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# Taq Polymerase Reverses Inhibition of Quantitative Real Time Polymerase Chain Reaction by Humic Acid

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**Aim** To investigate the dose-response effect of humic acid (HA) on the quantitative real time polymerase chain reaction (QRT-PCR) inhibition and the efficiency of Taq polymerase increment in preventing inhibition by HA in DNA extracted from ancient bones.

**Methods** DNA was isolated from bone samples and DNA quantification was conducted with the real-time 5′ exonuclease detection assay (TaqMan®), using the ABI PRISM® 7000 instrument.

**Results** The addition 10-75 ng of synthetic HA inhibited QRT-PCR, whereas the addition of 100 ng of synthetic HA completely inhibits QRT-PCR. The addition of 1.25 Unit (U) of Taq polymerase per assay appeared to be the optimum amount in overcoming the HA inhibition. The best results were obtained when crude DNA extracts containing humic substances were quantified by QRT-PCR with the addition of 1.25 Unit (U) of extra Taq polymerase per assay.

**Conclusion** The modified procedure with increased Taq polymerase concentration should allow more effective QRT-PCR analysis in samples containing HA.

Human DNA quantification has became an essential method for ensuring the quality of polymerase chain reaction (PCR)-based studies performed from low copy and/or highly damaged DNA samples (1).

Theoretically, PCR makes it possible to amplify a DNA sequence from any biological sample. In practice, however, compounds that inhibit PCR may co-purify with the DNA template, making the amplification impossible.

The problems that forensic scientists most often face while working with DNA extracted from bones and teeth samples recovered from mass graves or mass disasters are DNA quantity, DNA degradation, DNA contamination, and presence of inhibitor/s (2,3). Inhibition is an especially significant problem when extracting DNA from old and ancient material. Post-mortem DNA changes may also prevent amplification (4).

When the template DNA is extracted directly from soil, humic acid (HA) can inhibit PCR (5). Also, total DNA extraction from soil always results in co-extraction of other soil components, mainly humic acid (HA) or other humic substances, which negatively interfere with DNA detecting processes (6-10). Characteristic features of humic substances are their structural heterogeneity, their property to bind metal ions by complex formation, and their property to interact with a variety of organic compounds (11). Humic substances are ubiquitous in soil and water and can contaminate any material exposed to those environments, which causes false negative results (10,12).

DNA quantification ensures the optimal use of limited amounts of DNA found in the majority of forensic material evidence, so that DNA is not wasted in expensive repetitive endpoint PCR typing analysis, performed with inappropriate amounts of DNA templates (13). Furthermore, some DNA quantification designs could also provide additional information about DNA degradation and the presence of possible DNA inhibitors (1,14).

Quantitative Real Time PCR (QRT-PCR) can fail because of the presence of inhibitors or the absence of adequate template DNA (4). It is useful to distinguish between two categories of inhibition: 1) inhibitors of Taq DNA polymerase may co-purify with DNA; and 2) the modification of DNA template makes it unrecognizable as a substrate for QRT-PCR. Before concluding that amplifiable DNA is not present in a sample, it is necessary to confirm that the inhibitors of Taq polymerase are not present in the preparation.

Quality control PCR can detect inhibitors that could be present in templates. DNA quantification based on the real-time 5′ exonuclease detection assay (TaqMan®), using the ABI PRISM® 7000 instrument (Applied Biosystems), has gained a widespread use in many areas for low-copy DNA quantification (14,15). Real-time detection is a very fast and accurate technology that does not require post-PCR processing, since detection is done during each PCR cycle. The TaqMan assay is based on the cleavage of a target-specific probe by the 5′→3′ exonuclease activity of Taq DNA polymerase, resulting in an increased intensity of reporter emission (16). The threshold cycle ($C_T$) is inversely proportional to the final target sequence concentration. Furthermore, a multiplex analysis of multiple targets is possible using dyes with a large difference in emission wavelengths (16).

QRT-PCR methods have the ability to quantitate trace amounts of human DNA isolated from old bone samples (17,18). To enhance the PCR efficiency in samples containing inhibitors, 0.16 mg/mL bovine serum albumin (BSA) and 3.75 U extra AmpliTaq DNA polymerase were included in the reactions (14). Altered amplification plots were observed during the analysis of 50-year old saliva stains on stamps and envelopes due to the presence of inhibitors. The addition of BSA and extra Taq amounts has been proven efficient in overcoming the effects of inhibitors (13).

In this article, we describe the dose-response effect of HA on QRT-PCR inhibition and the effect of Taq polymerase increment in overcoming the HA inhibition.

## Materials and Methods

Human genomic DNA 9947 at 0.1 and 200 ng/μL concentrations (Applied Biosystems, Foster City, CA, USA) was used as a DNA standard. HA was a product of Fluka Inc. (Taufkirchen, Germany). Quantifiler™ human DNA quantification kit and AmpliTaq Gold® were from Applied Biosystems. There were 10 DNA extracts from ancient bone samples, 5 of them retrieved from 10-50 years old bones and 5 obtained from about 1,300 years old bones recovered from the church of St. Duje in Split.

All of our samples were femur bones. After all traces of soft tissue and bone marrow had been removed, using razor blades and sandpaper, the bone was crushed into small fragments and stored in sterile polypropylene tubes at -20°C until analyzed. Further bone preparation and DNA extraction were done as previously described (2,18-21).

### *Humic Acid Measurements in DNA Samples from Ancient Bones*

HA was evaluated by spectrophotometer measurements (11). Making serial dilutions of commercial HA mixture created duplicate standard curves. Absorbances at 495 nm were measured in quartz cuvette with HA standards and crude extracts. Measurements were made on spectrophotometer Ultrospec 2000 (Pharmacia Biotech (Biochrom) Ltd. Cambridge, UK)

### *DNA Quantification*

The quantification assay was performed in the total volume of 25 μL, containing 2 μL of DNA extract, Quantifiler human primer mix, and Quantifiler PCR reaction mix according to the manufacturer's protocols (16). All reactions without templates served as negative controls.

The influence of HA on the QRT-PCR efficiency of DNA extracts was monitored by duplicate experiments performed both with and without an extra addition of Taq polymerase. The first experiment was conducted with increasing amounts of HA (from 1 to 100 ng) and the same amount of DNA (100 pg), whereas the second experiment was conducted with 100 ng HA and different amounts

of DNA (from 12 to 50,000 pg). Two-fold serial dilutions of the genomic DNA were included in each experiment to generate the standard curve for nuclear DNA. All reactions without templates served as negative controls.

The influence of HA on the QRT-PCR efficiency was also monitored by duplicate experiments performed both with and without the extra addition of 1.25, 2.5, and 3.75 U Taq polymerase per assay.

Each probe was labeled with a specific reporter. A TaqMan MGB probe (Applied Biosystems) was labeled with 6-FAM™ dye for detecting the amplified sequence and the other TaqMan MGB probe (Applied Biosystems) was labeled with VIC® dye (a synthetic sequence not found in nature) for detecting the amplified IPC template DNA. During the run, charge-coupled device (CCD) camera detected the fluorescence emission ($R_n$) between 500 and 660 nm from each well and collected them by Sequence Detection System (SDS) ABI PRISM 7000 (Applied Biosystems). The SDS software displayed cycle-by-cycle changes in normalized reporter signal ($\Delta R_n$, calculated as the difference between fluorescence signal of the reaction and fluorescence signal of the baseline emission).

The thermal cycling conditions were: 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The samples were analyzed according to the manufacturer's protocols. Data were collected using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Data analysis was performed with SDS v 1.0 to generate the individual standard curves from each experiment and to calculate the DNA amount from each unknown sample. The cycle treshold value ($C_T$, cycle number at which the $\Delta R_n$ crosses the treshold) value was set to a default threshold of 0.20 for all reactions and the copy number value for unknown samples was inferred from the regression line of standard curves.

## Results

### *In Vitro Tests on Genomic DNA*

The amplification plots of 100 pg human DNA with FAM-labeled probe (Fig. 1A) and with VIC-labeled probe (Fig. 1B) show dose-response effect of HA (from 1 to 100 ng) on QRT-PCR inhibition. Amplification plot (Fig. 1A) shows lower $\triangle R_n$ values and higher $C_T$ values as the concentration of HA increased. $C_T$ results and corresponding quantification results were relatively stable up to 10 ng HA. The final results were more affected at higher concentrations of HA. As the concentration of HA increased, the PCR efficiency in the Quantifiler kit reactions decreased. The IPC system is more sensitive to PCR inhibition, so in samples containing more than 100 ng HA, amplification of IPC detectors failed (Fig. 1B).

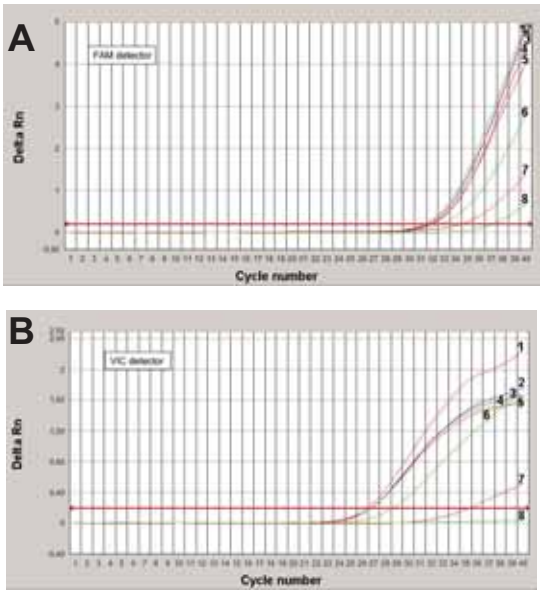


**Figure 1.** Humic acid inhibition of QRT-PCR. Human DNA (100 pg) was quantified both with and without addition of humic acid. Curve 1 – without addition of humic acid; 2 – addition of 1 ng of humic acid; 3 –addition of 2.5 ng of humic acid; 4 – addition of 5 ng of humic acid; 5 – addition of 10 ng of humic acid; 6 – addition of 50 ng of humic acid; 7 – addition of 75 ng of humic acid; 8 – addition of 100 ng of humic acid. **A.** Curves and $C_T$ values from experiments using FAM detector. **B.** Curves and $C_T$ values from experiments using VIC detector.

The results of this study (Table 1) showed higher $C_T$ values and lower amount of DNA as the concentration of HA increased, whereas the quantification of samples showed improved amplification efficiency and normal curve shape after adding extra Taq polymerase. Table 1 (sample 4) shows the results of the amplification plot of the human DNA (100 pg), detected with FAM and VIC labeled probes, with the addition of 100 ng of HA and 1.25 U of Taq polymerase. $C_T$ values of the FAM detector decreased from 37.81 to 31.80 and the final amount of DNA increased from 0.98 to

64.22 pg after adding extra Taq polymerase. The amplification of Internal PCR Control failed and $C_T$ value of the VIC detector was not detectable with the addition of Taq polymerase, with the $C_T$ value of 29.79.

We also investigated the effect of 100 ng of HA on different amounts of DNA on QRT-PCR inhibition (Fig. 2 and Table 2). Figure 2A shows amplification plots with normal curve shapes of serial dilutions of human DNA from 50,000 to 12 pg, without addition of HA and without addition of extra Taq polymerase. The results of $C_T$ values and DNA amounts are shown in Table 2A. Higher $C_T$ values correspond to lower amounts of DNA. The addition of HA (100 ng) was used to decrease the activity of Taq polymerase and reduce the amount of PCR products. Subsequently, the amplification plot had changed (Fig. 2B) and the amount of DNA drastically decreased with the increased $C_T$ values (Table 2B). In all samples, amplification of Internal PCR Control failed and $C_T$ values of the VIC detector were not detectable. Sometimes, these can be false negative results.

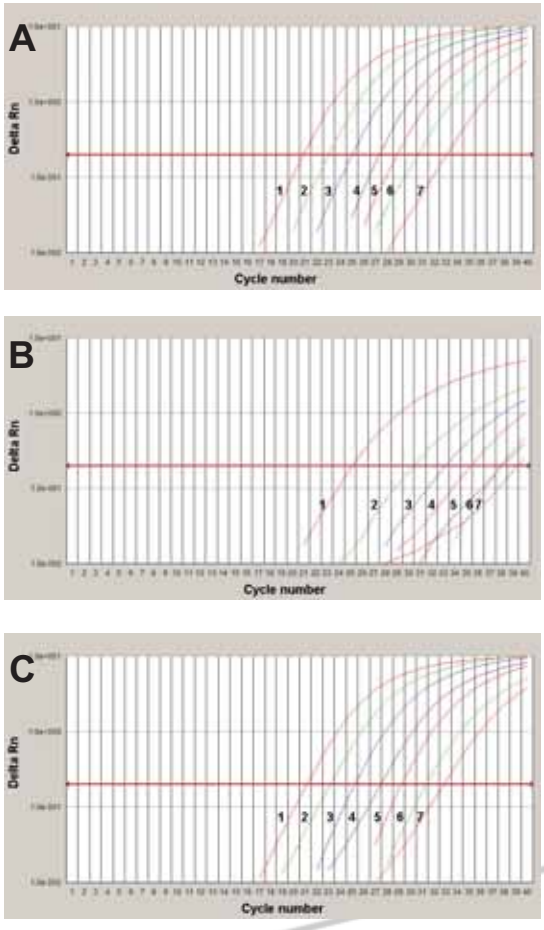


**Figure 2.** Amplification plot of serial dilutions of human DNA (50,000 to 12 pg shown in Table 2) with addition of 100 ng of humic acid and with/without extra addition of Taq polymerase displaying by FAM detector. (**A**) Amplification plots of human DNA without addition of humic acid and without extra addition of Taq polymerase. (**B**) Amplification plots of human DNA with addition of 100 ng of humic acid and without extra addition of Taq polymerase. (**C**) Amplification plot of human DNA with addition of 100 ng of humic acid and with extra addition of 1.25 U Taq polymerase.

**Table 1.** Quantification of 100 pg human DNA performed in an ABI Prism® 7000 Sequence Detection System (Applied Biosystems) with addition of different amounts of humic acid (HA)

| Amount (ng) of HA in the sample | Detector* | Cycle threshold value ($C_T$) with extra addition of Taq polymerase: none $C_T$ | none amount (pg) DNA† | 1.25 units $C_T$ | 1.25 units amount (pg) DNA† |
|---|---|---|---|---|---|
| 1 | VIC | 26.77 | | 26.54 | |
| | FAM | 31.22 | 96.49 | 31.48 | 80.48 |
| 5 | VIC | 26.89 | | 29.79 | |
| | FAM | 31.81 | 64.40 | 31.54 | 76.97 |
| 10 | VIC | 28.49 | | 27.12 | |
| | FAM | 32.42 | 42.27 | 31.62 | 72.65 |
| 100 | VIC | negative | | 29.79 | |
| | FAM | 37.81 | 0.98 | 31.80 | 64.22 |

*TaqMan MGB probe was labeled with 6-FAM™ dye for detecting the amplified sequence and with VIC® dye for detecting the amplified Internal PCR Control DNA.
†Amount (pg) of DNA after adding different amounts of humic acid in samples containing 100 pg DNA.

After the extra addition of 1.25 U of Taq polymerase into the reaction mixture, the curves shape became normal (Fig. 2C). $C_T$ values of the VIC detector were detectable and the amount of DNA rapidly increased (Table 2).

### *DNA Quantification from Ancient Bones*

The results obtained by the modified method (addition of extra Taq polymerase) are shown in Table 3 in comparison with different HA content (from 0 to 53.55 ng/μL). At HA concentrations below 5 ng/μL in the samples with extra Taq polymerase added, there was some or no effect of HA on DNA quantification. The higher concentrations of HA (samples 4 and 10) produced false negative results, which were reversed by adding extra Taq polymerase. In 5 DNA extracts (samples 1, 5, 6, 7, and 9), there was no DNA and no influence of HA as an inhibitor. In 3 DNA extracts (samples 2, 3, and 8) the quantification was improved after extra Taq polymerase had been added.

**Table 2.** Quantification of serial dilutions of human DNA performed in an ABI Prism® 7000 Sequence Detection System (Applied Biosystems) with addition of 100 ng of humic acid (HA)

| | | Threshold cycle value ($C_T$) with addition of HA and extra addition of Taq polymerase | | | | | |
|---|---|---|---|---|---|---|---|
| | | no HA, no extra Taq | | 100 ng HA, no extra Taq | | 100 ng HA, 1.25 units Taq | |
| Sample | Detector | $C_T$ | Amount (pg) DNA† | $C_T$ | amount (pg) DNA‡ | $C_T$ | amount (pg) DNA‡ |
| 1 | VIC | 38.77 | | negative | | 39.02 | |
| | FAM | 20.78 | 50,000.00 | 25.14 | 2675.97 | 21.13 | 43,954.30 |
| 2 | VIC | 29.82 | | negative | | 35.72 | |
| | FAM | 22.97 | 12,500.00 | 30.41 | 67.33 | 23.13 | 10,858.75 |
| 3 | VIC | 26.70 | | negative | | 29.71 | |
| | FAM | 24.98 | 3,125.00 | 33.16 | 9.90 | 25.18 | 2,597.53 |
| 4 | VIC | 26.31 | | negative | | 27.96 | |
| | FAM | 27.24 | 781.00 | 38.05 | 0.33 | 27.38 | 558.10 |
| 5 | VIC | 26.41 | | negative | | 27.53 | |
| | FAM | 28.78 | 195.00 | 35.34 | 2.16 | 29.17 | 160.60 |
| 6 | VIC | 26.53 | | negative | | 28.09 | |
| | FAM | 30.52 | 49.00 | 37.93 | 0.35 | 31.29 | 36.55 |
| 7 | VIC | 26.47 | | negative | | 27.27 | |
| | FAM | 33.07 | 12.00 | 39.36 | 0.13 | 32.18 | 12.64 |

*TaqMan® MGB probe was labeled with 6-FAM™ dye for detecting the amplified sequence and with VIC® dye for detecting the amplified Internal PCR Control DNA.
†Different amounts of input DNA (12 to 50,000 pg).
‡Measured amounts of DNA after addition of 100 ng of humic acid in samples with different input DNA concentrations.

## Discussion

Modified procedure of DNA amplification with the addition of extra Taq polymerase allows more effective QRT-PCR analysis in HA-containing samples. We demonstrated that the addition of 10-75 ng of synthetic HA (inhibited QRT-PCR, whereas the addition of 100 ng of synthetic HA completely inhibits QRT-PCR. The addition of 1.25 U of Taq polymerase per assay appeared to be the optimum amount in overcoming the HA inhibition. Weak amplification (high $C_T$ and low $\Delta R_n$ value) of the human DNA and no amplification of the IPC may indicate a partial PCR inhibition in the sample.

We also described the dose-response effect of HA on QRT-PCR inhibition and the effect of adding extra Taq polymerase in overcoming the HA inhibition.

The standard curves display plotted $C_T$ values from experiments versus the log of the initial genomic DNA concentrations (pg). Decreasing amounts of input DNA, consistently revealed higher $C_T$ numbers. In most experiments, the target was detected down to an amount of DNA in the dilutions series for the standard curve, which indicated that the system is highly sensitive. HA, as a PCR inhibitor, can interfere with the reaction and cause varying levels of reduced PCR efficiency (interfering with polymerase activity), including complete inhibition of QRT-PCR (16). The signal is detected and decreases in direct proportion to the decrease of PCR product. We showed that HA really inhibited QRT-PCR if present in more than 100 ng per reaction mix. In samples containing less HA, the amplification of IPC detectors was inhibited. In our study, the degree of inhibition by adding the HA was in agreement with the successful/unsuccessful amplification of the human DNA. The extra addition of 1.25 U of Taq polymerase to the reaction mixture has proven to be efficient in overcoming the inhibitor. In all samples, the overcoming effect was in a similar range: with extra addition of Taq polymerase about 80% of input DNA was amplified.

In the second part of the study, we tested QRT-PCR method with the addition of extra Taq polymerase for DNA samples extracted from ancient bones. The amplification of samples containing inhibitors showed increased amplification efficiency after the addition of extra Taq polymerase. The samples containing HA were usually not amplified when analyzed without extra Taq polymerase, depending upon the concentration of HA. We demonstrated that large amounts of HA could interfere with Taq polymerase, rendering false negative results on human DNA quantification. A relatively high incidence of PCR inhibitors could be predicted when analyzing bone and ancient DNA samples and other forensic samples. Partial inhibition of Taq polymerase may produce a situation similar to that of low concentration of DNA in a sample. Therefore, the use of extra Taq polymerase seems to be an efficient procedure to overcome false negative results, but our results showed that the extra addition of Taq polymerase in DNA

**Table 3.** Quantification of human DNA extracted from 10 ancient bones performed in an ABI Prism® 7000 Sequence Detection System (Applied Biosystems)

| | | | Threshold cycle value (CT) with extra addition of Taq polymerase | | | |
|---|---|---|---|---|---|---|
| | | | none | | 1.25 units | |
| Sample | Amount (ng/μL) of HA* | Detector† | $C_T$ | amount (pg) DNA‡ | $C_T$ | amount (pg) DNA |
| 1 | 0.05 | VIC | 29.43 | | 27.80 | |
| | | FAM | negative | ND | negative | ND |
| 2 | 5.30 | VIC | 30.42 | | 27.89 | |
| | | FAM | 34.14 | 32.11 | 33.66 | 47.29 |
| 3 | 1.33 | VIC | 33.68 | | 28.17 | |
| | | FAM | 34.42 | 25.63 | 33.64 | 47.96 |
| 4 | 18.65 | VIC | negative | | 28.85 | |
| | | FAM | 36.78 | 3.90 | 32.13 | 159.89 |
| 5 | 0.07 | VIC | negative | | 37.26 | |
| | | FAM | negative | ND | negative | ND |
| 6 | 0.0 | VIC | negative | | negative | |
| | | FAM | negative | ND | negative | ND |
| 7 | 0.02 | VIC | 29.83 | | 27.73 | |
| | | FAM | negative | ND | negative | ND |
| 8 | 0.03 | VIC | negative | | negative | |
| | | FAM | negative | ND | 29.45 | 2.90 |
| 9 | 0.0 | VIC | negative | | negative | |
| | | FAM | negative | ND | negative | ND |
| 10 | 53.55 | VIC | negative | | 30.55 | |
| | | FAM | negative | ND | 27.42 | 6,883.77 |

*Measured amounts of humic acid in DNA samples extracted from ancient bones.
†TaqMan® MGB probe was labeled with 6-FAM™ dye for detecting the amplified sequence and with VIC® dye for detecting the amplified internal PCR control DNA.
‡Measured amounts of DNA; ND – not detected.

extracts from ancient bones effectively overcame the inhibition of QRT-PCR only in some cases. This indicates that the HA is not the only inhibitor in DNA samples from ancient bones. Because of that, we evaluated the HA concentration in DNA from ancient bones by spectrophotometer and our results correlated with the finally amplified DNA amount (Table 3). In sample No. 10, which contained the highest concentration of HA, extra addition of Taq polymerase produced the best results when quantified with QRT-PCR.

Alonso et al (13) included BSA to prevent the action of inhibitory compounds present in some bone DNA samples. Andreasson et al and Giambernardi et al (14,15,22) showed that the addition BSA proved efficient in overcoming the effects of the inhibitors. In comparison with our study, the addition of BSA was not as good as the addition of Taq polymerase (data not shown).

Forensic DNA analysis of biological evidence materials containing very small amounts of DNA is becoming widely used in criminal investigations. Therefore, a reliable and highly sensitive DNA quantification system is necessary to ensure the optimum use of limited material available. Before concluding that amplifiable DNA is not present in a sample, it is necessary to confirm with QRT-PCR that inhibitors of Taq DNA polymerase are not present in the sample. The QRT-PCR quantification system has been proven to be sensitive, reliable, and very useful in routine forensic DNA analysis. Modified procedure (with addition of extra Taq polymerase) should allow more effective QRT-PCR analysis in HA-containing samples. Moreover, the assay will ensure that a minimum amount of DNA is used for a successful amplification and that DNA is retained for repeated analysis.

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