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# A rapid column-based ancient DNA extraction method for increased sample throughput

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**Abstract**

**Genetic analyses using museum specimens and ancient DNA from fossil samples are becoming increasingly important in phylogenetic and especially population genetic studies. Recent progress in ancient DNA sequencing technologies has substantially increased DNA sequence yields and, in combination with barcoding methods, has enabled large-scale studies using any type of DNA. Moreover, more and more studies now use nuclear DNA sequences in addition to mitochondrial ones. Unfortunately, nuclear DNA is, due to its much lower copy number in living cells compared to mitochondrial DNA, much more difficult to obtain from low-quality samples. Therefore, a DNA extraction method that optimizes DNA yields from low-quality samples and at the same time allows processing many samples within a short time frame is immediately required. In fact, the major bottleneck in the analysis process using samples containing low amounts of degraded DNA now lies in the extraction of samples, as column-based methods using commercial kits are fast but have proven to give very low yields, while more efficient methods are generally very time-consuming. Here, we present a method that combines the high DNA yield of batch-based silica extraction with the time-efficiency of column-based methods. Our results on Pleistocene cave bear samples show that DNA yields are quantitatively comparable, and in fact even slightly better than with silica batch extraction, while at the same time the number of samples that can conveniently be processed in parallel increases and both bench time and costs decrease using this method. Thus, this method is suited for harvesting the power of high-throughput sequencing using the DNA preserved in the millions of paleontological and museums specimens.**

*Keywords*: ancient DNA, columns, extraction, museum specimens, silica

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## Introduction

The field of ancient DNA has grown substantially during the last decade, moving away from the analysis of individual samples in phylogenetic analyses to population genetic analyses (Leonard *et al.* 2000) that require dozens (Barnes *et al.* 2002; Hofreiter *et al.* 2004; Weinstock *et al.* 2005) and sometimes hundreds of samples (Shapiro *et al.* 2004). This and the emerging fields like palaeogenomics (Green *et al.* 2006; Poinar *et al.* 2006; Miller *et al.* 2008) and phenotypic analyses using nuclear single-nucleotide polymorphisms (Ludwig *et al.* 2009) require testing many samples (Pennisi 2009) and optimizing DNA yields. A similar trend is taking place in the analysis of museum specimens, which are becoming increasingly important for genetic analyses (Wandeler *et al.* 2007) with population genetic studies now often being extensively (Godoy *et al.* 2004) or even exclusively (Miller *et al.* 2006; Krystufek *et al.* 2007) based on museum specimens. Finally, in forensic science, DNA analyses are, among others, used for the identification of criminals and victims of crime or war. Often such analyses also have to be performed on degraded bone or teeth samples (Iwamura *et al.* 2004), and when dealing with war or natural disaster victims, analyses have to be performed on large numbers of degraded samples (Holland *et al.* 2003; Deng *et al.* 2005; Lehrman 2006).

Like many fields in molecular biology, research on ancient and museum specimen DNA is becoming

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completely transformed by the introduction of high-throughput DNA-sequencing methods (Margulies *et al.* 2005; Schuster 2008), often referred to as next-generation sequencing (NGS). The large number of reads obtained and the fact that each read represents a clonal sequence make them ideally suited for ancient DNA research (Gilbert *et al.* 2007; Miller *et al.* 2008), whereas the short sequencing length of NGS is no disadvantage given the fragmented nature of ancient DNA templates (Pääbo *et al.* 2004; Poinar *et al.* 2006). Moreover, barcoding methods (Meyer *et al.* 2007; Erlich *et al.* 2009) allow sequencing of hundreds of specimens in a single-sequencing run raising the possibility of large-scale population studies using ancient or museum specimens.

However, to harvest the power of NGS technology for the analysis of ancient and historical specimens, a DNA extraction methodology that is efficient with regard to both DNA yields and the number of samples that can be processed in parallel is immediately required. Unfortunately, commercial column-based methods give insufficient yields of DNA (Rohland & Hofreiter 2007b) and, moreover, are usually not amenable to use with large sample volumes. Silica- (Rohland & Hofreiter 2007b), precipitation- (Vigilant *et al.* 2001) and microfilter-based methods (Leonard *et al.* 2000; Schwarz *et al.* 2009) are time-consuming and labour-intensive. In fact, the extraction of DNA from low DNA quality and quantity samples is now the rate-limiting step in the data production process using such samples. Therefore, we set out to optimize and combine the high DNA yields of a previously published silica extraction method with the more convenient and faster handling of column-based extractions.

## Materials and methods

We used two to three Pleistocene cave bear samples (samples e, g and i; for sample information, see Table S1) from three different caves in Austria for initial comparisons of a variety of parameters during DNA extraction. The DNA extraction protocol, referred to as batch method, we started with (Rohland & Hofreiter 2007a) uses a simple extraction buffer (consisting of 0.45 M EDTA, pH 8.0 and 0.25 mg/mL proteinase K) to demineralize and digest bone or tooth powder. After overnight incubation at RT, the supernatant (1 volume) is added to four times the volume of binding buffer [contrary to the protocol in Rohland & Hofreiter (2007a,b), the binding buffer we used consists solely of 5 M guanidinium thiocyanate (GuSCN)] and 100 μL of silica suspension, and the pH is adjusted to ~4 with hydrochloric acid. This mixture is incubated for 3 h under agitation. During this incubation, DNA molecules bind to the silica surface. Subsequent resuspensions of the silica pellet with washing buffer (50% ethanol, 125 mM NaCl, 1× TE) are required to remove salts and PCR-inhibiting agents, which get co-extracted from the specimen. Finally, the silica pellet is dried and DNA is eluted with 1× TE.

We investigated modifications of the method at all three stages of the protocol, the sample digestion step, the DNA binding step to silica and, finally, the washing step.

First, we tested whether it is possible to reduce the volume of extraction buffer used, as extremely large volumes are difficult to handle with flow-through columns. Therefore, we tested the DNA yield per gram of sample powder using different ratios of sample powder to buffer volume, varying from 50 to 400 mg/mL in a total volume of 5 mL.

Second, we tested whether it is possible to change the ratio of binding buffer to extraction buffer. We initially tested ratios of binding to extraction buffer ranging from the initial ratio of 4:1 down to 1:2. Due to the results of this test, we did a further set of tests using ratios between 1:1 and 1:10. In all experiments, we used 100 μL of silica suspension. To make the protocol more robust against handling variation, we also eliminated the pH adjustment step after combining binding buffer, silica and extraction buffer by buffering the binding buffer using sodium acetate (pH 5.2) with a final concentration in the binding buffer of 300 mM. Thus, the binding buffer now solely consists of 5 M GuSCN and 300 mM sodium acetate, pH 5.2. The incubation of extraction buffer together with binding buffer and silica is still performed in suspension for 3 h as we previously found that this length of time is necessary for optimal results and shorter incubation times result in a reduction of DNA yields (Rohland & Hofreiter 2007b).

Third, to simplify all subsequent washing and elution steps, we then immobilize the silica particles on a filter (e.g. glass microfibre binder-free Grade GF/B: 1.0 μm

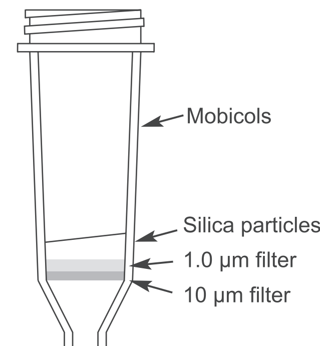


**Fig. 1 Set up of the columns:** Mobicols can be ordered with the 10 μm filter already applied to the column (MobiTec, Germany, product # M1002S). A filter with smaller pore size (e.g. Whatman GF/B 1.0 μm) needs to be placed on top of the large filter to prevent the small silica particles from being washed through during vacuum or centrifugation steps.

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from Whatman) that can be fitted into commercially available columns (Mobicols with 10 μm filter, M1002S; MoBiTec GmbH) by using a 7-mm hole punch (see Fig. 1 for column assembly). The addition of the 1-μm filter is necessary because the silica particles used to bind DNA are up to 10 μm in diameter and the majority would therefore not be retained by the 10-μm filter already applied to the columns. The silica suspension from the binding step, including extraction and binding buffer, is centrifuged for 2 min at 5000 *g*, the supernatant is discarded and the silica pellet is resuspended in 400 μL binding buffer. The suspension is loaded on top of the filter columns and the columns are applied to a vacuum manifold. Washing of the silica, to remove inhibitors originating from the specimens as well as traces of GuSCN, is carried out at least twice using 450 μL of washing buffer by adding the washing buffer on top of the silica and applying vacuum. We recommend a short centrifugation step of the columns before the first and after the last washing step to remove any remaining salt and ethanol, which could lead to the inhibition of downstream applications. After this centrifugation step, DNA is eluted by pipetting 1× TE on top of the silica layer, short incubation and subsequent centrifugation into a new labelled tube.

A detailed description of the new protocol can be found in the Supporting Information.

The performance of the different extraction variants investigated was tested using quantitative real-time PCR (qPCR) on a 110-bp fragment of the mitochondrial DNA with a cave bear-specific fluorescently labelled TaqMan® probe exactly as in Rohland & Hofreiter (2007b). Usually the copy number was measured from two different concentrations of the extract (undiluted and 1:10 dilution) in two independent amplifications per concentration; results with standard deviations are given in Tables S2–S4. The effect of different parameters on extraction performance (copy number per gram) compared with the best performing set up was calculated for each experiment and a paired Student's *t*-test was applied to investigate whether the observed differences are significant.

After we had established the protocol for column-based extraction, we tested its performance on two different sets of samples. First, we tested the method in direct comparison by qPCR to a silica batch method, which we had found previously to give optimal results with regard to ancient DNA yields (Rohland & Hofreiter 2007b). This comparison was performed on nine Pleistocene cave bear bone and teeth samples in duplication. Second, we extracted DNA from 13 chimpanzee teeth museum samples with a specimen age ranging from 4 to 22 years. These samples derive from chimpanzees that died in the wild and have been buried for *c*. 1 year at the field site for tissue removal. We attempted amplification of nuclear markers, the amelogenin locus as well as 19 microsatellite markers described for chimpanzees, ranging in length from 104 to 256 bp (Arandjelovic *et al.* 2009). From all teeth, we cut off a 130- to 230-mg piece of the tooth root and ground it into a fine powder using a freezer mill. For these samples, DTT and Triton X-100 were added to the extraction buffer in final concentrations of 50 mM and 1% respectively. Due to the value of these samples, a direct comparison using an alternative extraction method was not possible. However, it should be noted that three of the samples had been extracted previously using a different method (Vigilant *et al.* 2001) and amplification of microsatellite markers had been unsuccessful.

## Results

We found that it is not possible to reduce the extraction buffer volume (0.45 M EDTA, pH 8, 0.25 mg/mL proteinase K), as both the DNA yield per gram of bone powder and the total DNA yield per extraction decrease if more than 50 mg of bone powder is used per 1 mL of extraction buffer (Table 1). When qPCR's on further dilutions of the same extracts were performed, the amount of DNA per gram of bone powder did not increase (Table S2b), as it would be expected if the decreasing yield with higher amounts of bone powder was due to co-extracted inhibitors.

However, we found that it is possible to reduce the ratio of binding buffer (5 M GuSCN) to extraction buffer from the ratio of 4:1 as usually used. Although the DNA yields remain constant from a ratio of 4:1 to 1:1 of binding

**Table 1** Results of qPCR comparing different amounts of sample powder in the extraction buffer (kept constant at 5ml). Absolute copy numbers (from two measurements of two dilutions, each) per gram of powder are shown together with relative numbers (in parenthesize) compared to the best performing method for each sample in this experiment. Significantly worse performing input sample amounts compared to the best performing ratio are marked with an asterisk (*, $P<0.05$, paired Student's *t*-test)

| Sample ID | e | g | i | |
|---|---|---|---|---|
| Approximate amount of sample powder (mg/mL) | Copy number per gram (relative to best method) | | | Average of relative performance |
| 50 | 29 795 (1) | 9 541 (1) | 86 (0.22) | 0.74 |
| 100 | 4 972 (0.17) | 3 889 (0.41) | 386 (1) | 0.53 |
| 200 | 1 464 (0.05) | 1 670 (0.18) | 30 (0.08) | 0.10* |
| 400 | 0 (0) | 0 (0) | 0 (0) | 0* |

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to extraction buffer, a ratio of 1 volume binding buffer to 2 volumes extraction buffer gave even better DNA yields. Below this ratio, DNA yields decrease rapidly (Table 2). As GuSCN represents the most expensive reagent in the extraction process, this modification has the added benefit that it reduces the costs per extraction by 50% compared with the initial batch method.

We tested the column method using ~250 mg bone powder in 5 mL extraction buffer with a ratio of binding buffer (5 M GuSCN, 300 mM sodium acetate, pH 5.2) to extraction buffer of 1:2 (2.5 mL binding buffer) and binding of DNA to silica for 3 h in suspension, followed by washing and elution on columns on nine Pleistocene cave bear bones and teeth in duplication (Table 3). In this comparison, the new protocol gave results comparable and in fact even better than our previously published silica batch method (Rohland & Hofreiter 2007a,b) using resuspension for all washing and elution steps (Fig. 2 and Table 3). Although we found considerable variation for some samples, and the overall difference is statistically not significant, the column-based method was superior in this test for six of the nine samples.

For the 13 chimpanzee teeth, we were able to amplify all 19 nuclear microsatellites from six of the samples (Table S5). A further two samples yielded partial profiles (15 and 18 loci respectively). For Amelogenin, we found a total success rate of 61% (8 of 13 samples, all loci amplified in duplicate). Three of the samples were teeth that had been previously extracted (Vigilant *et al.* 2001) and had failed to amplify. One of them could be amplified for 18 of the 19 loci, one for 15 loci and one failed again using this method.

**Table 2** Results of qPCR comparing different ratios of extraction to binding buffer (amount of extraction buffer kept constant at 5ml). Absolute copy numbers (from two measurements of two dilutions, each) per gram of powder are shown together with relative numbers (in parenthesize) compared to the best performing method for each sample in the respective experiment (a and b). Significantly worse performing buffer ratios compared to the best performing ratio are marked with an asterisk (*, P<0.05, Student's *t*-test). **a)** initial experiment, varying the ratio from 4:1 to 1:2. **b)** second experiment, varying the ratio from 1:1 to 1:10

| Sample ID | e | g | |
|---|---|---|---|
| Ratio of binding to extraction buffer | Copy number per gram (relative to best method) | | Average of relative performance |
| (a) | | | |
| 4:1 | 75 459 (0.27) | 14 953 (0.72) | 0.5 |
| 3:1 | 77 986 (0.28) | 15 041 (0.73) | 0.51 |
| 2:1 | 53 273 (0.19) | 17 905 (0.87) | 0.53 |
| 1:1 | 18 657 (0.07) | 18 128 (0.88) | 0.48 |
| 1:2 | 281 309 (1) | 20 686 (1) | 1 |
| (b) | | | |
| 1:1 | 20 129 (0.07) | 16 887 (0.75) | 0.41 |
| 1:1.25 | 58 540 (0.21) | 19 066 (0.85) | 0.53 |
| 1:1.67 | 268 207 (0.94) | 18 968 (0.85) | 0.9 |
| 1:2.5 | 284 140 (1) | 22 437 (1) | 1 |
| 1:5 | 10 757 (0.04) | 1 452 (0.06) | 0.05* |
| 1:10 | 26 (0) | 886 (0.04) | 0.02* |

## Discussion

The aim of this study was to develop a DNA extraction method for paleontological and historical (museums specimens) bone and teeth samples that maximizes DNA retrieval and reduces the time and workload required. Both, the comparison between the column-based method developed and the batch-based silica extraction using nine Pleistocene cave bear samples, and the extraction of 13 historical chimpanzee teeth, show that the column extraction gives consistently high DNA yields. Although the column-based and original batch-based methods do not differ significantly in yields for the cave bear samples, this is the first column-based method that, in our experience, is not worse than batch-based silica extraction, but rather, if anything, better. It is notable that this method also works well on younger museum specimens, as shown by the results on the chimpanzee teeth for nuclear marker. For such younger samples, we recommend using

**Table 3** Results of qPCR comparing the initial extraction technique with the new column-based extraction method on nine cave bear samples. Absolute copy numbers (from two independent extractions and two measurements of two dilutions, resulting in 8 measurements per sample per method) per gram of powder are shown together with relative numbers (in parenthesize) compared to the best performing method for each sample in this experiment. The difference between both methods is not significant after a Student's *t*-test (P<0.05)

| Sample ID | a | b | c | d | e | f | g | h | i | |
|---|---|---|---|---|---|---|---|---|---|---|
| Extraction method | Copy number per gram (relative to best method) | | | | | | | | | Average of relative performance |
| A initial | 63 209 (1) | 2 432 (0.52) | 1 385 (0.6) | 8 331 (0.93) | 45 498 (0.1) | 165 (1) | 9 724 (0.35) | 165 (1) | 2 027 (0.51) | 0.67 |
| B columns | 31 091 (0.49) | 4 659 (1) | 2 293 (1) | 8 957 (1) | 467 461 (1) | 22 (0.13) | 28 165 (1) | 74 (0.45) | 3 966 (1) | 0.79 |

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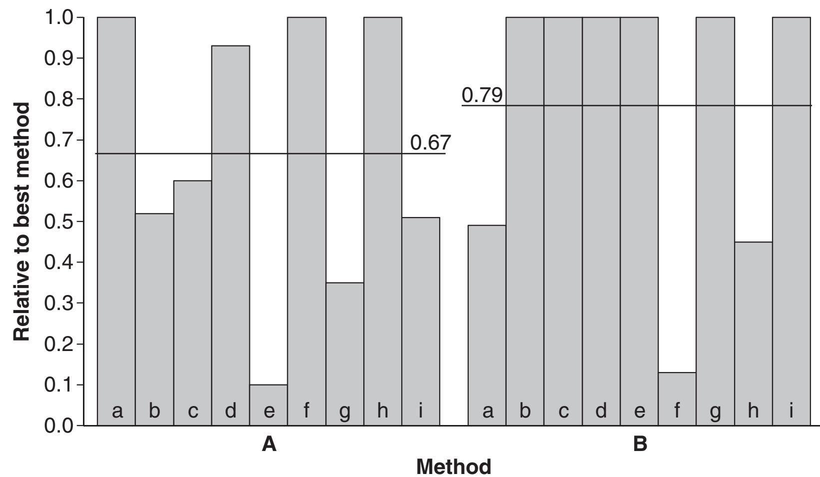


**Fig. 2 Relative results of the initial (A) and the new column-based method (B).** Copy number values are given relative to the copy number of the best performing method for each of the nine cave bear samples (a-i, see supporting online information, Table S1). The horizontal line indicates the average of the respective method over the nine samples, but is not significantly different after a Student's *t*-test ($P<0.05$). A, initial extraction method as previously described (Rohland & Hofreiter, 2007a; Rohland & Hofreiter, 2007b), B, new column-based method.

DTT and Triton X-100 in the concentrations as described for the extraction of the chimpanzee teeth. Although we did not find any beneficial effect on the extraction of Pleistocene specimens, neither of these reagents had a negative effect (Rohland & Hofreiter 2007b), and with younger specimens with more intact tissue structure, the addition of these two chemicals may have a positive effect on DNA yields. With regard to such younger specimens, it is especially encouraging that we were able to extract DNA from samples that had previously failed. In summary, it is likely that our protocol is suitable for the extraction of DNA from any degraded hard tissue sample, including not only fossil and museums specimens but also historical and forensic samples (Capelli *et al.* 2003; Holland *et al.* 2003; Deng *et al.* 2005; Lehrman 2006; Coble *et al.* 2009).

As we showed previously that the batch-based silica method gives higher DNA yields for ancient samples than commercially available kits, the modified extraction protocol presented in this study should outperform these kits. This difference between different column-based extractions is most likely explained by the fact that, in our protocol, binding of DNA is performed for 3 h in suspension whereas with commercial kits DNA can only bind during the few seconds while it is being washed through the silica matrix (Rohland & Hofreiter 2007b). This step provides a major difference to commercial column-based methods and may at least partially explain the different yields, as we found previously that an extended binding time is critical for high DNA yields. The change in the ratio of binding to extraction buffer from 4:1 to 1:2 seems to also contribute to the on average better performance of the column-based extraction compared with the previous protocol (Table 2). An interesting side effect of this change (apart from the slightly higher DNA yields) lies in the reduced costs, as GuSCN is the most expensive item in the protocol. Thus, compared to the previous batch-based extraction protocol, the total costs per extraction of 250 mg bone powder are reduced approximately by half (already taking the costs for the columns into account) using the column protocol.

A further important result of our tests lies in the discovery that adding too much bone powder to a certain amount of extraction buffer is highly detrimental for the DNA yield. Interestingly, this was not only the case for yields per milligram of bone but in fact for total yields per extraction (Table 1). Quantitative PCR on a dilution series of the extracts showed that this failure of large amounts of bone powder to yield DNA is not due to inhibition of the PCR, as up to a dilution of 1:250, extractions using smaller amounts of bone powder generally yielded higher copy numbers, both per millilitre of extract and per gram of bone powder (Table S2b), in accordance with previous findings on forensic specimens (Loreille *et al.* 2007). We suggest two mutually nonexclusive explanations for this observation. First, substances inhibiting proteinase K activity may be released. Second, ancient DNA may mostly be preserved in the inner parts of the bone powder granules. Thus, at a high bone powder to extraction buffer ratio, decalcification is insufficient to release the majority of the DNA in the powder, at least using the incubation temperatures and times in this protocol. As EDTA alone has been shown to allow DNA extraction from ancient bone, albeit with reduced efficiency (Rohland & Hofreiter 2007b), we believe that the second mechanism or a combination of both mechanisms is the more likely explanation. However, further experiments would be necessary to clarify this issue. In any case, more is clearly not always better, and researchers should refrain from using too much bone powder per extraction. However, using smaller samples has the positive side effect that damage to specimens is limited; an issue that is growing in importance due to increasing interest of

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geneticists in museums collections (Collins *et al.* 2009; Nicholls 2009). On the other hand, when larger samples are available, our method has the advantage that, in contrast to commercial kits for which binding to silica is already performed on the column, it is scalable to almost any degree. Even if large amounts of extraction and correspondingly binding buffer are used, after the binding of DNA to the silica, it can be resuspended in a small volume and applied to the columns.

Although the binding of DNA is still performed in batch, the use of columns for all steps after the binding step reduces the workload substantially and reduces the risk of cross-contamination compared to resuspension by extensive pipetting. When using standard vacuum manifolds or table-top centrifuges, in case no vacuum manifold is accessible, it is possible to process up to 24 samples per extraction. We recommend including at least two negative controls resulting in 22 specimens that can be processed in parallel. The original batch method uses suspension throughout the whole procedure, involving many time-consuming resuspension steps. Although it is possible to process up to 24 samples in parallel using the batch protocol, in our experience just the washing and elution steps take at least 4 h constant pipetting for an experienced person, whereas using the new protocol this part of the protocol requires only *c*. 1 h for 24 samples, as no time-consuming resuspension steps of the silica pellet are necessary anymore. Thus, the bench time required is reduced substantially. Furthermore, by omitting the resuspension steps during washing and elution, no pipetting of DNA-containing solutions is needed, reducing the possibility of cross-contamination. Finally, a further simplification of the protocol lies in the omission of the pH adjustment after extraction buffer, silica and binding buffer are combined, and its substitution by buffering the binding buffer using sodium acetate. Although this modification results in a pH between 5 and 6, above the ideal value for DNA binding of pH 4 (Rohland & Hofreiter 2007b), the results show that this does not harm DNA yields. We cannot exclude that using a buffer system at pH 4 would further increase DNA yields. However, using a buffer system simplifies handling and eliminates the risk of adjusting the pH to below 4, which results in a rapid loss of DNA (T. Maricic, pers. comm.).

The protocol developed is robust, easy to handle and combines high DNA yields with reduced bench time per sample. It also facilitates the parallel processing of larger sample numbers and is therefore also well suited for harvesting the power of NGS, which allow obtaining gigabases of DNA sequences in a single-sequencing run, for samples containing degraded DNA. This is becoming increasingly important, as various barcoding methods have been introduced that allow sequencing large numbers of specimens in parallel (Meyer *et al.* 2007; Craig *et al.* 2008; Cronn *et al.* 2008; Illumina 2008; Stiller *et al.* 2009).

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Table S1** Cave bear samples used in this study.

**Table S2a** Results of qPCR comparing different amounts of sample powder in the extraction buffer (kept constant at 5 mL).

**Table S2b** Results of a second qPCR using two of the extracts as in Table S2a, but using further dilution steps comparing different sample powder input and several dilutions.

**Table S3a** Results of qPCR comparing different ratios of extraction to binding buffer (amount of extraction buffer kept constant at 5 mL).

**Table S3b** Results of qPCR comparing different ratios of extraction buffer to binding buffer (amount of extraction buffer kept constant at 5 mL) reducing the amount of binding buffer even further compared to Table S3a.

**Table S4** Results of qPCR comparing the initial extraction technique with the new column-based extraction method on nine cave bear samples.

**Table S5** Results for the chimpanzee samples.

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