

Research Reports

A quantitative approach to detect and overcome PCR inhibition in ancient DNA extracts

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Inhibition is problematic in many applications of PCR, particularly those involving degraded or low amounts of template DNA, when simply diluting the extract is undesirable. Two basic approaches to monitoring inhibition in such samples using real-time or quantitative PCR (qPCR) have been proposed. The first method analyzes the quantification cycle (C_q) deviation of a spiked internal positive control. The second method considers variations in reaction efficiency based on the slopes of individual amplification plots. In combining these methods, we observed increased C_q values together with reduced amplification efficiencies in some samples, as expected; however, deviations from this pattern in other samples support the use of both measurements. Repeat inhibition testing enables optimization of PCR facilitator combinations and sample dilution such that DNA yields and/or quantitative accuracy can be maximized in subsequent PCR runs. Although some trends were apparent within sample types, differences in inhibition levels, optimal reactions conditions, and expected recovery of DNA under these conditions suggest that all samples be routinely tested with this approach.

Introduction

Since the advent of PCR, many inhibitory substances that interfere with the activity or availability of particular reaction components have been identified (1,2). The presence of inhibitors introduces a number of problems, ranging from reduced amplification capacity and reduced assay sensitivity to complete reaction failure. Thus, in situations where high (or simply consistent) amplicon concentrations or maximal sensitivity are desired, or when drawing conclusions from negative results, it is important that PCR inhibition be detected and circumvented as much as possible. Inhibition of qPCR presents additional concerns, as slight variations in amplification efficiency between samples can drastically affect the accuracy of template quantification (3).

When PCR inhibition is suspected, the simplest course of action is to dilute the template (and inhibitors), and make use of the sensitivity of PCR (1); however, for applications involving heavily degraded or otherwise low-copy templates, this

solution is often undesirable—and indeed sometimes impossible—due to the further reduction of template amounts. In these situations, which are commonly encountered in the forensic and ancient DNA fields, routine detection and quantification of PCR inhibition is necessary.

In standard PCR experiments, negative results or unexpectedly low product yields may be indicative of inhibition, provided that the template is known to be present; alternatively, a known amount of non-endogenous DNA can be added to the sample and amplified as an internal positive control (IPC). These controls may be used in qPCR assays as well, enabling quantitative assessments of their performance. Based on modeling individual reaction kinetics and/or the calculation of amplification efficiency, qPCR also allows inhibited samples to be identified without additional IPC amplifications.

Internal positive controls

A number of studies have incorporated IPC assays for inhibition detection (4–9), as have some commercially available systems such

as the Quantifiler Human DNA Quantification Kit (Applied Biosystems, Foster City, CA, USA). Typically, problematic samples are identified based on the shift in C_q (ΔC_q) observed relative to an uninhibited reaction. When using this type of control, one assumes that the effects of inhibitors on the IPC are predictive of those on other targets in subsequent PCR assays from the same sample. This assumption has been challenged, as some authors have noted differential susceptibility to PCR inhibition between assays (10,11).

Huggett et al. (11) found quantitatively small but significant differences between assays, with no correlation between the extent of inhibition and any particular characteristic of the template or primer sequences. Ståhlberg et al. (10) hypothesize that this may result from indirect inhibition, through competition for particular reaction components. If, for example, Mg^{2+} ions became limiting by the presence of EDTA in a sample, an assay with a greater Mg^{2+} requirement would be more susceptible to the same level of EDTA inhibition.

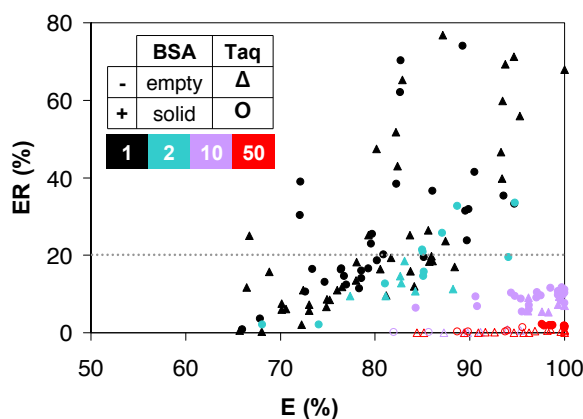


Figure 1. Effects of PCR facilitators and dilution factors on optimization criteria. Results of all successful IPC amplifications from the soil extracts are shown, with the minimum ER level of 20% noted. The use of BSA and additional *Taq* are indicated by the fill and shape (respectively) of each data point, with colors varying by dilution factor. ER, expected recovery; E, relative amplification efficiency.

Table 1. Sample information

Material	Species	Location	Extract	Age ^a (years BP ^b)
Bone	<i>M. primigenius</i>	Taimyr Peninsula, Siberia	B1	Unknown
			B2	55,800 ± 4500–2900
Feces	<i>Neotoma</i> sp.	Death Valley, CA, USA	F1	1942 ± 35
			F2	Unknown
Hair	<i>M. primigenius</i>	Taimyr Peninsula, Siberia	H1	20,380 ± 140
			H2	
Soil	—	Yukon, Canada	P1–10	ca. 25,300
			P11–20, P33–34	ca. 80,000–90,000
			P21–32	ca. 740,000

^aAges of soil samples based on proximity to volcanic tephra. ^bBP, before present.

Thus, it is important that both IPC and target reactions perform well under (ideally) the same conditions. In particular, the concentrations of components known to facilitate PCR in the presence of inhibitors (e.g., BSA, *Taq* polymerase, etc.) must be the same. Furthermore, IPC and target assays with different enzymatic requirements may not be comparable; for instance, if inhibition of the exonuclease activity of *Taq* is also measured (e.g., by a *Taq*Man IPC assay), target assays performed without such probes may be less susceptible to inhibition than the IPC predicts.

Amplification efficiency

Based on the potential for differential susceptibility to inhibition, the Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines (12) instead advocate measuring amplification performance using a dilution series of each sample [e.g., Ståhlberg et al. (10)]. As noted previously, this may not be feasible for low-copy templates.

Building on the 2002 publications of Liu and Saint (13,14), various mathematical models that describe individual

reaction kinetics have been developed and compared (3,15–25). Regardless of the particular model, it is clear that kinetic outliers can be identified when inhibitors influence amplification efficiency; however, certain inhibitory mechanisms may not be identified this way (20). Furthermore, highly inhibited samples cannot be differentiated from those having no template, or from a combination of low-copy template and moderate inhibition when there is simply no amplification observed.

To the best of our knowledge, no studies have incorporated the use of an IPC and amplification efficiency measurements in order to contrast their relative contribution in monitoring PCR inhibition. In this study, we present a comprehensive approach to detecting and quantifying PCR inhibition that incorporates both features, and recommend its use in routine sample processing (26), particularly when working with low-copy templates. Beyond simply identifying problematic samples, we demonstrate how quantifying inhibition effects can be used to determine an optimal combination of PCR facilitators (and dilution in some cases) for every DNA

extract that maximizes quantitative accuracy and/or template recovery.

Materials and methods

Samples and DNA extraction

The tests described herein were performed on 47 DNA extracts from a diverse set of ancient samples (Table 1), including permafrost soil, mammoth bone and hair, and packrat paleofeces. DNA was extracted from the soil (~250 mg) according to Willerslev et al. (27), but using 25 mM TCEP [Tris (2-carboxyethyl) phosphine hydrochloride] in place of β -mercaptoethanol and with a 10-min vortexing step in Lysing Matrix E tubes (MP Biomedicals, Solon, OH, USA) for sample disruption. The extracts were purified either using columns as indicated or using a modification of the protocol of Boom et al. (28). In this case, the extracts were mixed with 4 mL binding buffer [5 M guanidinium thiocyanate (GuSCN), 50 mM Tris-Cl pH 8, 22.5 mM NaCl, 20 mM EDTA, 1.25% Triton-X 100] that had been previously incubated with 50 μ L size-fractionated silicon dioxide (30 min with rotation at room temperature). Additional GuSCN was added to maintain a 5 M concentration upon addition of the extract and the pH was adjusted to 4.5–5.5 with glacial acetic acid to maximize binding efficiency (29). Following a minimum 1-h (room temperature) incubation, the silica-bound DNA was washed twice with 1 mL buffer (5 M GuSCN, 50 mM Tris-Cl pH 8, 22.5 mM NaCl) and once with 1 mL 80% ethanol (in 1 \times TE pH 7.5). The pellet was dried at 56°C for 5 min and the DNA eluted in 100 μ L Buffer EB (Qiagen, Hilden, Germany) as per the procedure performed by Willerslev et al. (27). A subset of the soil samples were processed using the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's alternative protocol for maximum yields. DNA was extracted from the bone samples (~100 mg) according to Poinar et al. (30). As part of a separate study, the same protocol (without a demineralization step) was used for the feces samples. Although commonly used silica-based methods may offer high DNA purity, we have noted considerable DNA loss in exchange (unpublished results) and wished to evaluate whether an alternative protocol might yield enough DNA to outweigh the effects of increased PCR inhibition. The hair samples (54 mg and 2 mg) were processed as per Gilbert et al. (31).

Inhibition assay

To assess the levels of PCR inhibition in the samples, we monitored the effect of each

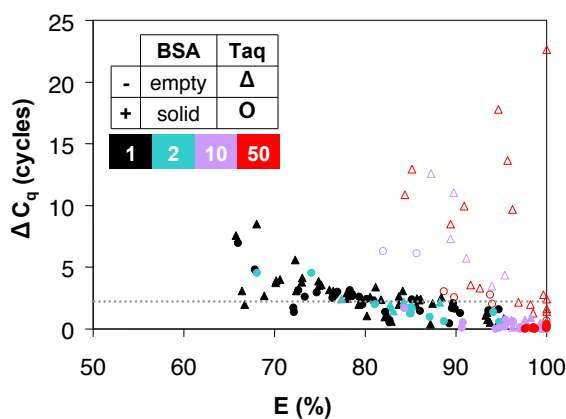


Figure 2. Effects of PCR facilitators and dilution factors on inhibition levels. Results of all successful IPC amplifications from the soil extracts are shown, with the maximum ΔC_q value of 2.3 (i.e., ER = 20% prior to any dilution) noted. The use of BSA and additional *Taq* are indicated by the fill and shape (respectively) of each data point, with colors varying by dilution factor. ΔC_q , cycle shift; E, relative amplification efficiency.

Table 2. Effects of PCR facilitator combinations at optimal dilution

Extract	Dilution factor	PCR facilitators				
		None	BSA	<i>Taq</i>	Both	
Bone	B1	1	2.4, 94	1.1, 100	0.5, 85	0.7, 100
	B2	1	6.1, 94	1.1, 100	1.5, 88	0.5, 100
Feces	F1	100	—	1.4, 86	—	1.0, 94
	F2	50	—	2.0, 82	—	1.3, 84
Hair	H1	50	—	0.5, 92	4.6, 78	0.4, 93
	H2	50	—	0.7, 90	0.4, 93	0.3, 97

Inhibition test results [ΔC_q (cycles), E (%)], with results under the optimal PCR facilitator combinations bolded. (—), complete inhibition.

purified extract on the amplification of an IPC during qPCR. As amplification of any endogenous templates would distort the quantification results, we selected an assay targeting the human β -2-microglobulin gene (*B2M*), which should not be present in these extracts, and used an IPC template derived from the *B2M* cDNA sequence (GenBank accession no. NM_004048), such that amplification of any contaminating human genomic DNA could be distinguished. The primers used in this assay (5′–3′: TGACTTTGTCACAGC-CCAAGATA and AATCCAAATGCGGCATCTTC) flank two intronic regions (~2 kb total), such that amplification of the cDNA-derived template yields an 85-bp product that is easily differentiated from amplification of longer *B2M* genomic DNA (32). As the assay is primarily used for quantification of human cDNA, the template (5′–3′: GAACCATGTGACTTTGTCACAGC-CCAAGATAGTTAAGTGGGATC-GAGACATGTAAGCAGCATCATG-GCGGTTTGAAGATGCCCGCATTTGGATTGGATGA) was synthesized to include an internal base modification

(underlined; primer binding sites are italicized) so that it may be distinguished from human cDNA contamination by sequencing as well, although this feature is not important for these experiments. We used an available synthetic ssDNA version of this template for convenience. As the same ssDNA template was used in all reactions, we perceive no disadvantages in its use compared to dsDNA template, nor must any corrections be made to the inhibition measurements.

Following gradient optimization (data not shown) of the annealing temperature and Mg^{2+} concentration, the assay demonstrated highly sensitive and reproducible amplification of serial dilutions of the IPC. Additional details are included in the MIQE checklist (Supplementary Table 1).

In each 20 μ L reaction, 12,500 copies of the IPC were amplified with 1 \times PCR Buffer II (10 mM Tris-Cl pH 8.3 and 50 mM KCl; Applied Biosystems), 2.5 mM $MgCl_2$, 300 nM primers, 400 μ M (each) dNTP, 1 U of *AmpliTaq* Gold DNA Polymerase (Applied Biosystems), and 0.167 \times SYBR Green I solution (Invitrogen, Carlsbad, CA, USA). Following an

initial denaturation at 95°C for 7 min, the reactions were subject to 50 cycles of 95°C, 59°C, and 72°C (30 s each), with data collection at the end of the annealing step. To verify that the cDNA-derived template was amplified, the melting temperature (T_m) of the product was monitored during a final cycle of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s.

Three reaction types were used in these tests: sample reactions, which included the IPC and one DNA extract at 10% of the final PCR volume; standard reactions, which included only the IPC; and no template control (NTC) reactions.

Inhibition measurement

Two measurements of inhibition were determined using the qPCR data for each sample reaction relative to the standard reactions. For the first, the Hill slope of each amplification plot was calculated by fitting a variable-slope sigmoidal dose-response curve to the raw fluorescence data using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA). Under a sigmoidal model, this is the only parameter affecting amplification efficiency calculations in the early cycles of PCR (14,24) and these calculations of initial amplification efficiency are highly consistent with those derived from standard curves (19). Since an absolute measure of amplification efficiency was not required in this analysis, we compared the Hill slope values directly; the Hill slope of each sample reaction was expressed as a percentage of the average of the two corresponding standard reactions. For simplicity, this value (E) is referred to in the text as “amplification efficiency” or just “efficiency,” with any deviations from 100% due to the effects of PCR inhibitors in the extract; however, we acknowledge that it is in fact a relative measurement of the shape of an amplification plot. For the second measurement, the C_q of each reaction was automatically calculated by the MxPro – Mx3000P QPCR System (Stratagene, La Jolla, CA, USA) using the amplification-based threshold-determination algorithm; C_q shifts (ΔC_q) were measured as the difference between the sample C_q and the average C_q of the two standard reactions.

To quantify the total effect of inhibition and any dilution of the extract on subsequent PCRs, we calculated the expected recovery (ER) value, where ER = (dilution factor $\times 2^{\Delta C_q}$)⁻¹ \times 100%. For example, from an extract showing $\Delta C_q = 1$ when used directly in PCR (dilution factor = 1), we expect other assays to recover 50% of the true yield. Likewise, for an extract

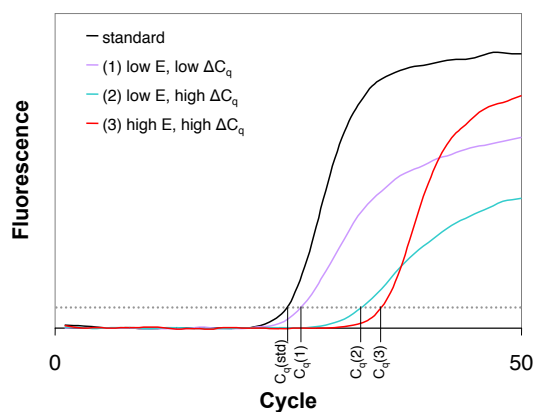


Figure 3. IPC amplification plots illustrating the observed efficiency- C_q shift relationships. Although high ΔC_q values were typically associated with low E values (blue), inhibition may not be apparent from either single measurement in other instances.

showing no inhibition ($\Delta C_q = 0$) when tested at a 1/10 dilution, we can only expect to recover 10% of the templates available in the original extract.

Circumventing inhibition

For the soil samples, our goal was to optimize the PCR conditions (in terms of amplification facilitators) for each extract in order to minimize inhibition and reduce,

if not eliminate, the need to dilute these extracts (i.e., maximize ER). Therefore, the inhibition tests were repeated with combinations of 0.75 mg/mL BSA, increased amounts of *Taq* (2.5 U per reaction), and using various dilutions of the extracts (in their corresponding elution buffer). Based on the performance of the most inhibited samples, the conditions were considered optimized when the ER was $\geq 20\%$. The

Table 3. Inhibition measurements by extraction method

Soil Sample	Extraction Method		
	a	b	c
P5	2.6, 88	3.4, 81	7.6, 66
P10	1.1, 93	2.5, 86	5.6, 72
P25	0.6, 100	1.3, 93	NA
P30	0.7, 93	8.5, 68	NA

Inhibition test results [ΔC_q (cycles), E (%)] for undiluted extracts with BSA; (a) Willerslev et al. (27) extraction and purification protocol, (b) Willerslev et al. (27) extraction with alternative purification protocol, (c) UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). NA, not attempted.

bone, feces, and hair samples were tested with all combinations of BSA, additional *Taq*, and a range of dilutions. In order to preserve the extracts, sample reactions were performed only once with the exception of four inter-run duplicates used to assess experimental variation. One NTC and two standards reactions were included for every set of PCR conditions in every run.

Results

All of the NTC reactions were negative, while all standard and positive sample

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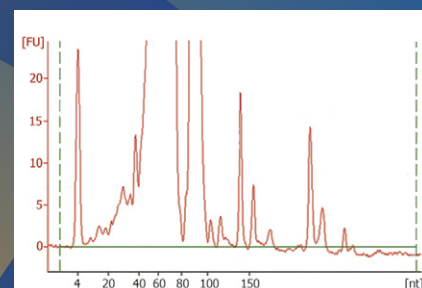
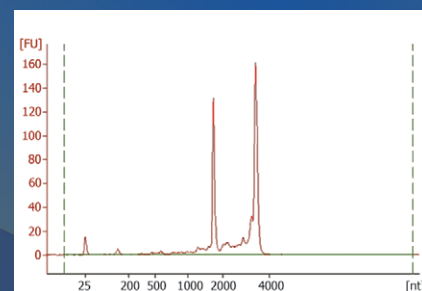
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reactions had T_m values of 81.8°C ($SD_{T_m} = 0.5^\circ\text{C}$). These results indicate that no unspecific primer binding or contamination with human DNA had occurred. Based on the data from duplicate standard reactions, the mean intra-assay variation in C_q and Hill slope measurements was low, with $SD_{C_q} = 0.12$ cycles and $CV_{HS} = 1.3\%$; between runs, CV_{HS} increased to 3.8% . Although inter-assay comparisons of C_q values for standard reactions are not possible, four sample reactions duplicated in different runs indicated that $SD_{\Delta C_q}$ was also low (0.08 cycles), with $SD_E = 2.1\%$. No combination of PCR facilitators produced levels of variation significantly different from the mean values presented here. Based on this precision, we presume that the data from single sample reactions is reliable, although there is a risk that aberrant reactions may have gone unnoticed in the absence of technical replicates.

Initial inhibition tests (using undiluted extracts and no PCR facilitators) showed complete inhibition in all soil, feces, and hair extracts, with only bone extracts permitting any amplification of the IPC (Supplementary Table 2). Both bone extracts (B1, B2) had good amplification efficiency ($E = 94\%$), but with ΔC_q values of 2.4 or 6.1 cycles (Table 2); thus, even the least inhibited extract limited our expected recovery to $<20\%$ under these conditions.

As indicated in Table 2, the use of additional *Taq* improved the ΔC_q values (increasing the ER), but led to a decrease in amplification efficiency for the bone extracts. The cause of this decrease is unknown; however, adding BSA improved the ΔC_q values comparably and with optimal efficiency. Incorporating both facilitators brought the ΔC_q values to <1 (62 and 69% ER), such that no dilution would be beneficial in subsequent PCR runs with these extracts.

For the feces (F1, F2) and hair (H1, H2) extracts, both facilitators and dilution were required to reduce inhibition sufficiently, although BSA alone was responsible for the majority of the improvement for extracts F1, F2, and H1. Even at very high dilutions, additional *Taq* was unable to facilitate amplification in the feces extracts, but was particularly effective against inhibitors in H2. Unfortunately, the dilutions necessary to overcome inhibition in these extracts drop the maximum ER values below 2% , which may render the optimization process trivial for highly degraded and other low-copy templates.

In terms of reaction success versus complete inhibition among the soil extracts, *Taq* had no significant effect as a facilitator in contrast to BSA ($P = 3 \times 10^{-15}$). Not surprisingly, BSA had a less noticeable effect on reaction success when the extracts were diluted ($P = 3 \times 10^{-3}$ and $P = 5 \times 10^{-1}$ with $1/10$ and $1/50$ dilutions, respectively). Optimal ER values (from 20 to 77%) were achieved with BSA alone in $\sim 31\%$ of the extracts (Figure 1, solid black triangles), while the other 69% benefited from additional *Taq* as well (Figure 1, solid black circles). A subset of those more inhibited extracts showed optimal ER values using a $1/2$ dilution in PCR (Figure 1, solid blue circles). Although the amplification efficiencies were improved by diluting the soil extracts further (Supplementary Table 2), conditions providing higher ER values were more relevant for subsequent PCR runs.

A comparison of inhibition levels across soil DNA extraction methods is shown in Table 3. In all cases, the method of Willerslev et al. (27) demonstrated the lowest inhibition levels. Although we expect some variation in inhibition levels between these extracts due to the heterogeneity of soil, comparable tests of duplicate extracts (i.e., biological replicates) of sample P15 (Supplementary Table 2) indicate that this variation is minimal, with $SD_{\Delta C_q} = 0.3$ cycles and $SD_E = 1.6\%$.

From the data in Figure 2, there is an apparent correlation between E and ΔC_q , which is expected given the threshold-based method of calculating C_q ; as the amplification efficiency decreases, the placement of the fluorescence threshold becomes more sensitive, and the resulting ΔC_q values more variable. Thus, many of these poor efficiency reactions have deceptively low ΔC_q values, even though they are highly inhibited.

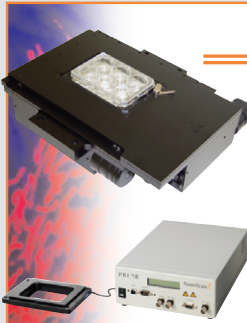
Interestingly, many reactions display a high ΔC_q value without a corresponding drop in reaction efficiency (Figure 2). This effect of inhibition is apparently overcome by the addition of BSA, as it is only visible in reactions lacking BSA, which required considerable dilution of the extracts to facilitate any amplification. Examples of these atypical efficiency- C_q relationships are shown in Figure 3.

Discussion

By incorporating both ΔC_q and efficiency measurements in our experiments, and by analyzing their results through pairwise comparisons, we have detected inhibition with various responses to PCR facilitators. We have typically observed C_q shifts in response to decreased amplification efficiency, as expected using threshold-based quantification. In some instances, however, a low ΔC_q and high ER can be associated with poor efficiency due to placement of the threshold. In these cases, and in any reaction where the efficiency differs from 100% (relative to a standard reaction), the ER value should only be considered an upper limit, but whose accuracy becomes greater as E approaches 100% .


In contrast, we observed many instances in which high ΔC_q values were associated with highly efficient reactions. This can be explained by the presence of thermolabile inhibitors that diminish with cycling; they may inhibit during early cycles of PCR, but the amplification efficiency will eventually recover and produce only a delayed C_q value. Alternatively, inhibitors that degrade or capture DNA (e.g., nucleases and DNA binding substances such as silica)

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may reduce the number of amplifiable IPC templates in an otherwise efficient PCR. This observation illustrates the importance of using an IPC for inhibition testing; without it (i.e., using efficiency calculations alone) these C_q shifts and large drops in ER would remain undetected.

Amplification of damaged templates may create the same pattern due to a reduction in amplification efficiency in the early cycles of PCR until newly synthesized/undamaged templates predominate (33). This is unlikely to affect our IPC, but may be an unavoidable source of error when quantifying damaged DNA relative to a pristine standard.

With repeat inhibition tests, we were able to optimize the PCR facilitator combinations and dilutions that result in the highest possible ER for each extract. For the bone and most soil samples, dilution could be avoided altogether; in contrast, inhibition in the feces and hair extracts could not be overcome with BSA and additional *Taq*, but instead required that 1/50 or even 1/100 dilutions be used in PCR. Although the resulting ER values are indeed optimal within the parameters of this study, the values are so low for these diluted extracts that additional PCR facilitators should be considered before subsequent PCR runs are attempted. If the values cannot be improved this way, additional purification or even alternative extraction procedures may be beneficial. As we have demonstrated, inhibition testing can also be useful in evaluating these protocols.

Although we recommend testing every extract for inhibition, it may not be necessary to test every combination of PCR facilitators. Some general trends exist within sample types using a particular extraction method: for instance, compared with BSA, additional *Taq* appeared to provide limited improvement for PCR results from our feces and soil extracts. This suggests that their major inhibitors do not act directly on the enzyme, provided that enough *Taq* was used to observe such an effect. In contrast, improvements in solely *Taq*-supplemented hair and bone reactions indicate that direct inhibitors of the enzyme are present. It is not surprising that BSA was able to overcome inhibition in all sample types, based on its combined enzyme-stabilizing and inhibitor-binding (34) abilities.

Recommendations

If the samples are to be used in subsequent quantitative comparisons, several considerations must be made to ensure accuracy. For threshold-based quantification, reaction conditions producing equivalent amplification efficiencies between samples and/or standards should be favored, even if ER

is low (e.g., with dilution). If this is not feasible, differences in efficiency can be applied as correction factors (13). Relative measurements of efficiency are sufficient in the former situation, while the latter requires highly accurate, absolute values. Alternatively, the recently published Cy0 method (21) obviates the need to assess amplification efficiency separately from quantification, and may be particularly useful when efficiency is highly variable; furthermore, $\Delta Cy0$ values determined from IPC reactions should be analogous to ΔC_q , with their incorporation in ER calculations producing a convenient, single-parameter correction factor.

The accuracy of correction factors derived from IPC data also depends on the original assumption that the IPC and target assays are equally affected by inhibition. Intuitively, large differences in the amplicon sizes should be avoided; however, no particular sequence characteristics are clear predictors of inhibition susceptibility (11). Huggett et al. (11) suggest that assay performance be compared in the presence of a suspected inhibitor; however, this approach may be ineffective when the inhibitors are of unknown types and amounts, and additionally impractical when inhibitors vary between samples. If inhibition susceptibility is instead related to competition for reaction components (10), it should be sufficient to demonstrate that the assays behave similarly (i.e., within the accuracy requirements of the experiment) under a variety of conditions (e.g., concentration gradients of Mg^{2+} , primers, and facilitators).

Based on the data presented here, we suggest that inhibition testing be regularly incorporated in procedures involving low amounts of DNA, such as in the forensic and ancient DNA fields. While the results are generally helpful for improving PCR success, they should be considered essential in experiments involving qPCR comparisons and when interpreting negative results. These tests should incorporate an IPC, which allows for simultaneous measurement of amplification efficiency and ΔC_q . When employed routinely, this form of pre-PCR processing ensures both maximum access to template DNA and more accurate quantification of template amounts.

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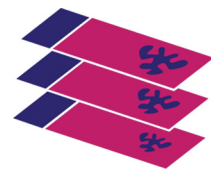
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Competing interests

The authors declare no competing interests.

References

1. Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* 63:3741-3751.
2. Rossen, L., P. Nørskov, K. Holmström, and O.F. Rasmussen. 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *Int. J. Food Microbiol.* 17:37-45.
3. Ramakers, C., J.M. Ruijter, R.H. Deprez, and A.F. Moorman. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* 339:62-66.
4. Cloud, J.L., W.C. Hymas, A. Turlak, A. Croft, U. Reischl, J.A. Daly, and K.C. Carroll. 2003. Description of a multiplex *Bordetella pertussis* and *Bordetella parapertussis* LightCycler PCR assay with inhibition control. *Diagn. Microbiol. Infect. Dis.* 46:189-195.
5. Sutlovic, D., M. Definis Gojanovi, S. Andelinovi, D. Gugi, and D. Primorac. 2005. *Taq* polymerase reverses inhibition of quantitative real time polymerase chain reaction by humic acid. *Croat. Med. J.* 46:556-562.
6. Deagle, B.E., J.P. Eveson, and S.N. Jarman. 2006. Quantification of damage in DNA recovered from highly degraded samples—a case study on DNA in faeces. *Front. Zool.* 3:11.
7. Nolan, T., R.E. Hands, W. Ogunkolade, and S.A. Bustin. 2006. SPUD: a quantitative PCR assay for the detection of inhibitors in nucleic acid preparations. *Anal. Biochem.* 351:308-310.
8. Swango, K.L., M.D. Timken, M.D. Chong, and M.R. Buonocristiani. 2006. A quantitative PCR assay for the assessment of DNA degradation in forensic samples. *Forensic Sci. Int.* 158:14-26.
9. Niederstätter, H., S. Köchl, P. Grubwieser, M. Pavlic, M. Steinlechner, and W. Parson. 2007. A modular real-time PCR concept for determining the quantity and quality of human nuclear and mitochondrial DNA. *Forensic Sci. Int.; Genet.* 1:29-34.
10. Stahlberg, A., P. Aman, B. Ridell, P. Mostad, and M. Kubista. 2003. Quantitative real-time PCR method for detection of B-lymphocyte monoclonality by comparison of kappa and lambda immunoglobulin light chain expression. *Clin. Chem.* 49:51-59.
11. Huggett, J.F., T. Novak, J.A. Garson, C. Green, S.D. Morris-Jones, R.F. Miller, and A. Zumla. 2008. Differential susceptibility of PCR reactions to inhibitors: an important and unrecognized phenomenon. *BMC Res Notes* 1:70.



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12. **Bustin, S.A., V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, et al.** 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55:611-622.
13. **Liu, W. and D.A. Saint.** 2002. A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. *Anal. Biochem.* 302:52-59.
14. **Liu, W. and D.A. Saint.** 2002. Validation of a quantitative method for real time PCR kinetics. *Biochem. Biophys. Res. Commun.* 294:347-353.
15. **Tichopad, A., A. Dzidic, and M.W. Pfaffl.** 2002. Improving quantitative real-time RT-PCR reproducibility by boosting primer-linked amplification efficiency. *Biotechnol. Lett.* 24:2053-2056.
16. **Bar, T., A. Stahlberg, A. Muszta, and M. Kubista.** 2003. Kinetic Outlier Detection (KOD) in real-time PCR. *Nucleic Acids Res.* 31:e105.
17. **Peirson, S.N., J.N. Butler, and R.G. Foster.** 2003. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Res.* 31:e73.
18. **Tichopad, A., M. Dilger, G. Schwarz, and M.W. Pfaffl.** 2003. Standardized determination of real-time PCR efficiency from a single reaction set-up. *Nucleic Acids Res.* 31:e122.
19. **Rutledge, R.G.** 2004. Sigmoidal curve-fitting redefines quantitative real-time PCR with the prospective of developing automated high-throughput applications. *Nucleic Acids Res.* 32:e178.
20. **Kontanis, E.J. and F.A. Reed.** 2006. Evaluation of real-time PCR amplification efficiencies to detect PCR inhibitors. *J. Forensic Sci.* 51:795-804.
21. **Guescini, M., D. Sisti, M.B. Rocchi, L. Stocchi, and V. Stocchi.** 2008. A new real-time PCR method to overcome significant quantitative inaccuracy due to slight amplification inhibition. *BMC Bioinformatics* 9:326.
22. **Rutledge, R.G. and D. Stewart.** 2008. Critical evaluation of methods used to determine amplification efficiency refutes the exponential character of real-time PCR. *BMC Mol. Biol.* 9:96.
23. **Shain, E.B. and J.M. Clemens.** 2008. A new method for robust quantitative and qualitative analysis of real-time PCR. *Nucleic Acids Res.* 36:e91.
24. **Swillens, S., B. Dessars, and H.E. Housni.** 2008. Revisiting the sigmoidal curve fitting applied to quantitative real-time PCR data. *Anal. Biochem.* 373:370-376.
25. **Ruijter, J.M., C. Ramakers, W.M. Hoogaars, Y. Karlen, O. Bakker, M.J. van den Hoff, and A.F. Moorman.** 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* 37:e45.
26. **Rådström, P., R. Knutsson, P. Wolffs, M. Lövenklev, and C. Löfström.** 2004. Pre-PCR processing: strategies to generate PCR-compatible samples. *Mol. Biotechnol.* 26:133-146.
27. **Willerslev, E., A.J. Hansen, J. Binladen, T.B. Brand, M.T. Gilbert, B. Shapiro, M. Bunce, C. Wiuf, et al.** 2003. Diverse plant and animal genetic records from Holocene and Pleistocene sediments. *Science* 300:791-795.
28. **Boom, R., C.J. Sol, M.M. Salimans, C.L. Jansen, P.M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* 28:495-503.
29. **Rohland, N. and M. Hofreiter.** 2007. Comparison and optimization of ancient DNA extraction. *BioTechniques* 42:343-352.
30. **Poinar, H.N., C. Schwarz, J. Qi, B. Shapiro, R.D.E. MacPhee, B. Buigues, A. Tikhonov, D.H. Huson, et al.** 2006. Metagenomics to paleogenomics: large-scale sequencing of mammoth DNA. *Science* 311:392-394.
31. **Gilbert, M.T., L.P. Tomsho, S. Rendulic, M. Packard, D.I. Drautz, A. Sher, A. Tikhonov, L. Dalen, et al.** 2007. Whole-genome shotgun sequencing of mitochondria from ancient hair shafts. *Science* 317:1927-1930.
32. **Gilbert, M.T., T. Haselkorn, M. Bunce, J.J. Sanchez, S.B. Lucas, L.D. Jewell, E. Van Marck, and M. Worobey.** 2007. The isolation of nucleic acids from fixed, paraffin-embedded tissues-which methods are useful when? *PLoS One* 2:e537.
33. **Sikorsky, J.A., D.A. Primerano, T.W. Fenger, and J. Denvir.** 2004. Effect of DNA damage on PCR amplification efficiency with the relative threshold cycle method. *Biochem. Biophys. Res. Commun.* 323:823-830.
34. **Kreider, C.A.** 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl. Environ. Microbiol.* 62:1102-1106.

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