in the Quantifiler reactions

[b] STR genotypes obtained from each DNA sample are presented

Table 3 MiniFiler results with DNA samples containing various HA concentrations

Locus	HA concentration in MiniFiler reactions (ng/μl)													
	0–34.2		38		45.6		53.2		60.8		68.4		76	
	K562	Raji	K562	Raji	K562	Raji	K562	Raji	K562	Raji	K562	Raji	K562	Raji
D16S539	11,12[a]	8,11[a]	+	−	−	−	−	−	−	−	−	−	−	−
CSF1PO	9,10	10,12	+	+	+	+	+	+	+	+	+	−	−	−
Amelogenin	*X*	*X,Y*	+	+	+	−	+	−	−	−	−	−	−	−
D13S317	8	13	+	+	+	+	+	+	+	−	−	−	−	−
D2S1338	17	22	+	+	+	+	+	+	+	+	+	−	−	−
D18S51	15,16	17	+	+	+	+	+	+	−	−	−	−	−	−
D7S820	9,11	10	+	+	−	−	−	−	−	−	−	−	−	−
FGA	21,24	19,27	+	+	+	−	−	−	−	−	−	−	−	−
D21S11	29,30,31	28,31	+	+	+	+	−	−	−	−	−	−	−	−
Numbers of amplified loci	9	9	9	8	7	5	5	4	3	2	2	0	0	0

+ when all alleles on each STR locus are repeatedly amplified in duplicated amplification,− when all alleles on each STR locus are not repeatedly amplified

[a] STR genotypes obtained from each DNA sample are presented

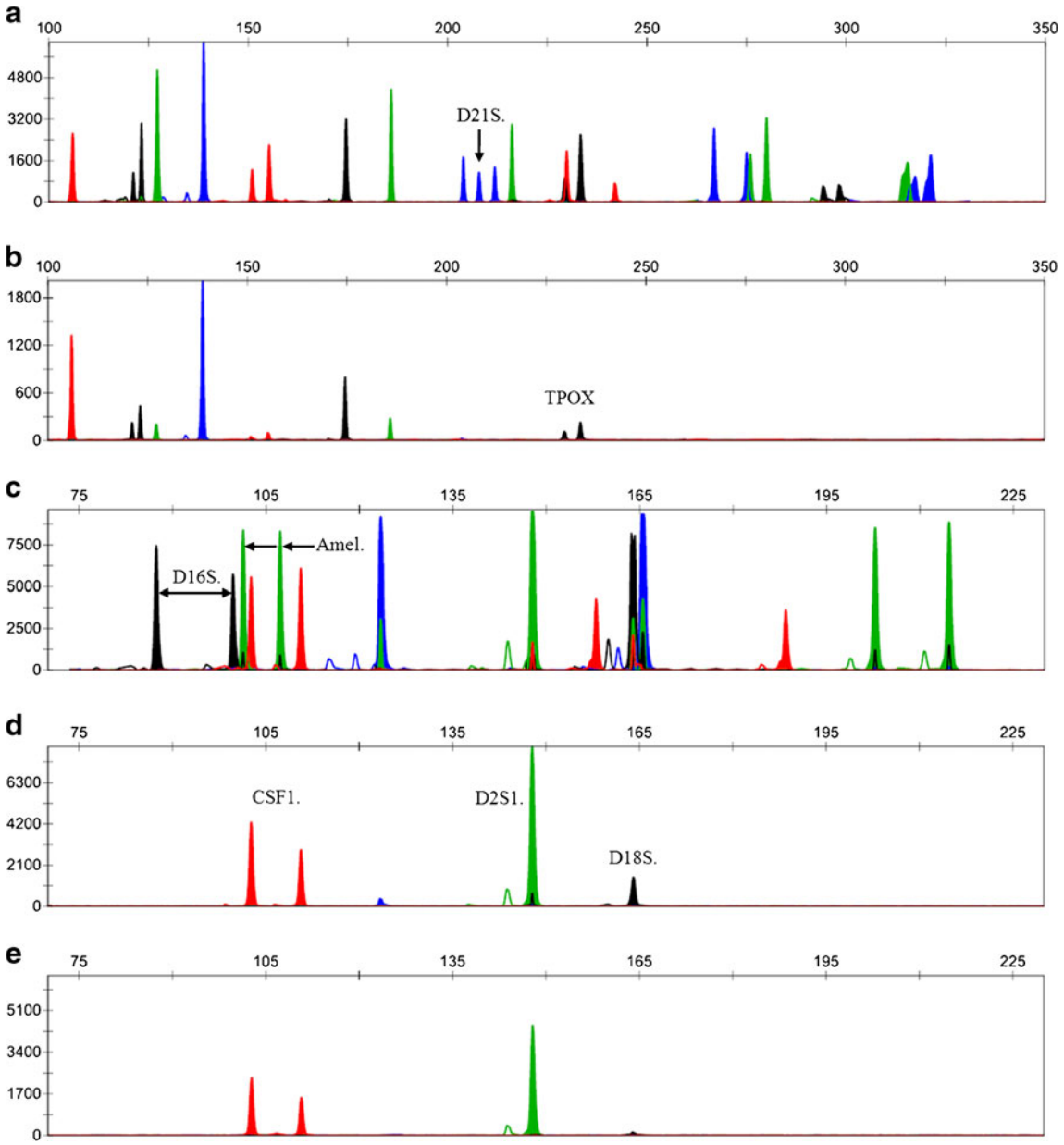

Fig. 2 Representative electropherograms obtained from DNA samples containing HA by using the Identifiler and MiniFiler kits. The electropherogram of (**a**, **b**) was obtained from K562 DNA samples containing 0 and 11.4 ng/μl HA by using the Identifiler kit. Electropherogram of (**c**–**e**) was obtained from Raji DNA samples containing 0, 53.2, and 60.8 ng/μl HA by using the MiniFiler kit, respectively

than any other loci at a high concentration of HA in the MiniFiler results. The results indicate that HA does not exert the same effect on different STR loci. Opel et al. [20] have reported that the PCR inhibition level in STR loci can be affected by primer character and that certain primers with a high melting temperature are less affected by PCR inhibition. Based on these results, it is conceivable that primers designed to generate short amplicons and to have high melting temperatures may lead to less interference with STR amplification by HA.

Conclusion

We examined DNA quantification values estimated by using a Quantifiler kit in DNA samples containing HA as a PCR inhibitor. DNA concentrations were reliably estimated in the DNA samples containing a low concentration of HA. However, as HA concentrations increased, the C_T values of the IPC reached 32; DNA quantification values were underestimated. These results suggest that researchers should be cautious when interpreting DNA quantification

results with the high C_T values of the IPC. Our study showed the DNA quantification results as well as the effects of HA on commercially available STR kits that have been widely used. A better understanding of the various effects of HA will help researchers recognize and manipulate samples containing PCR inhibitors, such as HA.

Acknowledgements This work was supported by grant no 04-2010-0500 from the Seoul National University Hospital (SNUH) Research Fund and by Future-based Technology Development Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. 2010-0020631).

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