Supplementary Material For:

# Comparison and optimization of ancient DNA extraction

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*BioTechniques 42:343-352 (March 2007)*

## Supplementary Materials and Methods

### 1 Initial comparison

*1a) Initial comparison of different methods using ~40mg sample material*

1a.1 Silica method (1)

40 mg of powder were digested in 1ml buffer composed of 0.45M EDTA pH 8.0, 0.5% N-lauroylsarcosine, 1% PVP, 50mM DTT, 2.5mM PTB and 0.25 mg/ml proteinase K overnight at 37°C in the dark. After centrifugation, the supernatant was added to 4ml binding buffer (L2: 5M GuSCN, 25mM NaCl, 50mM Tris (pH 8.0)) plus 15µl silica suspension (prepared according to (2), using silica from Sigma [ordering number: S5631]) and incubated under agitation for one hour at room temperature. The silica pellet was collected via centrifugation, the supernatant discarded and the pellet washed once with binding buffer and once with NewWash solution (see 3.8 for preparation, Qbiogene, USA). Subsequently, the pellet was dried at 56°C for 7min. DNA was eluted with 50µl 1xTE at 56°C for 10min, and after centrifugation, the aqueous solution was transferred to a new tube.

1a.2 Centricons

40 mg of powder were incubated as described above. After centrifugation, the supernatant was added to 1ml phenol (pH 8.0), gently mixed and centrifuged for 3min. This step was repeated once. The aqueous phase was added to 1ml Ready Red (Chloroform / Isoamylalcohol, Qbiogene), mixed and centrifuged again for 3min. Thereafter, the aqueous phase was added to Centricon YM 30 (MWCO 30,000; Millipore, USA) filter devices and centrifuged at 13,200 rpm until ~ 50µl remained. This solution was washed twice with 100µl 1xTE by centrifugation. The remaining ~50µl extract were transferred using inverse centrifugation of the filter into a new tube.

1a.3 Phenol chloroform (3)

40 mg of powder were decalcified in 2ml 0.5M EDTA (pH 8.0) at room temperature overnight. After centrifugation, the supernatant was discarded and the remaining pellet was digested by incubation at 37°C overnight in 1ml extraction buffer composed of 10mM Tris (pH 8.0), 10mM NaCl, 0.1% SDS, 50mM DTT, 0.25mg/ml proteinase K and 2.5mM PTB (concentrations of DTT, proteinase K and PTB were taken from (1), as the concentrations are omitted in (3)). After centrifugation, the supernatant was added to 1ml phenol (pH 8.0), gently mixed and centrifuged for 3min. This step was then repeated. The aqueous phase was added to 1ml Ready Red, gently mixed and centrifuged for 3min. Then the aqueous phase was added to Centricon YM 30 filter devices, and the solution was concentrated via centrifugation at 5,000 rpm. The solution was washed twice with 200µl 1xTE. The remaining ~50µl extract were transferred by inverse centrifugation of the filter using a new tube.

1a.4 User-developed protocol: Isolation of genomic DNA from compact bone using the DNeasy Tissue Kit (Qiagen, Germany)

40 mg of powder were decalcified in 2ml 0.5M EDTA (pH 8.0) at room temperature overnight. After centrifugation, the supernatant was discarded and the pellet washed with 1ml dd$H_2O$, centrifuged and again the supernatant discarded. 360µl buffer ATL and 40µl proteinase K were added to the pellet. This mixture was incubated under agitation for one hour at 56°C until lysis of the pellet was complete. After vortexing, 400µl buffer AL were added, and the mixture was incubated at 70°C for 10min. 400µl ethanol absolute and 3ng of DNA size marker serving as a carrier were added, and the samples were gently mixed. The mixture was pipetted onto DNeasy spin columns and centrifuged at 8,000 rpm for 1min. The flow-through was discarded, and the remaining mixture was pipetted to the columns and centrifuged again. Following this step, the column was washed once with 500µl buffer AW1, once with 500 µl AW2, and then centrifuged for 3min at 14,000rpm until it was dry. 50µl of buffer AE were added, incubated for 1min at room temperature and centrifuged for 1min at 8,000rpm. This flow-through represents the extract.

1a.5 "DNA-Präparation aus Knochen" with All-tissue DNA-Kit (GEN-IAL GmbH, Germany)

40 mg of bone powder were decalcified overnight in 2ml 0.5M EDTA (pH 8.0) at room temperature. After centrifugation, the supernatant was discarded and the pellet was washed with 1ml dd$H_2O$, centrifuged and the supernatant again discarded. 1ml Lyse1 buffer, 100µl Lyse 2, 3mg DTT and 15µl proteinase K were added to the pellet and

incubated for about one hour at 56°C under agitation until the pellet was completely dissolved. 750µl Lyse 3 buffer were added, vortexed, incubated at -20°C for 5min and centrifuged at 13,000rpm for 20min. The supernatant (~1.8ml) was transferred to a fresh tube and 0.8 volume of isopropanol, plus 10ng of a DNA size marker as a carrier were added. To precipitate the DNA, the mixture was centrifuged for 15min at 13,000rpm.The supernatant was removed and 300µl ice cold 70% ethanol were added and centrifuged at 13,000rpm for 5min. The liquid was discarded and the DNA pellet dried at 56°C for 5min. The DNA was dissolved in 50µl 1xTE.

1a.6 Bone Extraction Protocol to be used with the DNA IQ System (Promega, USA).

40 mg of powder were incubated in 1ml proteinase K digestion buffer composed of 10mM Tris (pH 8.0), 10mM NaCl, 50mM EDTA (pH 8.0), 0.5% SDS and 1mg/ml proteinase K for one hour at 56°C under agitation. 2ml Lysis buffer (including 10mM DTT) and 15µl of Resin (magnetic particles) were added to the supernatant. After incubation at room temperature for 10min with frequent mixing of the solution, the Resin was collected using the Magnetic Stand. The supernatant was removed and again 100µl Lysis buffer were added, vortexed, the Resin separated, the solution removed and 100µl Wash buffer added. After vortexing, separation and removal of the liquid, this washing step was repeated twice. The Resin was then air dried for 15min in the Magnetic Stand. 50µl 1xTE were added, vortexed and DNA was eluted for 5min at 65°C. The separated supernatant represents the extract.

*1b) Initial comparison of "silica" with "DNA IQ" using 200mg sample material*

1b.1 Silica method (1)

The method is the same as described above (1a.1), except that the amount of powder was increased to 200mg, the volume of extraction buffer increased to 5ml, the incubation conditions were modified from 37°C overnight to 56°C for one hour (the same as for the DNA IQ Kit) and the volumes of the binding buffer and the silica suspension were increased to 20ml and 50µl, respectively. All other steps including concentrations, times and temperatures remained identical.

1b.2 Bone Extraction Protocol to be used with the DNA IQ System (Promega, USA).

The method is the same as described above (1a.6), except that the amount of powder was increased to 200mg and the volumes of proteinase K digestion buffer and of the Lysis buffer were increased to 1.5ml and 3ml, respectively. All other steps including concentrations, times and temperatures remained identical.

## 2 Extraction buffer components, incubation temperature and time.

*2.1 Surfactants*

The "silica" method was used as described under 1b.1, except that the temperature of the initial incubation was again set to 37°C, the amount of silica suspension was 40µl and aside from the 0.5% N-lauroylsarcosine (sarcosyl) used in the original protocol, the performance of other detergents (non-ionic: 1% Tween-20, 1% Triton X-100; anionic: 0.5% SDS; cationic: 1% CTAB, 2M Urea, 2M GuCl) as well as the absence of detergent was tested.

*2.2 Incubation temperature and time*

Extraction was performed as described under 2.1, without adding detergents to the extraction buffer. Incubation temperature and time of the powder in extraction buffer were varied (56°C for 1hour; 37°C for 1hour; 37°C overnight; room temperature overnight; room temperature for four days, room temperature for four days plus the addition of 0.25mg/ml proteinase K after two days).

*2.3 Remaining chemicals in the extraction buffer in all combinations*

Extraction was performed as described under 2.2. Incubation was at room temperature overnight and the effects of both the presence and absence of all remaining ingredients in the extraction buffer were tested in all possible combinations. Results were compared for each possible extraction buffer composition in pairwise comparisons of presence versus absence of the four ingredients except EDTA (PTB, PVP, DTT and PK).

*2.4 Presence vs. absence of 0.05M Tris (pH 8.0)*

Extraction was performed as described under 2.3, reducing the composition of the extraction buffer to 0.45M EDTA, 0.25mg/ml proteinase K and 50mM DTT and testing the presence and absence of 0.05M Tris (pH 8.0) in the extraction buffer.

*2.5 EDTA concentration*

Extraction was performed as described under 2.4 without adding Tris, varying the EDTA concentration between 0.45M, 0.2M, 0.1M and 0.05M in the extraction buffer.

*2.6 Reducing agents*

Extraction was performed as described under 2.5 with an EDTA concentration of 0.45M in the buffer. We tested the effect of different reducing agents besides DTT, all in the same concentration of 50mM (Thioglycolic acid; 2-Mercaptobenzothiazole; 2-Naphthalenethiol; 2-Amino-5-mercapto-1,3,4-thiadiazole; 5,5□-Dithiobis(2-nitrobenzoic acid); 2-Mercaptoethanol; Dodecanthiol), as well as the absence of any reducing agent.

*2.7 Sample preparation technique*

Extraction was performed as described under 2.6 without any reducing agent. The difference in this experiment was how the sample was prepared prior to incubation in the extraction buffer. Either the powder was prepared using a freezer mill or part of the compact sample was ground to a more or less coarse-grained “powder” using a mortar and pestle. It should be noted that the different samples could be ground to varying

granularities using the mortar and pestle. For example, sample 891 is extremely compact, and particles with varying diameters of up to several millimeters were used in this experiment, whereas other samples were much easier to grind using mortar and pestle, so that a fine powder was obtained. After completing this experiment, the remaining sample material was extracted a second time using the same procedure as described above, except that it was incubated for 2 hours at 56°C until almost all powder was digested.

## 3 Binding buffer, silica age, pH during binding, binding time, silica preparation technique, wash solutions

*3.1 Age of the silica suspension*

Extraction was performed as described under 2.6, again using a fine, homogeneous powder. In this experiment the age of the silica suspension varied from one week to approximately one year.

*3.2 pH of the binding buffer (including the extraction buffer plus silica)*

Extraction was performed as described under 3.1 using silica suspension not older than three months, and adjusting the pH between 4.0, 6.0 and 8.0 with hydrochloric acid after adding both silica suspension and extraction buffer to the binding buffer.

*3.3 Amount of silica suspension*

Extraction was performed as described under 3.2 (pH was not adjusted to 4.0, but was ~ 8.0) using different amounts of silica suspension (5µl, 10µl, 20µl, 40µl, 100µl).

*3.4 Different binding salts tested on DNA size marker*

In this experiment, we used a 100bp DNA size marker (NEB, USA) instead of an extraction buffer containing ancient DNA. The size marker was diluted with 0.5M EDTA (total 400µl) and 8µl silica suspension plus 1.6ml of the respective salt solution (saturated salts: $KH_2PO_4$, NaCl, NaI, $NH_4SO_4$, CsCl, KCl, $MgSo_4$, GuCl, NaAc, $NH_4Ac$; 5M solution: $NaClO_4$, GuSCN) were added. The mixtures were incubated for 1 hour at room

temperature under constant agitation. After centrifugation the supernatant was discarded and the silica pellet was washed once with the respective binding salt and once with NewWash solution. After completely removing the liquid, the pellet was dried for 5min at 56°C and DNA was eluted in 15µl 1xTE. 10µl were loaded on a gel together with a size marker in the amount expected at 100% recovery.

*3.5 Performance of different binding salts using ancient DNA samples*

Extractions were performed as described under 3.2 adjusting the pH to 4.0 and using 100µl silica suspension. Instead of using the usual binding buffer, different salts (saturated KCl, saturated GuCl, 5M GuSCN, 5M $NaClO_4$, saturated NaCl, saturated NaI) were used as binding buffer, as these salts gave the best results in the size marker test (3.4). After binding to silica and centrifugation, the silica was washed first with the respective binding solution and then with NewWash buffer. When GuSCN was used as a binding salt, two washing variations were performed. In the first variation, the silica was washed once with NewWash and in the second, it was washed twice before it was dried and the DNA eluted.

*3.6 Binding time*

Extractions were performed as described under 3.5 using 3M NaCl. Washing steps were done once with 3M NaCl and twice with NewWash buffer. This experiment was done using only two samples from 100mg sample material each, because the remaining material from the third sample was needed for the final comparison. The low amounts of bone powder resulted in highly variable outcomes. The binding time was varied in the

first trial (a) between 15 minutes to overnight (0.25h, 1h, 3h, overnight) and in the second trial (b) between 1.5h, 3h and 6h.

*3.7 Silica preparation technique*

Extractions were performed as described under 3.6 using an incubation time of 3h for binding the DNA to silica. Silica suspensions were prepared using two different techniques. While the "normal" silica was prepared as described in Boom et al. (2), the second preparation technique ("new") was slightly different: 4.8g Silica were suspended in $ddH_2O$ to a final volume of 40ml. After sedimentation for 1 hour, 39ml were transferred into a new tube and left to settle for additional 4 hours. Thereafter 35ml of the supernatant were removed and 48µl 30% HCl were added to the sediment in order to adjust the pH. After dividing the silica into 400 l aliquots, the solution was stored in the refrigerator.

*3.8 Testing the efficacy of different wash solutions*

Extractions were performed as described under 3.7 using the new silica preparation technique. Four different possible alternatives for the commercial NewWash solution (1ml NewWash concentrate [Qbiogene], 20ml $ddH_2O$, 23ml Ethanol absolute) were tested:

Alternative 1: 50% Ethanol, 0.1M NaCl, 1mM EDTA (pH 8.0), 10mM Tris (pH 8.0)

Alternative 2: 50% Ethanol, 0.3M NaCl, 1mM EDTA (pH 8.0), 10mM Tris (pH 8.0)

Alternative 3: 60% Ethanol, 0.1M NaCl, 1mM EDTA (pH 8.0), 10mM Tris (pH 8.0)

Alternative 4: 70% Ethanol, 0.3M NaCl, 10mM EDTA (pH 8.0), 100mM Tris (pH 8.0)

## 4 Final comparison

### *4.1 Silica method (1)—method A*

Extractions were performed as described under 1a.1 using 500 mg powder and an extraction buffer volume of 10ml. The binding buffer volume was increased to 35ml, the silica suspension to 50µl and the DNA was eluted in 100µl 1xTE.

A 1:10 and 1:50 dilution were prepared immediately after the extraction, and a standard PCR (see 5.2) was performed with these two dilution steps before the extracts were frozen.

### *4.2 Modified silica method with NaCl as binding salt—method B*

500 mg of powder were incubated in 10ml extraction buffer (0.45M EDTA and 0.25mg/ml proteinase K) at room temperature overnight. After centrifugation, the supernatant was added to 35ml 5M NaCl, 90µl silica suspension prepared using the new preparation technique was added and the pH was adjusted to 4.0. This mixture was incubated for three hours at room temperature under rotation. After centrifugation the supernatant was discarded, the silica pellet was washed with 1ml 5M NaCl and then transferred into a 2ml tube. After short centrifugation, the NaCl was removed with a pipette, the pellet resuspended in 1ml wash solution, which is a combination between alternatives 1 and 2 (51.3% Ethanol, 125mM NaCl, 1mM EDTA (pH 8.0), 10mM Tris (pH 8.0)), shortly centrifuged, and the supernatant removed with a pipette. The last washing step was repeated. Subsequently, the silica pellet was air dried at room temperature for 15min, resuspended in 100µl 1xTE and after 8min incubation at RT, the

silica was collected by centrifugation at maximum speed (16,000 g). The supernatant, representing the extract, was transferred into a fresh tube. 1:10 and 1:50 dilutions were prepared immediately after the extraction, and a standard PCR was performed using these two dilutions before the extracts were frozen.

*4.3 Modified silica method using GuSCN binding solution—method C*

This extraction method is exactly the same as described above (4.2), except that the 5M NaCl was exchanged by the binding solution used in the initial silica method (L2; see 1a.1). 1:10 and 1:50 dilutions were prepared immediately after extraction, and a standard PCR was performed using these dilutions before the extracts were frozen.

*4.4 Phenol chloroform (3)—method D*

Extractions were performed as described under 1a.3 using 500 mg bone powder and 25ml 0.5M EDTA for decalcification. The remaining protein pellet was digested in 10ml extraction buffer at 53°C. The volume of phenol and Ready Red was adjusted to 10ml each and the volume of washing with 1xTE was, in total, 650µl. The remaining extract was ~100µl as for all other methods. 1:10 and 1:50 dilutions were prepared immediately after extraction, and a standard PCR was performed using these two dilutions before the extracts were frozen.

*4.5 Ethanol precipitation (4)—method E*

500 mg of sample powder were digested in 1.1ml extraction buffer composed of 0.1M EDTA (pH 8.0), 0.5% N-lauroylsarcosine, 0.25mg/ml proteinase K at 37°C overnight.

After centrifugation, the approximately 750µl of supernatant were divided into 3 aliquots à 250µl (the remaining liquid was bound to the bone powder). To each 250µl aliquot, 250µl 4M $NH_4$-Acetat, 3.5µl Blue Dextran (1µg/µl) and 500µl 96% ethanol were added and this mixture vortexed. After incubation for 10min at -80°C, the DNA was precipitated at 13,200rpm for 15min, dried at 56°C for 10min and dissolved in 33.3µl 1xTE for each of the 3 parts, resulting in 100µl per sample. 1:10 and 1:50 dilutions were prepared immediately after extraction and a standard PCR was performed with these two dilutions before the extracts were frozen.

*4.6 User-developed protocol: Isolation of genomic DNA from compact bone using the DNeasy Tissue Kit (Qiagen, Germany)—method F*

These extractions were performed as described under 1a.4 using 500mg powder and 18ml 0.5M EDTA for decalcification. Washing the protein pellet with $ddH_2O$ was omitted. 3.8ml buffer ATL and 200µl proteinase K (10mg/ml) were added to the pellet and incubated for about 2 hours at 55°C until the pellet was dissolved. The volume of both buffer AL and ethanol absolute was increased to 4ml and no carrier was added. This mixture was added step by step (each 650µl maximum) to the columns and centrifuged for 1min after each addition of buffer. The volume of the elution buffer AE was increased to 100µl. 1:10 and 1:50 dilutions were prepared immediately after extraction and a standard PCR was performed with these two dilutions before the extracts were frozen.

## 5 Quantitative PCR and standard PCR

*5.1 Real time quantitative PCR*

A probe based real time quantitative PCR assay was designed using sequences from *Ursus arctos* (AF303110), *U. maritimus* (NC003428) and *U. americanus* (Y08520) and the PrimerExpress software (Applied Biosystems). It is located in the 12S region of the mitochondrial genome and is 110bp long, including primers. As no information about cave bear 12S sequences were found in the database, the nucleotide sequences of both primers and probe were sequenced in four cave bear samples from three different caves, all showing the same sequence. The assay, furthermore, was designed not to amplify human contamination, as the forward primer contains two mismatches (5) at the 3´end and the probe has, in total, 7 mismatches to the corresponding human sequence. DNA from the above mentioned *Ursus* species should also not be detected, as all have a minimum of one mismatch to the probe. However, it was never tested if DNA from these species could be detected using this assay. Sequences of primer and probe were as follows:

CB TM 12S F 5´-AAA ATG CCC TTT GGA TCT TAA T-3´

CB TM 12S R 5´-ACT GCT GTA TCC CGT GGG-3´

CB TM 12S Probe 5´-VIC-CGA TTT GAA GGA GCG GGC A-TAMRA-3´

As the quantitative PCR assay is probe-based, potential nonspecific amplification products are not measured, as they would lack a sequence complementary to the probe. Thus, even if amplification of nonspecific products occurred, it would not influence the results. We used eleven dilution steps (between 25 and 50,000 copies) of a PCR product about 399bp in length amplified from a cave bear extract from Gamssulzen cave, Austria,

as template DNA in the standard. Each dilution step was amplified in triplicate for the same 110 bp fragment as the ancient DNA extracts alongside the samples. The copy number of the standard was estimated via UV absorption using a spectrophotometer (Nanodrop ND-1000).

5µl of the extracts or dilutions of extracts were used for each quantitative PCR in a final volume of 20µl consisting of 1x TaqMan® Buffer A, 4.5mM $MgCl_2$, 0.4mM dUTP, 0.2mM dATP, dCTP and dGTP each, 1U / 2U AmpliTaq® Gold DNA Polymerase (1U was used for the "initial comparisons" and the "detergent" test, all subsequently performed real time quantitative PCR runs were performed using 2U), 0.3µM each forward and reverse primer and 0.2µM probe. Initial denaturation and activation of AmpliTaq® Gold were run for 5min at 95°C, followed by 50 to 60 cycles at 95°C for 15sec and 60°C for 45sec.

Standard curves were generated from the dilution series, and copy numbers of the extracts were extrapolated from the equation of the standard curve. $R^2$ of standard curves were between 0.96 and 0.99, with 73% of all runs having values above 0.98.

*5.2 Standard PCR after final extraction*

After finishing each final extraction protocol, a standard PCR using bear specific primers (CBL164 and CBH221 from (1)) was performed, amplifying a 103bp long fragment of the mitochondrial HVR I. 5µl of 1:10 and 1:50 dilutions were used in a final volume of 20µl consisting of 1x GeneAmp® PCR Buffer II , 4mM $MgCl_2$, 625µM of each dNTP, 2.5U AmpliTaq Gold® and 500nM of forward and reverse primer, respectively. Initial denaturation and activation of AmpliTaq Gold® were run at 94°C for 4min, followed by

60 cycles 94°C for 20sec, 52°C for 30sec, 72°C for 30sec and a final elongation at 72°C for 10min.

## 6 Inhibition test

To assess the degree of inhibition originating from the different extraction methods in the final comparison, a quantitative human mitochondrial PCR assay (6) was spiked with 5µl of a 1:10 dilution of the extracts. In a 20µl reaction the concentrations of the various ingredients were as follows: 1x TaqMan buffer A, 4.5 mM $MgCl_2$ , 200 pM probe, 300 pM forward and reverse primer, 2U AmpliTaq Gold® and (except for the negative controls and the standard) 30 pg of human DNA. The standard dilution was done in quadruplicate from the same human DNA, beginning with 10,000 pg down to 1pg in 10 fold dilutions, and was amplified alongside the inhibition test. The quantitative PCR was performed on a Stratagene Mx3000p (Stratagene, The Netherlands), with 10min activation of AmpliTaq Gold® at 95°C, followed by 45 cycles at 95°C for 15sec and 60°C for 1min. In total, 23 positive controls (without spiking with ancient DNA) were performed.

Two different approaches for testing for inhibition were used. The first approach calculates the inhibition from the differences of the mean of all positive PCR controls (containing ~ 30pg human DNA), and the mean of the six measurements per sample for each extraction method using the absolute amount of DNA measured. If this difference deviated more than two times the standard deviation from the mean of the positive controls, we scored the extract as inhibitory. To understand the degree of inhibition, these differences were divided by the mean value of the positive controls. This procedure

produced the inhibition of each sample and method as percentages (i.e. 90% inhibition indicates that the copy number measured was only 10% of the mean copy number measured for the positive controls).

As it is assumed that inhibition influences the efficiency of the PCR, so that there is less than doubling of the target sequence per cycle, thereby rendering the slope of the qPCR curve to be less steep compared to uninhibited PCR, the second approach calculates the inhibition from the efficiencies of each individual qPCR. The efficiencies of each individual PCR were calculated using the software LinRegPCR (7). Next, we calculated the difference between the mean of the efficiencies from all positive controls (and the standards), and the mean of the six measurements per sample and extraction method. If the differences were larger than two standard deviations of the positive controls, inhibition was assumed. Again, to get an idea of the degree of inhibition, the differences were divided by the mean of the positive controls, and the result was treated as inhibition percentages for each sample and method.

## 7 Measuring PCR performance

### *7.1 Presence vs. absence of BSA*

Five extracts from different cave bear samples were tested in triplicate with the same 103bp fragment mentioned above (5.2), in both the presence and absence of a final volume of 1mg/ml BSA (bovine serum albumin) per PCR reaction using different amounts of AmpliTaq Gold® (between 1U and 1/16U). 5µl of 1:10 dilution of extract were used in a final volume of 20µl using 1x GeneAmp® PCR Buffer II, 2.5mM $MgCl_2$, 200µM of each dNTP, 200nM of forward and reverse primer, respectively, both with and

without 1mg/ml BSA and varying concentrations of AmpliTaq Gold® (1U, 0.5U, 0.25U, 0.125U, 0.0625U) using the temperature profile described above (5.2).

*7.2 Efficacy of different Taq DNA Polymerases*

7.2.1 General consideration

If not stated otherwise (e.g. in case of pre-mixed solutions), in the following tests, the same dilution of one cave bear sample, 1U of the respective Taq DNA polymerase, 0.2µM of each primer and 200µM each dNTP were always used.

7.2.2 SUPER TAQ (HT-Biotechnology LTD, UK) + TaqStart™ Antibody (BD Bioscience, USA)

As this polymerase is not a hot start enzyme and the handling in clean rooms is much easier with hot start polymerases not requiring ice, this polymerase was mixed 2:1 with a monoclonal antibody that binds to and inactivates Taq DNA polymerases below 70°C. PCR was performed under the following conditions: 1x PCR Buffer, 2.375mM $MgCl_2$ ($MgCl_2$ from the PCR buffer is included). The temperature profile of the PCR was: initial denaturation 94°C for 2min, 60 cycles 94°C for 20sec, 50°C for 30sec, 72°C for 30sec, and final extension at 72°C for 10min.

7.2.3 AmpliTaq Gold® (Applied Biosystems, USA)

PCR was completed using 1x GeneAmp® PCR buffer II and 2.5mM $MgCl_2$ in a final volume of 20µl, according to a temperature profile of 94°C for 5min, 60 cycles 94°C for 20sec, 50°C for 30sec, 72°C for 30sec and a final extension of 72°C for 10min.

7.2.4 Platinum® *Taq* DNA Polymerase (Invitrogen, USA)

PCR was performed using 1x PCR Buffer and 1.5mM $MgCl_2$ in a final volume of 20µl and according to a temperature profile of 94°C for 2min, followed by 60 cycles at 94°C for 20sec, 50°C for 30sec, 72°C for 30sec and final extension at 72°C for 10min.

7.2.5 Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen)

PCR was completed using 1x High Fidelity PCR Buffer and 2mM $MgSO_4$ in a final volume of 20µl according to the following temperature profile: 94°C for 2min, 60 cycles 94°C for 20sec, 50°C for 30sec, 68°C for 30sec and final extension at 70°C for 10min.

7.2.6 Platinum® PCR SuperMix (Invitrogen)

This is a ready to use mixture of polymerase, buffer and dNTP´s. To adjust the amount of polymerase (1U) for all other experiments, the $MgCl_2$ concentration in the final volume of 50µl was 1.5mM and each dNTP 200µM. Initial denaturation was done at 94°C for 2min, followed by 60 cycles at 94°C for 20sec, 50°C for 30sec, 70°C for 30sec and a final extension at 72°C for 10min.

7.2.7 AccuPrime™ *Taq* DNA Polymerase (Invitrogen)

PCR was executed using 1x AccuPrime PCR Buffer I (already including $MgCl_2$, resulting in a final concentration of 1.5mM) in a final volume of 20µl and according to the following temperature profile: 94°C for 2min, 60 cycles 94°C for 20sec, 50°C for 30sec, 68°C for 30sec and a final extension at 70°C for 10min.

7.2.8 BD Advantage™ 2 Polymerase Mix (BD Bioscience, USA)

PCR was performed using 1x Advantage 2 SA PCR Buffer (resulting in a final concentration of 2mM $MgCl_2$) in a final volume of 20µl and according to the following temperature profile: initial denaturation at 94°C for 2min, 60 cycles 94°C for 20sec, 50°C for 30sec, 68°C for 30sec and a final extension at 70°C for 10min.

7.2.9 SuperTaq™* Plus (Ambion, USA)

PCR was completed using 1x Long PCR Buffer and 1.5mM $MgSO_4$ in a final volume of 20µl with a temperature profile as follows: initial denaturation at 94°C for 2min, followed by 60cycles at 94°C for 20sec, 50°C for 30sec, 68°C for 30sec and a final extension at 70°C for 10min.

7.2.10 HotMaster™ Taq DNA Polymerase (Eppendorf, Germany)

PCR was executed using 1x HotMaster Taq Buffer with 25mM $Mg^{2+}$ (resulting in an end concentration of 2.5mM $Mg^{2+}$) in a final volume of 20µl and according to a temperature profile with initial denaturation at 94°C for 2min, followed by 60 cycles at 94°C for 20sec, 50°C for 30sec, 65°C for 30sec and a final extension at 70°C for 10min.

7.2.11 MasterTaq® Kit (Eppendorf)

PCR was completed using 1x Taq Buffer with $Mg^{2+}$ (resulting in an end concentration of 1.5mM $Mg^{2+}$) and 1x TaqMaster PCR Enhancer in a final volume of 20µl and according to the following temperature profile: initial denaturation at 94°C for 2min, followed by

60 cycles at 94°C for 20sec, 50°C for 30sec, 72°C for 30sec and a final extension at 72°C for 10min.

7.2.12 BD™*Taq* Full Hot Start DNA Polymerase Enzyme Mix (BD Bioscience)

PCR was completed using 1x Taq Full PCR Buffer (resulting in 2mM MgCl2) in a final volume of 20µl and according to the following temperature profile: 94°C for 2min, followed by 60 cycles at 94°C for 20sec, 50°C for 30sec, 68°C for 30sec and a final extension at 72°C for 10min.

**Results & Discussion**

To 1b):

After digestion of the powder at the same temperature and time, it is noteworthy that the powder was almost completely digested in the extraction buffer using the silica method, whereas almost no change of the powder volume could be observed for the proteinase K digestion buffer of the DNA IQ Kit. This difference could be explained by the overall smaller amount of buffer or the lower concentration of EDTA in the IQ extraction buffer (only 50mM vs. 450mM for the silica extraction buffer), both of which are too small to demineralize (or decalcify) the bone material. As the amount of proteinase K is the same in both buffers, proteinase K is unlikely to be the limiting factor.

To 2.3):

Apart from EDTA and sarcosyl, which were tested in separate experiments, the initial extraction buffer contained proteinase K, DTT, PTB and PVP. The later four reagents were tested in all possible combinations in a single experiment. Thus, for each reagent, we had eight different buffers containing the reagent and eight corresponding buffers lacking the reagent (Table S5). We compared both all means for the corresponding pairs from a certain reagent and the individual relative values for each sample from each buffer pair. Using a paired t-Test, only proteinase K showed a statistically significant improvement for both comparisons ($P<0.01$ and $P<0.05$, respectively). DTT showed a tendency of a positive trend (average performance with DTT = 0.86, without = 0.72), but neither comparison was statistically significant ($P=0.08$ in both cases). PTB showed no effect in either direction ($P>0.1$ in both cases; see also table S5), while PVP even had an

adverse effect with statistical significance for the comparison of the means ($P<0.05$) and close to statistical significance for the individual values ($P=0.07$). Due to these results, we retained proteinase K in the buffer and performed a more in-depth evaluation of the effects of reducing agents.

**Supplementary Tables:**

| Sample ID | Type | Origin | Country | $^{14}$C age | Dating No. | Citation |
|---|---|---|---|---|---|---|
| 233 | bone | Potocka zijalka | Slovenia | 26,900 +/- 110 BP | Beta - 143240 | (1) |
| 243 | bone | Vindija layer H | Croatia | 42,660 +/- 900 BP | Beta - 156101 | (1) |
| 322 | bone | Ramesch cave | Austria | - | | |
| 323 | bone | Ramesch cave | Austria | - | | |
| 330 | bone | Winden cave | Austria | - | | |
| 576 | bone | Gailenreuth | Germany | - | | |
| 748 | tooth | Wildkirchli | Switzerland | - | | |
| 751 | tooth | Ossenyaga cave | Russia | - | | |
| 891 | bone | Gamssulzen cave | Austria | 44,160 (+1400/-1190) | KIA25287 | (8) |
| 1050 | bone | Harz | Germany | - | - | - |

**Table S1**: Samples used in this study. Laboratory internal sample ID, type of material, cave in which the sample was found (origin), country, $^{14}$C age, dating number and citation are given.

| | 323 | 330 | 748 | Sample ID |
|---|---|---|---|---|
| **Extraction** | **Copy Number per gram** (Standard Deviation) | | | |
| **Method** | ***Relative to Best Method*** | | | ***Average*** |
| Silica | 0 | 1,953 (1,933) | 206 (504) | |
| | *0* | *1* | *0.07* | ***0.36*** |
| Centricon | 0 | 0 | 2,894 (3,314) | |
| | *0* | *0* | *1* | ***0.33*** |
| Phenol Chloroform | 0 | 399 (978) | 363 (414) | |
| | *0* | *0.2* | *0.13* | ***0.11*** |
| DNeasy | 16 (38) | 0 | 173 (316) | |
| tissue kit | *0.03* | *0* | *0.06* | ***0.03*** |
| All-tissue | 72 (177) | 19 (47) | 599 (771) | |
| DNA-kit | *0.12* | *0.01* | *0.21* | ***0.11*** |
| DNA IQ | 626 (626) | 13 (32) | 2,872 (2,558) | |
| system | *1* | *0.01* | *0.99* | ***0.67*** |

**Table S2a**: Results of quantitative PCR comparing different extraction methods using 40 mg of sample powder. Absolute copy numbers per gram of powder (standard deviation in parenthesis) are shown together with relative numbers compared to the best performing method in this test series, calculated separately for each sample. This is the same table as Table 1a, including standard deviations for the absolute copy numbers.

| | 323 | | | 330 | | | 748 | | | 891 | | | Sample ID | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Extraction** | **Copy Number per gram** (Standard deviation) | | | | | | | | | | | | | | |
| **Method** | ***Relative to Best Method*** | | | | | | | | | | | | ***Average*** | | |
| | original | 1:10 | total | original | 1:10 | total | original | 1:10 | total | original | 1:10 | total | original | 1:10 | total |
| Silica | 0 | 0 | 0 | 0 | 70 (53) | 35 (51) | 36 (19) | 151 (145) | 93 (115) | 22 (100) | 11,065 (1,164) | 5,555 (5,809) | | | |
| | *0* | *0* | *0* | *0* | *1* | *0.21* | *0.2* | *1* | *1* | *0.01* | *1* | *1* | ***0.05 **** | ***0.75*** | ***0.4*** |
| DNA IQ | 98 (140) | 48 (117) | 73 (131) | 330 (382) | 0 | 165 (318) | 178 (203) | 0 | 89 (173) | 2,142 (260) | 420 (171) | 1,281 (927) | | | |
| System | *1* | *1* | *1* | *1* | *0* | *1* | *1* | *0* | *0.96* | *1* | *0.04* | *0.23* | ***1*** | ***0.26*** | ***0.63*** |

**Table S2b:** Results of quantitative PCR comparing the two best performing extraction methods using 200 mg of sample powder. Absolute copy numbers per gram of powder (standard deviations are given in parentheses) are shown for the pure extract and a 1:10 dilution. Relative numbers for pure extract, dilution and total were separately calculated for each sample, and were compared to the best performing method in this test series. This is the same table as Table 1b, including standard deviations for the absolute copy numbers.

| Surfactant | 330 | 748 | 891 | Sample ID |
|---|---|---|---|---|
| | Copy Number per gram (Standard Deviation) | | | |
| | *Relative to Best Method* | | | *Average* |
| 1% Tween® 20 | 94 (33) | 11,496 (3,105) | 585,456 (158,150) | |
| | *0.07* | *0.67* | *1* | ***0.58*** |
| 1% Triton®-X 100 | 982 (354) | 13,266 (4,057) | 443,401 (69,532) | |
| | *0.78* | *0.78* | *0.76* | ***0.77*** |
| 0.5% N-lauryl-sarcosine | 7 (10) | 4,893 (4,695) | 65,566 (815) | |
| | *0.01* | *0.29* | *0.11* | ***0.17 **** |
| 0.5% SDS | 0 | 5,207 (1,113) | 89,645 (14,791) | |
| | *0* | *0.30* | *0.15* | ***0.15 **** |
| 1% CTAB | 1,263 (1,786) | 1,839 (635) | 13,453 (4,327) | |
| | *1* | *0.11* | *0.02* | ***0.38*** |
| 2 M Urea | 249 (7) | 3,577 (995) | 277,575 (10,349) | |
| | *0.20* | *0.21* | *0.47* | ***0.29 **** |
| 2 M GuCl | 23 (1) | 10,322 (2,706) | 355,536 (23,555) | |
| | *0.02* | *0.60* | *0.61* | ***0.41*** |
| without | 72 (88) | 17,108 (213) | 450,806 (3,736) | |
| | *0.06* | *1* | *0.77* | ***0.61*** |

**Table S3.** Results of quantitative PCR comparing different surfactants in the extraction buffer. Absolute copy numbers per of gram powder (standard deviations are given in parenthesis) are shown together, with relative numbers separately compared to the best performing method for each sample in this test series. This is the same table as Table 2, including standard deviations for the absolute copy numbers.

| Temperature and Duration of Incubation | 233 | 748 | 891 | Sample ID |
|---|---|---|---|---|
| | Copy Number per gram (Standard Deviation) | | | |
| | *Relative to Best Method* | | | *Average* |
| 56°C 1h | 0 | 5,771 (3,142) | 113,569 (10,851) | |
| | *0* | *0.65* | *0.41* | ***0.35*** |
| 37°C 1h | 120 (170) | 8,868 (6,114) | 97,507 (27,340) | |
| | *0.06* | *1* | *0.35* | ***0.47*** |
| 37°C Overnight | 0 | 7,508 (2,805) | 172,780 (51,569) | |
| | *0* | *0.85* | *0.63* | ***0.49*** |
| Room Temperature Overnight | 92 (131) | 6,779 (4,120) | 275,869 (19,782) | |
| | *0.05* | *0.76* | *1* | ***0.6*** |
| Room Temperature 4 Days | 1,876 (2,654) | 1,443 (2,041) | 139,473 (18,298) | |
| | *1* | *0.16* | *0.51* | ***0.56*** |
| Room Temperature 4 Days (+ PK) | 356 (342) | 2,129 (2,934) | 128,746 (53,787) | |
| | *0.19* | *0.24* | *0.47* | ***0.3*** |

**Table S4.** Results of quantitative PCR comparing different times and temperatures of incubation of samples in extraction buffer. Absolute copy numbers per gram of powder (standard deviations are given in parentheses) and relative numbers compared to the best performing method in this test series are shown. This is the same table as Table 3, including standard deviations for the absolute copy numbers.

| Buffer Components | PTB | 233 | 748 | 891 | Sample ID |
|---|---|---|---|---|---|
| | | Copy Number per gram (Standard Deviation) | | | |
| | | *Relative to Best Method* | | | Average |
| EDTA | + | 16,714 (9,729) | 5,552 (184) | 438,896 (30,686) | |
| | | *1.00* | *0.44* | *1.00* | ***0.81*** |
| | - | 7,758 (4,842) | 12,660 (11,673) | 240,806 (3,547) | |
| | | *0.46* | *1.00* | *0.55* | ***0.67*** |
| EDTA PVP | + | 3,797 (805) | 2,952 (2,433) | 383,168 (77,086) | |
| | | *0.99* | *0.42* | *1.00* | ***0.81*** |
| | - | 3,817 (5,398) | 6,955 (4,217) | 299,251 (99,495) | |
| | | *1.00* | *1.00* | *0.78* | ***0.93*** |
| EDTA DTT | + | 12,394 (5,676) | 3,681 (5,206) | 568,282 (79,271) | |
| | | *0.69* | *0.35* | *1.00* | ***0.68*** |
| | - | 17,894 (729) | 10,536 (8,379) | 244,619 (32,337) | |
| | | *1.00* | *1.00* | *0.43* | ***0.81*** |
| EDTA PK | + | 14,505 (481) | 14,433 (5,544) | 563,521 (78,607) | |
| | | *0.34* | *1.00* | *1.00* | ***0.78*** |
| | - | 42,504 (26,406) | 29 (40) | 402,450 (177,654) | |
| | | *1.00* | *0.00* | *0.71* | ***0.57*** |
| EDTA PVP DTT | + | 11,173 (6,503) | 9,772 (1,860) | 523,813 (48,100) | |
| | | *0.92* | *1.00* | *1.00* | ***0.97*** |
| | - | 12,122 (8,640) | 5,766 (4,157) | 238,216 (75,879) | |
| | | *1.00* | *0.59* | *0.45* | ***0.68*** |
| EDTA PVP DTT PK | + | 48,846 (21,563) | 676 (925) | 447,990 (32,968) | |
| | | *1.00* | *0.04* | *0.93* | ***0.66*** |
| | - | 28,113 (5,656) | 17,724 (13,628) | 482,233 (276,241) | |
| | | *0.58* | *1.00* | *1.00* | ***0.86*** |
| EDTA PVP PK | + | 23,295 (1,457) | 8,553 (5,978) | 277,632 (71,779) | |
| | | *0.70* | *0.56* | *1.00* | ***0.75*** |
| | - | 33,105 (366) | 15,344 (8,884) | 238,616 (120,162) | |
| | | *1.00* | *1.00* | *0.86* | ***0.95*** |
| EDTA DTT PK | + | 42,690 (3,141) | 2,641 (2,896) | 381,364 (35,063) | |
| | | *0.93* | *0.29* | *0.53* | ***0.59*** |
| | - | 45,722 (5,710) | 8,953 (6,838) | 715,096 (174,680) | |
| | | *1.00* | *1.00* | *1.00* | ***1.00*** |

**Table S5a**: Results of quantitative PCR comparing each possible extraction buffer composition in pairwise comparisons to presence (+) or absence (-) of PTB. Absolute copy numbers per gram powder (standard deviations are given in parenthesis) and relative numbers compared to the best performing method in this test series are shown. In three out of eight cases the presence, and in five out of eight cases the absence of PTB performed better in the pairwise comparisons. However, neither the individual nor the summed differences are statistically significant after a paired t-Test ($P>0.1$). PTB: N-phenacyl thiazolium bromide (2.5mM); PVP: Polyvinylpyrrolidone (1%); DTT: Dithiothreitol (50mM), PK: proteinase K (0.25mg/ml).

| Buffer Components | PVP | 233 | 748 | 891 | Sample ID |
|---|---|---|---|---|---|
| | | Copy Number per gram (Standard Deviation) | | | |
| | | *Relative to Best Method* | | | Average |
| EDTA | + | 3,817 (5,398) | 6,955 (4,217) | 299,251 (99,495) | |
| | | *0.49* | *0.55* | *1.00* | ***0.68*** |
| | - | 7,758 (4,842) | 12,660 (11,673) | 240,806 (3,547) | |
| | | *1.00* | *1.00* | *0.80* | ***0.93*** |
| EDTA PTB | + | 3,797 (805) | 2,952 (2,433) | 383,168 (77,086) | |
| | | *0.23* | *0.53* | *0.87* | ***0.54*** |
| | - | 16,714 (9,729) | 5,552 (184) | 438,896 (30,686) | |
| | | *1.00* | *1.00* | *1.00* | ***1.00*** |
| EDTA DTT | + | 12,122 (8,640) | 5,766 (4,157) | 238,216 (75,879) | |
| | | *0.68* | *0.55* | *0.97* | ***0.73*** |
| | - | 17,894 (729) | 10,536 (8,379) | 244,619 (32,337) | |
| | | *1.00* | *1.00* | *1.00* | ***1.00*** |
| EDTA PK | + | 33,105 (366) | 15,344 (8,884) | 238,616 (120,162) | |
| | | *0.78* | *1.00* | *0.59* | ***0.79*** |
| | - | 42,504 (26,406) | 29 (40) | 402,450 (177,654) | |
| | | *1.00* | *0.00* | *1.00* | ***0.67*** |
| EDTA PTB DTT | + | 11,173 (6,503) | 9,772 (1,860) | 523,813 (48,100) | |
| | | *0.90* | *1.00* | *0.92* | ***0.94*** |
| | - | 12,394 (5,676) | 3,681 (5,206) | 568,282 (79,271) | |
| | | *1.00* | *0.38* | *1.00* | ***0.79*** |
| EDTA PTB DTT PK | + | 48,846 (21,563) | 676 (925) | 447,990 (32,968) | |
| | | *1.00* | *0.26* | *1.00* | ***0.75*** |
| | - | 42,690 (3,141) | 2,641 (2,896) | 381,364 (35,063) | |
| | | *0.87* | *1.00* | *0.85* | ***0.91*** |
| EDTA PTB PK | + | 23,295 (1,457) | 8,553 (5,978) | 277,632 (71,779) | |
| | | *1.00* | *0.59* | *0.49* | ***0.70*** |
| | - | 14,505 (481) | 14,433 (5,544) | 563,521 (78,607) | |
| | | *0.62* | *1.00* | *1.00* | ***0.87*** |
| EDTA DTT PK | + | 28,113 (5,656) | 17,724 (13,628) | 482,233 (276,241) | |
| | | *0.61* | *1.00* | *0.67* | ***0.76*** |
| | - | 45,722 (5,710) | 8,953 (6,838) | 715,096 (174,680) | |
| | | *1.00* | *0.51* | *1.00* | ***0.84*** |

**Table S5b**: Results of quantitative PCR comparing each possible extraction buffer composition in pairwise comparisons to presence (+) or absence (-) of PVP. Absolute copy numbers per gram powder (standard deviations are given in parenthesis) and relative numbers compared to the best performing method in this test series are shown. In two out of eight cases the presence, and in six out of eight cases the absence of PVP performed better in the pairwise comparisons. Although no individual comparison is statistically significant, depending on the way of comparison (see above) the addition of PVP results in overall statistically significantly worse results after a paired t-Test ($P<0.05$ and $P=0.07$, respectively). PTB: N-phenacyl thiazolium bromide (2.5mM); PVP: Polyvinylpyrrolidone (1%); DTT: Dithiothreitol (50mM), PK: Proteinase K (0.25mg/ml).

| Buffer Components | DTT | 233 | 748 | 891 | Sample ID |
|---|---|---|---|---|---|
| | | Copy Number per gram (Standard Deviation) | | | |
| | | *Relative to Best Method* | | | Average |
| EDTA | + | 17,894 (729) | 10,536 (8,379) | 244,619 (32,337) | |
| | | *1.00* | *0.83* | *1.00* | ***0.94*** |
| | - | 7,758 (4,842) | 12,660 (11,673) | 240,806 (3,547) | |
| | | *0.43* | *1.00* | *0.98* | ***0.81*** |
| EDTA PVP | + | 12,122 (8,640) | 5,766 (4,157) | 238,216 (75,879) | |
| | | *1.00* | *0.83* | *0.80* | ***0.88*** |
| | - | 3,817 (5,398) | 6,955 (4,217) | 299,251 (99,495) | |
| | | *0.31* | *1.00* | *1.00* | ***0.77*** |
| EDTA PTB | + | 12,394 (5,676) | 3,681 (5,206) | 568,282 (79,271) | |
| | | *0.74* | *0.66* | *1.00* | ***0.80*** |
| | - | 16,714 (9,729) | 5,552 (184) | 438,896 (30,686) | |
| | | *1.00* | *1.00* | *0.77* | ***0.92*** |
| EDTA PK | + | 45,722 (5,710) | 8,953 (6,838) | 715,096 (174,680) | |
| | | *1.00* | *1.00* | *1.00* | ***1.00*** |
| | - | 42,504 (26,406) | 29 (40) | 402,450 (177,654) | |
| | | *0.93* | *0.00* | *0.56* | ***0.50*** |
| EDTA PVP PTB | + | 11,173 (6,503) | 9,772 (1,860) | 523,813 (48,100) | |
| | | *1.00* | *1.00* | *1.00* | ***1.00*** |
| | - | 3,797 (805) | 2,952 (2,433) | 383,168 (77,086) | |
| | | *0.34* | *0.30* | *0.73* | ***0.46 **** |
| EDTA PVP PTB PK | + | 48,846 (21,563) | 676 (925) | 447,990 (32,968) | |
| | | *1.00* | *0.08* | *1.00* | ***0.69*** |
| | - | 23,295 (1,457) | 8,553 (5,978) | 277,632 (71,779) | |
| | | *0.48* | *1.00* | *0.62* | ***0.70*** |
| EDTA PVP PK | + | 28,113 (5,656) | 17,724 (13,628) | 482,233 (276,241) | |
| | | *0.85* | *1.00* | *1.00* | ***0.95*** |
| | - | 33,105 (366) | 15,344 (8,884) | 238,616 (120,162) | |
| | | *1.00* | *0.87* | *0.49* | ***0.79*** |
| EDTA PTB PK | + | 42,690 (3,141) | 2,641 (2,896) | 381,364 (35,063) | |
| | | *1.00* | *0.18* | *0.68* | ***0.62*** |
| | - | 14,505 (481) | 14,433 (5,544) | 563,521 (78,607) | |
| | | *0.34* | *1.00* | *1.00* | ***0.78*** |

**Table S5c**: Results of quantitative PCR comparing each possible extraction buffer composition in pairwise comparisons to presence (+) or absence (-) of DTT. Absolute copy numbers per gram powder (standard deviations are given in parenthesis) and relative numbers compared to the best performing method in this test series are shown. In five out of eight cases the presence, and in three out of eight cases the absence of DTT performed better in the pairwise comparisons. Only one pairwise comparison was significantly different after a paired t-Test (*, $P<0.05$), whereas the summed comparison shows no significant difference ($P=0.08$ in both comparisons), although the additions of DTT seems to show a slightly positive effect. PTB: N-phenacyl thiazolium bromide (2.5mM); PVP: Polyvinylpyrrolidone (1%); DTT: Dithiothreitol (50mM), PK: Proteinase K (0.25mg/ml).

| Buffer Components | PK | 233 | 748 | 891 | Sample ID |
|---|---|---|---|---|---|
| | | Copy Number per gram (Standard Deviation) | | | |
| | | *Relative to Best Method* | | | Average |
| EDTA | + | 42,504 (26,406) | 29 (40) | 402,450 (177,654) | |
| | | *1.00* | *0.00* | *1.00* | ***0.67*** |
| | - | 7,758 (4,842) | 12,660 (11,673) | 240,806 (3,547) | |
| | | *0.18* | *1.00* | *0.60* | ***0.59*** |
| EDTA PVP | + | 33,105 (366) | 15,344 (8,884) | 238,616 (120,162) | |
| | | *1.00* | *1.00* | *0.80* | ***0.93*** |
| | - | 3,817 (5,398) | 6,955 (4,217) | 299,251 (99,495) | |
| | | *0.12* | *0.45* | *1.00* | ***0.52*** |
| EDTA DTT | + | 45,722 (5,710) | 8,953 (6,838) | 715,096 (174,680) | |
| | | *1.00* | *0.85* | *1.00* | ***0.95*** |
| | - | 17,894 (729) | 10,536 (8,379) | 244,619 (32,337) | |
| | | *0.39* | *1.00* | *0.34* | ***0.58*** |
| EDTA PTB | + | 14,505 (481) | 14,433 (5,544) | 563,521 (78,607) | |
| | | *0.87* | *1.00* | *1.00* | ***0.96*** |
| | - | 16,714 (9,729) | 5,552 (184) | 438,896 (30,686) | |
| | | *1.00* | *0.38* | *0.78* | ***0.72*** |
| EDTA PVP DTT | + | 28,113 (5,656) | 17,724 (13,628) | 482,233 (276,241) | |
| | | *1.00* | *1.00* | *1.00* | ***1.00*** |
| | - | 12,122 (8,640) | 5,766 (4,157) | 238,216 (75,879) | |
| | | *0.43* | *0.33* | *0.49* | ***0.42 **** |
| EDTA PVP DTT PTB | + | 48,846 (21,563) | 676 (925) | 447,990 (32,968) | |
| | | *1.00* | *0.07* | *0.86* | ***0.64*** |
| | - | 11,173 (6,503) | 9,772 (1,860) | 523,813 (48,100) | |
| | | *0.23* | *1.00* | *1.00* | ***0.74*** |
| EDTA PVP PTB | + | 23,295 (1,457) | 8,553 (5,978) | 277,632 (71,779) | |
| | | *1.00* | *1.00* | *0.72* | ***0.91*** |
| | - | 3,797 (805) | 2,952 (2,433) | 383,168 (77,086) | |
| | | *0.16* | *0.35* | *1.00* | ***0.50*** |
| EDTA DTT PTB | + | 42,690 (3,141) | 2,641 (2,896) | 381,364 (35,063) | |
| | | *1.00* | *0.72* | *0.67* | ***0.80*** |
| | - | 12,394 (5,676) | 3,681 (5,206) | 568,282 (79,271) | |
| | | *0.29* | *1.00* | *1.00* | ***0.76*** |

**Table S5d**: Results of quantitative PCR comparing each possible extraction buffer composition in pairwise comparisons to presence (+) or absence (-) of proteinase K (PK). Absolute copy numbers per gram powder (standard deviations are given in parenthesis) and relative numbers compared to the best performing method in this test series are shown. In seven out of eight cases the presence, and in one out of eight cases the absence of proteinase K performed better in the pairwise comparisons. Only one pairwise comparison was significantly different after a paired t-Test (*, $P<0.05$). However, in the summed comparison, proteinase K shows a strong positive effect with statistical significance ($P<0.01$ and $P<0.05$, respectively). PTB: N-phenacyl thiazolium bromide (2.5mM); PVP: Polyvinylpyrrolidone (1%); DTT: Dithiothreitol (50mM), PK: Proteinase K (0.25mg/ml).

| Condition | 233 | 748 | 891 | Sample ID |
|---|---|---|---|---|
| | Copy Number per gram (Standard Deviation) | | | |
| | *Relative to Best Method* | | | *Average* |
| - 0.05M | 77,649 (9,460) | 7,454 (2,725) | 751,214 (31,530) | |
| Tris | *1* | *1* | *1* | ***1*** |
| + 0.05M | 66,618 (8,368) | 3,548 (2,833) | 615,327 (0) | |
| Tris | *0.86* | *0.48* | *0.82* | ***0.72*** |

**Table S6**: Results of quantitative PCR comparing absence (-) and presence (+) of 0.05M Tris (pH 8.0) in the extraction buffer. Absolute copy numbers per gram of powder and relative numbers compared to the best performing method in this test series are shown. The difference between the two methods is not significant, according to a paired t-Test ($P<0.05$).

| | 233 | 748 | 891 | Sample ID |
|---|---|---|---|---|
| **EDTA** | **Copy Number per gram** (Standard Deviation) | | | |
| **Concentration** | ***Relative to Best Method*** | | | ***average*** |
| 0.45 M | 1,356 (288) | 15,084 (3,372) | 1,482,244 (5,761) | |
| | *0.08* | *1* | *1* | ***0.69*** |
| 0.2 M | 16,765 (11,959) | 13,943 (1,568) | 847,416 (3,294) | |
| | *1* | *0.92* | *0.57* | ***0.83*** |
| 0.1 M | 1,046 (1,367) | 7,390 (1,116) | 358,338 (23,661) | |
| | *0.06* | *0.49* | *0.24* | ***0.12 **** |
| 0.05 M | 0 | 0 | 62,912 (1,467) | |
| | *0* | *0* | *0.04* | ***0.01 **** |

**Table S7**: Results of quantitative PCR comparing different EDTA concentrations in the extraction buffer. Absolute copy numbers per gram powder (and standard deviations in parenthesis) and relative numbers compared to the best performing method in this test series are shown. Significantly worse EDTA concentrations compared to the best performing one (0.2M EDTA) are indicated by an asterisk (*; $P<0.05$, paired t-Test).

| Reducing Agent | 233 | 748 | 891 | Sample ID |
|---|---|---|---|---|
| | Copy Number per gram (Standard Deviation) | | | |
| | *Relative to Best Method* | | | *Average* |
| without | 86,724 (19,891) | 1,105 (211) | 621,418 (31,671) | |
| | *1* | *0.15* | *0.94* | ***0.7*** |
| DTT | 20,469 (6,548) | 5,220 (5,118) | 553,495 (6,512) | |
| | *0.24* | *0.73* | *0.83* | ***0.6*** |
| 5,5´-Dithiobis-(2-nitrobenzoic acid) | 75,274 (4,426) | 7,130 (10,083) | 599,191 (39,923) | |
| | *0.87* | *1* | *0.9* | ***0.92*** |
| 2-Mercapto-ethanol | 19,743 (4,754) | 4,300 (605) | 559,805 (52,618) | |
| | *0.23* | *0.6* | *0.84* | ***0.56*** |
| 2-Mercaptobenzo-thiazol sodiumsalt | 55,768 (5,242) | 2,521 (3,565) | 505,898 (21,820) | |
| | *0.64* | *0.35* | *0.76* | ***0.58*** |
| 2-Naphtalin-thiol | 25,151 (1,380) | 0 | 201,472 (73,380) | |
| | *0.29* | *0* | *0.3* | ***0.2 **** |
| 2-Amino-5-mercapto-1,3,4-thiadiazol | 39,119 (6,112) | 2,939 (1,981) | 662,876 (33,784) | |
| | *0.45* | *0.41* | *1* | ***0.62*** |
| Thioglycolacid | 30,506 (239) | 4,397 (2,058) | 645,816 (58,180) | |
| | *0.35* | *0.62* | *0.97* | ***0.46*** |
| tert-Dodecanthiol | 21,926 (1,480) | 4,373 (4,595) | 582,423 (72,908) | |
| | *0.25* | *0.61* | *0.88* | ***0.58*** |

**Table S8**: Results of quantitative PCR comparing different reducing agents in the extraction buffer. Absolute copy numbers per gram of powder and relative numbers compared to the best performing method in this test series are shown. Only one reducing agent (2-Naphtalinthiol) performed significantly worse than the best performing one (5,5´-Dithiobis-(2-nitrobenzoic acid)), according to a paired t-Test (*, $P<0.05$)

| Condition | 233 | 748 | 891 | Sample ID |
|---|---|---|---|---|
| | Copy Number per gram (Standard Deviation) | | | |
| | *Relative to Best Method* | | | *Average* |
| Coarse | 38 (38) | 0 | 115,092 (11,840) | |
| 1.Extraction | *0* | *0* | *0.25* | ***0.08 ** ** |
| Fine | 13,803 (2,670) | 3,127 (4,422) | 455,327 (26,054) | |
| 1.Extraction | *1* | *1* | *1* | ***1*** |
| Coarse | 3,960 (2,633) | 0 | 182,488 (16,693) | |
| 2.Extraction | *1* | *0* | *1* | ***0.67*** |
| Fine | 0 | 0 | 53,084 (203) | |
| 2.Extraction | *0* | *0* | *0.29* | ***0.1*** |
| Coarse | 3,998 | 0 | 297,580 | |
| Sum | *0.29* | *0* | *0.59* | ***0.29 ** ** |
| Fine | 13,803 | 3,127 | 508,411 | |
| Sum | *1* | *1* | *1* | ***1*** |

**Table S9**: Results of quantitative PCR comparing fine powder (obtained via Freezer Mill) with coarse samples (obtained via mortar and pestle). Absolute copy numbers per gram of powder, and relative numbers compared to the best performing method in this test series are shown. Relative values are calculated separately for the first, the second and both extractions together. In the last two rows, the first and second extraction of the "coarse" and "fine" powder, respectively, were summed. A paired t-Test indicates that the coarsely ground samples performed more poorly statistically in the first extraction (*, $P<0.05$) and if both extractions were taken together. The finely ground samples in the 2$^{nd}$ extraction (re-extraction) were not statistically worse when compared to the coarsely ground samples.

| Salt (Concentration) | 233 | 748 | 891 | Sample ID |
|---|---|---|---|---|
| | Copy Number per gram (Standard Deviation) | | | |
| | *Relative to Best Method* | | | *Average* |
| KCl (saturated, about 4.6 M) | 1,222 (132) | 147 (207) | 82,566 (10,953) | |
| | *0.02* | *0.01* | *0.07* | ***0.03 ** ** |
| GuCl (saturated, about 22 M) | 54,956 (3,881) | 273 (78) | 547,916 (65,902) | |
| | *0.99* | *0.03* | *0.49* | ***0.50*** |
| GuSCN a) (5M) | 55,522 (14,601) | 3,324 (331) | 401,612 (10,017) | |
| | *1* | *0.34* | *0.36* | ***0.57*** |
| GuSCN b) (5M) | 6,927 (3,292) | 7,920 (5,653) | 1,088,570 (58,806) | |
| | *0.12* | *0.80* | *0.96* | ***0.63*** |
| $NaClO_4$ (5M) | 36,864 (10,873) | 5,510 (3,582) | 436,263 (14,508) | |
| | *0.66* | *0.56* | *0.39* | ***0.54*** |
| NaCl (saturated, about 6 M) | 40,266 (335) | 9,878 (2,756) | 763,410 (15,868) | |
| | *0.73* | *1* | *0.68* | ***0.80*** |
| NaI (saturated, about 12 M) | 631 (99) | 6,764 (4,375) | 1,128,573 (23,459) | |
| | *0.01* | *0.68* | *1* | ***0.56*** |

**Table S10:** Results of quantitative PCR comparing different salts for binding DNA to silica. Absolute copy numbers per gram of powder (standard deviation are given in parenthesis) and relative numbers compared to the best performing method in this test series are shown. This is the same table as Table 4, including standard deviations for the absolute copy numbers.

| Age of Silica Suspension | 233 | 748 | 891 | Sample ID |
|---|---|---|---|---|
| | Copy Number per gram (Standard Deviation) | | | |
| | *Relative to Best Method* | | | *Average* |
| 1 Week | 47,294 (3,169) | 1,289 (1,822) | 525,707 (74,405) | |
| | *1* | *0.2* | *0.93* | ***0.71*** |
| 2 Weeks | 14,817 (6,815) | 6,346 (1,347) | 547,922 (41,028) | |
| | *0.31* | *1* | *0.97* | ***0.76*** |
| 4 Weeks | 34,526 (6,632) | 5,861 (2,737) | 563,641 (62,129) | |
| | *0.73* | *0.92* | *1* | ***0.88*** |
| 8 Weeks | 173 (133) | 3,432 (3,320) | 448,145 (1,768) | |
| | *0* | *0.54* | *0.8* | ***0.45 **** |
| 12 Weeks | 9,339 (2,154) | 1,457 (308) | 324,794 (6,405) | |
| | *0.2* | *0.23* | *0.58* | ***0.34 **** |
| ~ 26 Weeks | 619 (41) | 824 (956) | 548,084 (10,809) | |
| | *0.01* | *0.13* | *0.97* | ***0.37*** |
| > 52 Weeks | 46 (65) | 0 | 455,788 (7,191) | |
| | *0* | *0* | *0.81* | ***0.27 **** |

**Table S11**: Results of quantitative PCR comparing the age of the silica suspension ranging from 1 week to 1 year. Absolute copy numbers per gram of powder and relative numbers compared to the best performing method in this test series are shown. Silica suspensions performing statistically worse when compared to the best (4 weeks) are indicated by an asterisk (*, $P<0.05$).

| pH | 233 | 748 | 891 | Sample ID |
|---|---|---|---|---|
| | Copy Number per gram (Standard Deviation) | | | |
| | *Relative to Best Method* | | | *Average* |
| 4.0 | 78,576 (11,754) | 12,082 (382) | 1,024,274 (20,234) | |
| | *1* | *1* | *1* | ***1*** |
| 6.0 | 73,574 (3,487) | 5,881 (5,608) | 770,886 (88,137) | |
| | *0.93* | *0.49* | *0.75* | ***0.72*** |
| 8.0 | 34,624 (821) | 7,095 (1,255) | 865,694 (17,101) | |
| | *0.44* | *0.59* | *0.85* | ***0.63 **** |

**Table S12**: Results of quantitative PCR comparing the pH in the binding buffer (L2, including silica suspension and extraction buffer of the respective sample). Absolute copy numbers per gram of powder and relative numbers compared to the best performing method in this test series are shown. The extraction method using a binding buffer (together with the extraction buffer) at pH 8.0 performed statistically worse as compared to the best (pH 4.0), according to a paired t-Test (*, $P<0.05$). See also Fig.2.

| Amount of Silica Suspension | 233 | 748 | 891 | Sample ID |
|---|---|---|---|---|
| | Copy Number per gram (Standard Deviation) | | | |
| | *Relative to Best Method* | | | *Average* |
| 5µl | 0 | 791 (1,118) | 149,603 (6,356) | |
| | *0* | *0.12* | *0.24* | ***0.12 ****** |
| 10µl | 4,583 (177) | 3,248 (151) | 232,573 (898) | |
| | *0.08* | *0.5* | *0.37* | ***0.32 ****** |
| 20µl | 6,438 (3,115) | 2,782 (161) | 243,496 (41,193) | |
| | *0.12* | *0.43* | *0.39* | ***0.31 ****** |
| 40µl | 34,127 (132) | 1,757 (2,485) | 624,831 (19,308) | |
| | *0.61* | *0.27* | *1* | ***0.63*** |
| 100µl | 55,525 (1,073) | 6,445 (2,614) | 428,116 (11,576) | |
| | *1* | *1* | *0.69* | ***0.9*** |

**Table S13**: Results of quantitative PCR comparing the amount of silica suspension used in the binding step. Absolute copy numbers per gram of powder and relative numbers compared to the best performing method in this test series are shown. 5, 10 and 20µl silica suspensions performed statistically worse (*, t-Test, $P<0.05$), as compared to the best performing method (100µl).

a)

| Time of Incubation | 748 | 891 | Sample ID |
|---|---|---|---|
| | Copy Number per gram (Standard Deviation) | | |
| | *Relative to Best Method* | | *Average* |
| 1/4 h | 26 (36) | 16,940 (7,772) | |
| | *0* | *0.03* | ***0.02 ****** |
| 1 h | 3 (5) | 241,154 (45,191) | |
| | *0* | *0.5* | ***0.25*** |
| 3 h | 5,773 (7,192) | 473,725 (82,187) | |
| | *1* | *0.98* | ***0.99*** |
| Overnight | 8 (11) | 484,848 (27,405) | |
| | *0* | *1* | ***0.5*** |

b)

| Time of Incubation | 748 | 891 | sample ID |
|---|---|---|---|
| | Copy Number per gram (Standard Deviation) | | |
| | *Relative to Best Method* | | *Average* |
| 1.5 h | 1,173 (1,659) | 579,012 (13,241) | |
| | *0.52* | *0.62* | ***0.57*** |
| 3 h | 162 (229) | 935,797 (72,696) | |
| | *0.03* | *1* | ***0.52*** |
| 6 h | 2,266 (2,510) | 627,813 (17,228) | |
| | *1* | *0.67* | ***0.84*** |

**Table S14**: Results of quantitative PCR comparing different incubation durations for binding DNA to silica using 100mg sample, 3M NaCl as binding buffer and adjusting pH to 4.0 (this test was done twice a) and b)). Absolute copy numbers per gram of powder and relative numbers compared to the best performing method in each test series are shown. Only ¼ h incubation in a) performed statistically worse, as compared to the best performing method (3h) after a t-Test (*, $P<0.05$). Sample 748 is extremely variable due to overall low copy numbers, and the results from this sample must be interpreted with caution. If only the higher, and therefore more reliable, numbers from sample 891 are

taken into account, the yield appears to level off after 3 hours, a reasonable processing time.

| | 748 | 891 | Sample ID |
|---|---|---|---|
| **Silica** | **Copy Number per gram** (Standard Deviation) | | |
| **Type** | ***Relative to Best Method*** | | ***Average*** |
| "Normal" | 4,212 (2,252) | 1,182,728 (27,048) | |
| | *0.7* | *0.99* | ***0.85*** |
| "New" | 6,045 (3,397) | 1,196,603 (109,317) | |
| | *1* | *1* | ***1*** |

**Table S15**: Results of quantitative PCR comparing the setup of the silica suspension. "Normal" was prepared after (2), "New" see supplement text (chapter 3.7). Absolute copy numbers per gram of powder and relative numbers compared to the best performing method in this test series are shown. The averages are not statistically significant according to a t-Test (*, $P<0.05$).

| | 748 | 891 | Sample ID |
|---|---|---|---|
| **Wash** | **Copy Number per gram** (Standard Deviation) | | |
| **Solution** | ***Relative to Best Method*** | | ***Average*** |
| Company | 6,045 (3,397) | 1,196,603 (109,317) | |
| "New Wash" | *0.6* | *0.91* | ***0.76*** |
| alternative | 10,015 (3,109) | 1,127,417 (61,852) | |
| 1 | *1* | *0.85* | ***0.93*** |
| alternative | 691 (784) | 1,320,877 (66,433) | |
| 2 | *0.07* | *1* | ***0.54*** |
| alternative | 2,846 (4,025) | 584,570 (69,147) | |
| 3 | *0.28* | *0.44* | ***0.36*** |
| alternative | 0 | 0 | |
| 4 | *0* | *0* | ***0 **** |

**Table S16**: Results of quantitative PCR comparing commercial and alternative wash solutions. See supplement text for composition (chapter 3.8).

Absolute copy numbers per gram of powder and relative numbers compared to the best performing method in this test series are shown. Wash solutions performing significantly worse statistically, as compared to the best performing one (alternative 1), are indicated with an asterisk (*, t-Test, $P<0.05$).

| | 233 | 243 | 322 | 576 | 748 | 751 | 891 | 1050 | Sample ID |
|---|---|---|---|---|---|---|---|---|---|
| | Copy Number per gram (Standard Deviation) | | | | | | | | |
| **Extraction Method** | ***Relative to Best Method*** | | | | | | | | ***Average*** |
| **A Silica** | 266,572 (76,109) | 466 (541) | 62 (123) | 18,458 (7,046) | 2,234 (1,381) | 2,454 (1,249) | 253,083 (119,662) | 1,515 (1,088) | |
| | *0.70* | *0.27* | *0.04* | *0.57* | *0.37* | *0.31* | *1* | *1* | ***0.53*** |
| **B Modified Silica - NaCl** | 15,868 (27,672) | 249 (382) | 0 | 4,835 (5,641) | 230 (445) | 844 (1,602) | 24,288 (26,966) | 0 | |
| | *0.04* | *0.15* | *0* | *0.15* | *0.04* | *0.11* | *0.10* | *0* | ***0.07 **** |
| **C Modified Silica - GuSCN** | 378,173 (31,423) | 1,719 (965) | 1,626 (1,514) | 32,142 (5,117) | 6,097 (3,522) | 8,057 (2,281) | 56,903 (12,434) | 1,116 (719) | |
| | *1* | *1* | *1* | *1* | *1* | *1* | *0.23* | *0.74* | ***0.87*** |
| **D Phenol Chloroform** | 133 (256) | 0 | 0 | 11,053 (9,550) | 563 (984) | 3,605 (4,950) | 149,439 (124,014) | 943 (842) | |
| | *0* | *0* | *0* | *0.34* | *0.09* | *0.45* | *0.59* | *0.62* | ***0.26 **** |
| **E Ethanol Precipitation** | 6,709 (6,209) | 0 | 0 | 0 | 0 | 0 | 6,878 (4,478) | 0 | |
| | *0.02* | *0* | *0* | *0* | *0* | *0* | *0.03* | *0* | ***0.01 **** |
| **F Qiagen Kit** | 0 | 0 | 0 | 317 (464) | 145 (288) | 2,054 (520) | 103,646 (59,696) | 112 (132) | |
| | *0* | *0* | *0* | *0.01* | *0.02* | *0.26* | *0.41* | *0.07* | ***0.10 **** |

**Table S17**: Results of quantitative PCR comparing six different extraction techniques using eight ancient cave bear samples each in duplicate. Copy numbers were calculated from three measurements of two dilutions of both extracts, per sample per method (12 data points per method altogether). Absolute copy numbers per gram of powder and relative numbers compared to the best performing method in this test series are shown. Extractions methods performing significantly worse as compared to the best one (C – modified Silica – GuSCN), according to a t-Test are indicated by an asterisk (*, $P<0.05$).

| | 233 | 243 | 322 | 576 | 748 | 751 | 891 | 1050 | Sample ID | Extraction Control |
|---|---|---|---|---|---|---|---|---|---|---|
| | Inhibition in % (derived from the amount of DNA) | | | | | | | | Average | |
| **Extraction Method** | *Inhibition in % (derived from the Efficiency of each PCR)* | | | | | | | | *Average* | |
| **A** | 0.25 | 3.62 | -0.75 | -0.14 | -3.06 | -6.64 | -5.91 | -3.19 | **-1.98** | -2.56 |
| **Silica** | *-2.96* | *-1.56* | *-1.50* | *-2.12* | *-1.23* | *-0.95* | *-0.64* | *-4.89* | **-1.98** | *-5.79* |
| **B** | 100.00 | 49.24 | 100.00 | 99.16 | 65.34 | 18.20 | 84.91 | 100.00 | **77.11** | 3.49 |
| **Modified Silica - NaCl** | *100.00* | *19.79* | *100.00* | *74.19* | *19.66* | *18.00* | *32.77* | *100.00* | **59.80** | *0.21* |
| **C** | 6.40 | -2.34 | 1.43 | -4.73 | -7.80 | -0.65 | -0.80 | -6.97 | **-1.93** | -12.05 |
| **Modified Silica - GuSCN** | *-0.57* | *-2.25* | *-0.50* | *0.49* | *0.78* | *-0.86* | *1.31* | *3.28* | **0.21** | *0.73* |
| **D** | 99.95 | 100.00 | 51.16 | 80.32 | 10.34 | 2.30 | 94.13 | 92.63 | **66.35** | 1.66 |
| **Phenol Chloroform** | *77.16* | *100.00* | *15.11* | *14.97* | *-1.47* | *-1.04* | *37.54* | *26.20* | **34.02** | *0.19* |
| **E** | 29.83 | -4.38 | 91.07 | -4.22 | -3.56 | -4.09 | 5.00 | -3.92 | **13.22** | 1.96 |
| **Ethanol Precipitation** | *5.33* | *1.44* | *26.08* | *0.89* | *1.54* | *-1.34* | *1.05* | *2.34* | **4.67** | *1.89* |
| **F** | 100.00 | 100.00 | 99.98 | 99.99 | 70.80 | 21.94 | 60.69 | 99.98 | **81.67** | -2.32 |
| **Qiagen Kit** | *100.00* | *100.00* | *25.32* | *59.68* | *5.03* | *1.23* | *1.46* | *23.89* | **40.44** | *-0.67* |

**Table S18**: Results of the inhibition test performed as described under 6 (Supplement: Material and Method) Ancient DNA extracts were used to spike a quantitative human PCR assay for each extraction in triplicate. The difference of the mean from six measurements (3 qPCR´s for each of the two extractions per method) per sample and the mean of the modern human DNA without spiking was calculated as a percentage (upper row for each method – Inhibition in % (derived from the amount of DNA)). Inhibition in % derived from the efficiency of each PCR (lower row and shaded) was calculated as a percentage of the difference between the

mean from six different measurements (3 qPCR´s for each of the two extractions per method) per sample and the mean from the efficiencies of the modern DNA without spiking. Numbers in red indicate an inhibition higher than the double standard deviations of the modern human DNA samples without spiking (for inhibition in % derived from the amount of DNA it is >11,42 %  and for inhibition derived from the efficiency of each PCR it is > 9,12%).

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