Establishing Molecular Methods for the Extraction of DNA from Beluga Whale (Delphinapterus leucas) Hard Tissues

Project 1: teeth

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INTRODUCTION

For over a decade, molecular genetic studies have been conducted on the beluga whale, *Delphinapterus leucas*, at the Southwest Fisheries Science Center. These studies have focused on the analysis of population structure and dispersal patterns of this species through the examination of variation within several genetic markers, and have relied primarily on the collection of tissue samples, typically skin, from harvested and stranded animals. Some skin samples have also been collected from live-caught whales during satellite tagging operations or from free-swimming whales via projectile biopsy.

Despite the success in the collection of large sample numbers from many locations in Alaska, sample coverage is incomplete. There are a number of key areas where samples are required and yet the prospect of getting samples in the near future are slim. Some of these areas were historically important hunting sites. In a few, biological samples were collected during past hunts. The soft tissues (e.g., reproductive tracts), however, were typically preserved in formalin, and an early study conducted at our laboratory revealed that tissues preserved in this manner not only yielded low amounts of DNA, but that the preservative may alter the structure of the DNA itself. Although future developments may allow the use of formalin-fixed tissues in molecular ecology studies, current technology renders these tissues unusable at present.

As well as the collection of soft tissue, many of these earlier beluga whale studies collected teeth for age determination. DNA occurs in very small amounts in teeth, but until recently, this tissue was inaccessible to study. Recent developments in the field of ‘ancient DNA analysis’ now provides the possibility of extracting DNA from teeth and bone, of enough quality and quantity, for use in our study of population structure.

The following report details a comprehensive study to test a range of methods for extracting DNA from hard tissues on beluga whale teeth. Because of the low quantities of DNA involved, strict policies were established to prevent contamination. A separate lab with dedicated equipment, buffers, compounds, etc. was set up to isolate the research from all other molecular projects at Southwest Fisheries.

This report provides information on methods as well as some preliminary results. Details of an expanded analysis of beluga whale teeth from a number of areas in Alaska will be provided in a future report.
# Report: Establishing methods for DNA extractions from hard tissues

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1 Goals

For future large scale DNA extraction from bone and tooth tissues, different extraction methods were to be evaluated for different kinds of samples. A new clean room was set up and guidelines and controls were established to avoid contamination. In the context of this work DNA was extracted from a set of less than 30 years old teeth from 33 beluga whale individuals (from Kotzebue Sound).

2 Introduction

Extracting DNA from hard tissues involves two steps. Physical pretreatment is carried out first, pulverizing the samples to create surface for chemical exploitation. Subsequently chemical treatment is used to release and extract DNA. The following methods were chosen for physical pretreatment:

- grinding
- drilling
- cutting with an isomet saw.

From a variety of DNA extraction protocols the following ones were selected. They are based on different extraction principles:

- Hoess & Paabo\textsuperscript{1} (silica based)
- Qbiogene 'GeneClean for aDNA' (silica based, kit)\textsuperscript{2}
- Bachmann/Pusch (electrophoresis based, gel elution)\textsuperscript{3}
- Rosenbaum/Lifton's buffer (sucrose, organic extraction)\textsuperscript{4}

The protocols as they were published or transmitted are given in section 9. Assuming that no kind of physical pretreatment in- or decreases the yield of only one particular extraction method, the focus was first laid on finding the best extraction method(s). The influence of physical pretreatment was investigated later on.
Comparison of extraction methods for teeth

3.1 Preliminary test of all extraction methods

To check whether successful extractions can in general be gained from all of the four selected methods, a set of recent beluga whale teeth (Z #12224, 1998) was chosen as the first sample. Varying amounts of powder from drilling as well as from grinding (for drilling and grinding protocols, see 11) were used. Two protocols had to be slightly changed concerning reaction volumes (Hoess & Paabo) or incubation conditions (Rosenbaum) because of lab limitations. Table 1 refers to the protocols as they were used and the extraction success, which was checked by amplification of a ~200 bp fragment from the mitochondrial D-Loop region with the primers DL1 & AD1 (for amplification protocol, see 12).

<table>
<thead>
<tr>
<th>method</th>
<th>protocol</th>
<th>amplification success</th>
<th>controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoess &amp; Paabo</td>
<td>10.1</td>
<td>yes</td>
<td>OK</td>
</tr>
<tr>
<td>w/o PTB</td>
<td>20 mg powder from drilling</td>
<td>yes</td>
<td>OK</td>
</tr>
<tr>
<td>Hoess &amp; Paabo</td>
<td>10.1</td>
<td>yes</td>
<td>OK</td>
</tr>
<tr>
<td>w/ PTB</td>
<td>200 mg powder from grinding</td>
<td>yes</td>
<td>OK</td>
</tr>
<tr>
<td>QBiogene Kit</td>
<td>9.2</td>
<td>yes</td>
<td>OK</td>
</tr>
<tr>
<td>w/ PK predig.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bachmann/Pusch (electrophoresis)</td>
<td>see 3.2</td>
<td>?</td>
<td>contamination</td>
</tr>
<tr>
<td>Rosenbaum/Lifton's buffer</td>
<td>9.4</td>
<td>yes</td>
<td>OK</td>
</tr>
</tbody>
</table>

Table 1: amplification success for first extractions

All methods were showing good results, except the electrophoresis based one. Therefore this method and its exclusion from further experiments are discussed in 3.2.

3.2 Excluding Bachmann/Pusch (electrophoresis)

The electrophoresis method (for protocol as published see 9.3) was carried out as follows: Fivefold stock-solutions of TBE/TBGE (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA / 5.72 g/l glycerol) were prepared from 54 g Tris-base, 27.5 g boric acid, 20 ml 0.5 M EDTA pH 8.0 (and 28.6 g Glycerol) with water added to 1 L. Gel loading buffer stock solution lacking DTT was prepared from 4.5 g glycerol, 9 ml 10 % SDS, 1.5 ml 50 mM Tris buffer pH 7.5 and water added to 30 ml. All stock solutions were made in glass bottles and UV irradiated for 12 hours under a hood for sterilization. Electrophoresis material was treated with bleach and UV sterilized...
as well.

100 ml agarose (2 % GenePur agarose in 1x TBE, containing 2 ml of 1 % EtBr solution) were cast into a large gel rack with a 13 slot sandwich comb, obtained by cutting a 26 slots comb in the middle. The rack was placed into the chamber and 1x TGBE was filled in without covering the top of the gel. DTT (23 mg/ml) was added to the gel loading buffer. 80 ml gel loading buffer were applied to each slot. Electrophoresis for equilibration was carried out for 15 minutes at 20 V. The gel was loaded as illustrated in figure 1. Electrophoresis was run for 24 minutes at 70 V until the blue dye had run completely into the gel.

Under UV visualization slices were cut from the gel and DNA recovered using the Quiaex II Gel Extraction Kit. Figure 2 shows the amplification success for every slice.

Analyzing the results shown in fig. 2, the electrophoresis method was excluded from further efforts mainly for two reasons:

1) Since all extraction blanks are contaminated, it is obviously hard to avoid contamination, despite all precautions.

2) The positive control proves that released DNA could have been recovered from the gel. Therefore only little or no DNA was released from the tooth powder. Continuing work on this protocol would require troubleshooting and support of those who published the method. The first results are not encouraging to take these steps.
3.3 **Further comparisons - excluding Rosenbaum/Lifton's**

After excluding the electrophoresis method, the three remaining methods were compared using teeth from 8 individuals of the Kotzebue Sound beluga teeth set. The samples were ground (see 11.1) and all three extraction methods carried out for each specimen (H & P 10.1 w/o PTB; QBiogene 9.2 w/ PK predigestion; Rosenbaum/Lifton's 10.3). Extraction success was checked by amplification of the ~200 bp AD1/DL1-fragment and additionally of the ~500 bp TRO/D-fragment, both within the mitochondrial D-loop region. Results are given in table 2.

<table>
<thead>
<tr>
<th>sample</th>
<th>mg powder</th>
<th>mg powder</th>
<th>mg powder</th>
<th>mg powder</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rosenbaum/Lifton's ampl. success</td>
<td>QBiogene ampl. success</td>
<td>H &amp; P ampl. success</td>
<td></td>
</tr>
<tr>
<td></td>
<td>~200 bp AD1/DL1</td>
<td>~500 bp TRO/D</td>
<td>~200 bp AD1/DL1</td>
<td>~500 bp TRO/D</td>
</tr>
<tr>
<td>#29786</td>
<td>53</td>
<td>+</td>
<td>+</td>
<td>53</td>
</tr>
<tr>
<td>#29787</td>
<td>76</td>
<td>+</td>
<td>+</td>
<td>84</td>
</tr>
<tr>
<td>#29788</td>
<td>98</td>
<td>+</td>
<td>--</td>
<td>79</td>
</tr>
<tr>
<td>#29789</td>
<td>35</td>
<td>+</td>
<td>--</td>
<td>22</td>
</tr>
<tr>
<td>#29790</td>
<td>50</td>
<td>+</td>
<td>+</td>
<td>61</td>
</tr>
<tr>
<td>LDL A</td>
<td>156</td>
<td>+</td>
<td>--</td>
<td>186</td>
</tr>
<tr>
<td>#35248</td>
<td>35</td>
<td>+</td>
<td>--</td>
<td>33</td>
</tr>
<tr>
<td>#35249</td>
<td>76</td>
<td>+</td>
<td>--</td>
<td>106</td>
</tr>
</tbody>
</table>

*table 2: extraction success for 8 specimens with different methods*

Quantitative PCR was not available at this time to quantify the extraction yield. But besides yield, fragment length is of particular interest for measuring extraction success and probably correlates with yield to a certain degree. Whereas Hoess & Paabo and the QBiogene kit yielded amplifiable long fragments for all samples, Lifton's failed to do so in 5 out of 8 cases and was therefore the second extraction method to be excluded from further experiments.

3.4 **Finishing beluga set - optimizing QBiogene Kit and H & P**

The remaining teeth samples from the Kotzebue Sound set were progressively extracted using the H & P method and the QBiogene kit in parallel. Several modifications were applied during this process, mainly to the QBiogene protocol.

3.4.1 **Determining impact of Proteinase K**

Proteinase K is routinely added to the Hoess & Paabo lysis buffer. The manual of the QBiogene kit also suggests a Proteinase K predigestion step. At least for the kit this step is quite costly and time consuming. Extractions were carried out for both methods, determining the impact of Proteinase K (Hoess & Paabo 10.1, w/ and w/o PK; QBiogene 9.2, w/ and w/o PK predigest.). An older orca tooth from 1887 was extracted together with the Kotzebue Sound beluga samples.
The results are given in table 3.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ground</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD1/DL1</td>
<td>92</td>
<td>+</td>
<td>80</td>
<td>+</td>
</tr>
<tr>
<td>TRO/D</td>
<td>183</td>
<td>+</td>
<td>144</td>
<td>+</td>
</tr>
<tr>
<td>AD1/DL1</td>
<td>112</td>
<td>+</td>
<td>98</td>
<td>+</td>
</tr>
<tr>
<td>TRO/D</td>
<td>140</td>
<td>+</td>
<td>144</td>
<td>+</td>
</tr>
<tr>
<td>#35252</td>
<td>144</td>
<td>+</td>
<td>136</td>
<td>+</td>
</tr>
<tr>
<td>ground</td>
<td>136</td>
<td>+</td>
<td>137</td>
<td>--</td>
</tr>
<tr>
<td>one piece</td>
<td>163</td>
<td>+</td>
<td>160</td>
<td>(+)</td>
</tr>
<tr>
<td>#34085 (orca, 1887)</td>
<td>228</td>
<td>+</td>
<td>210</td>
<td>--</td>
</tr>
<tr>
<td>ground</td>
<td>217</td>
<td>--</td>
<td>239</td>
<td>--</td>
</tr>
<tr>
<td>one piece</td>
<td>143</td>
<td>+</td>
<td>147</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>+&amp;PTB</td>
<td>220</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*table 3: Impact of PK on extraction success. (PTB discussed in 3.4.2) (+) indicates faint PCR products

For both methods Proteinase K is of significant benefit. The kit does not seem to be capable of extracting DNA from older teeth, at least not from this particular orca tooth.  
It is a surprising aspect that the impact of Proteinase K on extraction efficiency is even higher than that of grinding the sample. The intention for extracting from whole chunks of tooth actually was to prove that there is no surface contamination. It might be interesting to follow this idea vice versa later on, which means, omitting proteinase K to prove for no surface contamination. This should work for older samples (orca tooth), although it is just an assumption that surface contamination could be extracted without proteinase K, which would have to be proved.

### 3.4.2 Impact of PTB

During the preliminary extraction test the Hoess & Paabo method was working without PTB not worse than with. Therefore all following extractions were carried out without PTB. PTB was added a second time to one of the extractions from the old orca tooth (#34085, see 3.4.1, table 3). This extraction failed, whereas the ones without PTB were successful. From the statistical point
of view this hardly allows any conclusion. However, PTB (N-phenacylthiazolium bromide) has been proven to break bonds between DNA and sugars in sugar-rich paleofeces and prehistoric samples. Since these kind of samples are not of interest at present, PTB is not routinely added to the Hoess & Paabo lysis buffer. Nevertheless, it might be worth trying PTB for particular samples that are failing without.

3.4.3 Optimizing, troubleshooting, exclusion of QBiogene kit

Although the QBiogene kit initially was showing good results, the longer it was being used the more troubles were occurring. Among these the following ones could be resolved:

1) Referring to the QBiogene manual the amount of silica ('Glassmilk solution') must be increased 4x when using the proteinase K predigestion. Thus, the spin filter carries more silica than can be resuspended.

   This problem can easily be overcome by decreasing the volume of Glassmilk back to 0.3 ml (which by the way also decreases the costs for the kit back to normal). A positive effect could be verified.

2) Periodically the spin filters were soaking and breaking down whilst spinning. This problem occurs mostly when too many samples (> 15) are handled at one time.

3) The incubation temperature must not be less than 60 °C. Therefore the samples can not be rotated in an incubator, which only heats up to 55 °C.

However, the major problem could not be resolved. Whereas the kit was working reliably at the beginning, except for breaking filters, several extraction sets failed later on. Extraction positive controls did not gain the same good results as before. Although the 200 bp AD1/DL1-fragment could still be amplified nearly every time, the 500 bp TRO/D fragment periodically failed to amplify. Eventually it was not possible to gain the longer fragment anymore from kit extracts. This observation strongly indicates a decline of one or more chemicals. Subsequently Proteinase K, SDS solution (which are not part of the kit) and the kit itself were exchanged, always double checking extractions in parallel with the old chemicals if possible. The source of failure could not be located. The final conclusion may be that one of the chemicals of the first kit, which was more than two years old, aged to be the decisive factor and could not be equally replaced by a new bottle.

At this point the kit was excluded from further experiment. Its purpose is to simplify the extractions process and give reliable, reproducible results. Since it failed those demands, possible modifications with alternative buffers that are supplied with the kit were not tested anymore.

The QBiogene kit protocol is nevertheless given in section 10.2 as it gave good results for a while and could be useful for some sample types.

3.5 Conclusion - Hoess and Paabo best

The Hoess & Paabo method was proven to be the best method for tooth material, since it gave
reliable results and, estimated only by gel pictures, provide the highest extraction yields. However, the methods were only compared for tooth, not for bone or ancient samples. As the electrophoresis method and the QBiogene kit are not working, it would not be useful to test them on bone material. For a completion of the method comparison, Hoess & Paabo and Rosenbaum/Lifton's could be compared on bone and ancient samples. Another option would be to test a different protocol based on Phenol/Chloroform, which was developed for extracting nuclear DNA from bone\textsuperscript{5}. Rosenbaum/Lifton's method was originally designed for DNA extraction from baleen. Extraction success was checked by amplification of the 200 bp AD1/DL1-fragment and the 150 bp DL3/D-fragment.

4 Testing Hoess & Paabo on bone and baleen

After the Hoess & Paabo method was proven to be the best extraction method for teeth, its capability to extract DNA from bone and baleen tissue was tested using samples from different species. The samples were ground. Prior to grinding baleen, its surface layers were removed with a sterilized screwdriver. Extractions were carried out as given in table 4.

<table>
<thead>
<tr>
<th>sample number</th>
<th>species</th>
<th>type</th>
<th>age</th>
<th>mg powder</th>
<th>remarks</th>
<th>AD1/DL1 (200 bp)</th>
<th>DL3/D (150 bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#34523</td>
<td>orca</td>
<td>tooth</td>
<td>15y</td>
<td>57 / 145</td>
<td>+ / +</td>
<td>+ / +</td>
<td></td>
</tr>
<tr>
<td>WFP492</td>
<td>gray whale</td>
<td>bone</td>
<td>28y</td>
<td>250 / 290</td>
<td>inhib. / inhib.</td>
<td>inhib. / inhib.</td>
<td></td>
</tr>
<tr>
<td>HJB33</td>
<td>bone</td>
<td></td>
<td>17y</td>
<td>158 / 446</td>
<td>+ / +</td>
<td>+ / +</td>
<td></td>
</tr>
<tr>
<td>#28493</td>
<td>sperm whale</td>
<td>tooth</td>
<td>n/a</td>
<td>120 / 255</td>
<td>+ / +</td>
<td>+ / +</td>
<td>--</td>
</tr>
<tr>
<td>#7087</td>
<td>bryde's whale</td>
<td>bone</td>
<td>n/a</td>
<td>110 / 315</td>
<td>+ / +</td>
<td>+ / +</td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>blue whale</td>
<td>baleen</td>
<td>55y</td>
<td>46 / 170</td>
<td>+ / +</td>
<td>+ / +</td>
<td></td>
</tr>
</tbody>
</table>

\textbf{table 4: Results of duplicate extractions from different sample types with Hoess & Paabo (10.1 w/o PTB).}
\begin{itemize}
  \item \textit{inhib.} = no PCR product and no primer dimers visible
  \item \textit{--} = no product: DL3/D primer pair does not fit for sperm whales.
\end{itemize}

One of the bone samples, which was very oily inside, showed PCR inhibition. But in general the Hoess & Paabo method worked well for all tested materials and its results are consistent for duplicate extractions. To evaluate the extraction yields that could be gained from the different materials, qPCRs were conducted (together with the samples in chapter 5). The amplification plots are given in figure 3.
The shape of the curves indicate, that inhibition is a big issue when extractions are carried out from more than 100 to 200 mg of sample. This may partly be due to the fact, that the volume of lysis buffer was reduced to one third. The Hoess & Paabo method could not be tested on ancient samples, since none were available at the time. However, it was particularly developed for DNA extractions from historic and prehistoric samples.

5 Comparison of Sampling Methods

Three sampling method were conducted on a 15 year old orca tooth in replicates:
- grinding (11.1)
- drilling (11.2)
- cutting with isomet saw

(The powder suspension was treated different than given in 11.3. The suspension was decanted into 15 ml tubes after sawing. The powder was spun down and the supernatant discarded. The silky pellets were resuspended and united. (~ 5 ml overall). Definite volumes of the suspension were used for extracting. To estimate the ratio of powder weight per suspension volume, 1.5 ml tubes of equal weight were filled with 100, 200, 300 and 400 ?l suspension. The tubes were spun for two hours in a speedvac with maximum heating. The weight differences were 9, 7 and 9 mg. Therefore 100 ml contained an estimated 8 mg of powder.)

Varying amounts of powder obtained by the different sampling methods were used for DNA extraction (Hoess & Paabo, 10.1 w/o PTB). Extraction success was checked by amplifying the D/DL3-fragment. One extraction blank was contaminated. Nevertheless 5 ml of each extract were used for a Cybergreen qPCR assay. A standard curve, using dilutions of a beluga extract of known concentration (starting dil. 1:1000 of 120 ng/ml), was compiled first (figure 4). This was done to be able to estimate the DNA concentrations of the samples.
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fig. 4: qPCR standard curve

The x-axis is false. Starting concentration was not 5 ng/ml but 0.12 ng/ml. The Ct-value was measured in triplicates for each concentration. Concentrations lower than \( \sim 1.2 \times 10^{-4} \) (\( 5 \times 10^{-3} \) on false axis) cannot be estimated precisely.

Referring to the standard curve, the qPCR software analyzed the amplification plots (figure 5) and calculated DNA concentrations for each extract (table 5).

![fig. 5: Amplification plot for comparison of sampling methods](image)

<table>
<thead>
<tr>
<th>sampling method</th>
<th>amount of powder</th>
<th>amplification success</th>
<th>DNA conc. in extract [ng/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>D/DL3</td>
<td></td>
<td></td>
<td>calculated by qPCR</td>
</tr>
<tr>
<td></td>
<td>(~ 150 bp)</td>
<td>D/DL3</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>------------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>drilled</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22 mg</td>
<td>+</td>
<td>1.23E-03</td>
<td></td>
</tr>
<tr>
<td>44 mg</td>
<td>+</td>
<td>2.98E-03</td>
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<td>105 mg</td>
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<td>43 mg</td>
<td>+</td>
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<td>116 mg</td>
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<td>209 mg</td>
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<td>saw powder</td>
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<tr>
<td>20 ml (1.5 mg)</td>
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<td>4.25E-05</td>
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<tr>
<td>200 ml (8 mg)</td>
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<td>2.80E-04</td>
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<tr>
<td>300 ml (24 mg)</td>
<td>+</td>
<td>9.85E-04</td>
<td></td>
</tr>
<tr>
<td>1000 ml (80 mg)</td>
<td>+</td>
<td>2.97E-03</td>
<td></td>
</tr>
<tr>
<td>extraction blank</td>
<td>+</td>
<td>7.78E-06</td>
<td></td>
</tr>
</tbody>
</table>

*Table 5: Extracts and yield as estimated by qPCR. Statistical remarks: Concentration lower than 1.2*10^-4 are vague and written in italic. All other concentration are quite exact in relation to each other. The absolute concentrations are rough estimations, though. The contamination in the last extraction blank can be ignored this time, since it is far away from the range of all other concentrations.*

The DNA concentration should theoretically be straight proportional to the amount of sample that was used for extraction. The corresponding plot is given in figure 6.

*Figure 6: Plot of DNA concentration versus amount of sample. Two data points were excluded from the*
In theory, the sampling method showing the highest slope in the trend line should be most efficient. However, it is hard to conclude which method is best, mainly because there is only little data and some of it had to be excluded. For this particular sample, drilling seems to be slightly more efficient than grinding and sawing. In fact, the sample was rather soft and did not heat up. Especially for drilling, results may change with different samples. The powder from sawing shows the best linear correlation ($R^2 = 0.9985$). Although this method is extremely time-consuming and vulnerable to contamination, it can be very useful if more protocols or modifications of protocols have to be evaluated in the future. It grants a high level of reproducibility.

It can be concluded, that no sampling method was inefficient. Therefore the way of sampling might not be the decisive factor for successful extractions.

6  The contamination issue

Contamination is one of the major issues while extracting DNA from hard tissues or ancient samples. Extraction techniques for these kind of samples are usually more complex, i.e. they require higher volumes and more steps, which make them more vulnerable to contamination. Extractions yields are little and amplification conditions sensitive. Thus, it is unlikely that contamination will stay in the background.

6.1  How to avoid contamination

It is important to be aware of the possible sources of contamination, which basically are

- PCR products of all kinds,
- DNA extracts from cetaceans,
- tissue from cetaceans,
- cross-contamination within one extraction set.

Bone work is done in a clean-room to prevent contamination from outside. The clean-room guidelines have strictly to be followed (see 13). Use single capped PCR (strip) tubes.

6.2  How to control contamination

Unless appropriate contamination control is carried out at all time, extractions results are of no significance. Contamination can occur on the following levels:

- Are samples contaminated as they reach the lab?
  
  It should be investigated, where samples are coming from, how they were collected, treated, stored, and which kinds of contamination are to be expected (human, bacterial
OK; cetacean not). Sterilizing the sample surface with bleach and/or UV is a difficult process and bears the risk of destroying the sample. Therefore it should not be done without reasons. However, the sequence data should eventually found within the bounds of possibility.

- *Contamination while drilling, grinding, sawing, ...*
  There are no useful negative controls for this particular working step. It is crucial to do it most carefully.

- *Contamination while extracting*
  At least every 5th extraction should be a negative control (blank), with at least two for each extraction set. The blanks should not be the first or last samples of a set, but be arranged inbetween.

- *PCR Contamination*
  Controlled by several PCR blanks.

If possible, extraction sets should be assembled from different species. Background contamination can be determined by cloning PCR products and sequencing several clones. If extraction blanks amplify products, the whole extraction set must be discarded and the source of contamination eliminated.

## 7 Manual for extraction of DNA from hard tissues

Read the clean-room guidelines (see 13). Choose samples and arrange them in a way that helps backtracking cross-contamination in case it occurs. Pulverize samples as given in 11. Grinding is the easiest method. Add an appropriate number of extraction blanks and one extraction positive control.

Extract DNA following the Hoess & Paabo protocol (see 10.1). Do not add PTB to lysis buffer. Amplify desired locus (see 12) in single capped PCR tubes. Do not forget to add several blanks and one PCR positive control.

**Suggested trouble shooting**

In case no product was amplified:

- Check positive controls.
- Check for inhibition (primer dimers missing).
- If PCRs are not inhibited:
  - Test different primers. Try to amplify very short fragments. OR
  - Add more template to PCR. OR
  - Increase PCR volume and add even more. OR
  - Repeat extraction using larger amount of sample. OR
  - Repeat extraction eluting in less 1x TE. OR
Repeat extraction using PTB.

In case of inhibition:
- Add more Taq to PCR. OR
- Add less extract to PCR. OR
- Reextract extract. OR
- Repeat extraction using a larger volume of lysis buffer or less sample. OR
- Repeat extraction adding more washing steps. OR
- Try PTB.

In case extraction blanks are showing products:
- Check PCR blanks. If necessary repeat PCR. Load gel carefully.
- Discard extracts. Search source of contamination (cross-contamination or from outside).

If necessary sequence contamination.

In case PCR blanks are showing products:
- Search source of contamination and/or repeat PCR.

8 Unfinished jobs, perspectives

belugas:

9 Original protocols as published or received

9.1 Hoess & Paabo (2003 version MPI-EVAN)
- grind a piece of bone or tooth (freezermill or with mortar and pestill)
- put 0.5 g of that ground material (powder) into 10 ml buffer (0.45 M EDTA pH 8.0; 0.5 % Sarcosyl; 0.25 mg/ml Proteinase K; 2.5 mM PTB; 50 mM DTT [= 80 mg/10ml]; 1 % Polyvinylpyrrolidone)
- rotate it overnight at 37 °C
- centrifuge at full speed for 2 min
- ad supernatant to 35 ml (1 tube) L2 and add 50 ml silica
- rotate at RT for 15 min to 1 h
- centrifuge at full speed for 1 min
- discard supernatant (Keep in fridge until you know everything worked OK, then throw away.)
  · add 1 ml L2, resuspend and transfer to a fresh 2 ml tube
  · centrifuge for 5 sec. in minicentrifuge
  · take off supernatant, resuspend in 1 ml NewWash
  · centrifuge 10 sec. in minicentrifuge
  · take off supernatant, spin briefly, remove remaining liquid
  · dry at 56 °C for 5 min with open lids
  · add 100°C 1x TE, elute at 56 °C for 8 min with closed lids
  · spin at max. speed for 1 min, remove TE to a fresh tube, take care to avoid any traces of silica
  · freeze at -20 °C, use within 4-5 month (usually 5 ml per PCR)

Binding solutions, ancient DNA
1. L2 buffer
   · 26 g GuSCN 5.5 M
   · 16.5 ml Tris (pH 8.0; 0.1 M) 0.05 M
   · 180 ml NaCl (5 M) 0.025 M
   · add 200 ml silica, vortex, let sit (optional)

2. NewWash
   · 1 ml NewWash concentrate
   · 23 ml EtOH, 100 %
   · 20 ml water
   · store at RT

Silica for ancient DNA
   · take 4.8 g silica
   · add ddH2O to 40 ml, vortex and let sit for 24 h
   · take of 35 ml, add ddH2O to 40 ml, vortex
   · let sit for 6 h, take of 36 ml
   · add 48 ml 30 % HCl
   · resuspend, aliquote app. 200 - 400 ml
   · store dark at RT

9.2 QBiogene "Geneclean for aDNA" Kit Manual

Optional Proteinase K Preincubation
Starting samples are 240 - 400 mg of bone powder.
Overnight Soaking Solution
5 ml 0.5 M EDTA
200 ml 10 % SDS
200 ml 20 mg/ml Proteinase K
1) Samples were rotated and incubated at 37 °C for 12 - 15 h.
2) 1 ml DeHybernation Solution A was added to each sample and rotated for 2 - 4 hours at 60 °C.
3) Samples were spun in centrifuge to pellet particulate.
4) Supernatant was transferred to clean tube and 1.2 ml Ancient DNA Glassmilk and 3.0 ml DeHybernation Solution A were added.
5) Samples were rotated for 2 hours at 35 - 40 °C
6) Samples were centrifuged at 4,000 rpm for 1 minute to pellet Ancient DNA Glassmilk. Supernatant was discarded.
7) 0.5 ml Salton Wash #1 was added to resuspend pellet, which was then transferred to Spin Filter
8) The protocol, starting with Step 6, was followed from this point forward.

PROTOCOL
1) Add 100-500 mg homogenized or powdered sample to 1ml DeHybernation Solution* in a nucleic acid free microcentrifuge tube.
2) Incubate at 45 - 60 °C between 2 and 12 hours with mixing (longer incubation times may be necessary).
3) Centrifuge sample at high speed for 5 minutes to pellet particulate material. Transfer supernatant to a new nucleic acid-free microcentrifuge tube. Add 300 ml Ancient DNA Glassmilk suspension. Incubate at room temperature for 10 - 30 minutes with mixing.
4) Transfer suspension to a Spin Filter and Catch Tube. Centrifuge at 14,000 x g in microcentrifuge for 1 minute or until liquid is transferred to catch tube. (Empty Catch Tube as needed.)
5) Add 0.5 ml Salton Wash #1 and centrifuge at 14,000 x g to clean Glassmilk / DNA complex.
6) Add 0.5 ml Salton Wash #2 and centrifuge at 14,000 x g to clean Glassmilk / DNA complex.
7) Add 0.5 ml Ancient DNA Alcohol Wash and centrifuge to empty filter of Wash Solution. Repeat. [Important: Be sure to add alcohol prior to first use.]
8) Empty Catch Tube and centrifuge for 2 minutes to "dry" Glassmilk in Spin Filter.
9) Place filter into DNA-free Elution Catch Tube. Add 50 - 100 ml DNA-free Elution Solution. Resuspend pellet by hand or briefly vortex. (1-2 seconds, any longer will result in damage to filter.) Centrifuge for 1 minute to transfer eluate to Catch Tube.
Optional: Elute a second time.
10) Remove Spin Filter and discard. DNA is ready to use in amplification without further manipulation.

* There are two different Dehyb solutions supplied with this kit. They differ in composition and each is effective in the isolation of DNA. Because the materials from which the DNA is to be extracted vary so greatly we are not able to recommend one over another.

There is an additional detergent supplied with this kit that can be used in conjunction with DeHyb A. In certain applications this detergent (DeHyb A2) has resulted in improved DNA yields. If you choose to use DeHyb A2, then the amount of DeHyb A should be decreased to 850 ml, and 150 ml of DeHyb A2 must be added just prior to incubation or homogenization.
9.3 Bachmann & Pusch

We employed a new MiniSub DNA cell (Bio-Rad, Richmond, CA, USA) electrophoresis chamber (gel tray, 10 x 7 cm) that had not been used for DNA experiments previously. The chamber was carefully cleaned with DNAaway (Molecular Bio-Products, San Diego, CA); subsequent UV-irridiation for 2 h between experiments removed any DNA and/or DNase. A comb with a tooth size of 5 x 10 x 2 mm was selected to allow a loading volume of about 50 ml for each slot. 1x Tris-borate-EDTA (TBE) gels were cast using Separidel (Gibco BRL Life Technologies, Gaithersburg, MD, USA), a gel matrix dedicated to seperating small double-stranded DNA fragments. SeparideU has no detectable contamination activity as determined by DNA nicking assays. A 25 w/v gel matrix was used, as it provided good resolution of DNA fragments of 50 - 500 bp. Ethidium bromide (100 ng/mL) was added to the gel prior to casting. A 1x TBGE buffer (89 mM Tris, 89 mM boric acid, 5.72 g/L glycerol, and 2.5 mM EDTA; pH 8.0) was used as the running buffer. Prior to electrophoresis xylene cyanol/bromophenol blue was placed in an empty lane but not added to the samples to avoid masking the faint fluorescence of aDNA. A 1.5 x gel-loading buffer was prepared by combining 15 mL of 50 % w/v glycerol solution (final 15 %), 7.5 ml of a 20 % w/v SDS stock solution (final 3%: electrophoresis grade), and 2.5 mL of a 1 M Tris-HCl solution, pH 7.5 (final 50mM). Dithiothretiol (DTT; 150 mM) was added just prior to use. Gel-loading buffer lacking DTT can be stored at room temperature. Forty ml of gel-loading solution were applied to each slot, and the gel was prerun for 15 min at 2 V/cm prior to sample loading to allow for the equilibration of the Separidel matrix next to the slots.

A maximum of 25-30 µg of each sample was loaded into the bottom of a well using a minispatula. One slot was left empty between samples. An equal volume (-25 µL) of 1.5-fold loading buffer was added to each lane, including the empty lanes. After loading, the mixture was gently stirred with a crystal tip. The electrophoresis was run at 7 V/cm. After the dye front had moved into the gel, DNA was visualized on a short-wave (304 nm) UV-Transilluminator (Bachofer, Reutlingen, Germany). The DNA was quickly recovered from the gel following the technique of Pusch [Pusch CM (1997) Electrophoresis 18, 1103-1104], as commercially available spin columns are not recommended for the recovery of VIR-DNA from gel slices because of the severe degradation and nicking of aDNA. For efficient recovery of high quality aDNA, the DNA extraction solution was replaced by H2O.

9.4 Rosenbaum / Lifton's buffer

"...Each piece of baleen was then pulverized and transferred to an 1.5-mL tube to which 500 µl of extraction buffer (0.1 M Tris, 0.2 M sucrose, 0.05 M EDTA, 0.1 % SDS at pH 9.0) and 1.4 mg/mL proteinase K was added. Tube lids were wrapped with parafilm as a precaution against leakage. All samples and extraction negative controls were incubated for 24 h at 65 C while rocking. Phenol chloroform extraction followed by ethanol precipitation (Sambrook et al. 1989) proceeded under the aDNA conditions specified above. ..."
10 Extraction protocols as applied
10.1 Hoess & Paabo

MODIFICATIONS:
· volumes of lysis buffer, L2 buffer and silica suspension reduced to 1/3rd to fit into 15 ml tubes

reagents
· N-Lauroylsarcosine sodium salt
· Polyvinylpyrrolidone
· DTT
· 0.5 M EDTA solution (pH 8.0)
· 1x TE buffer (pH 8.0)
· 10 mg/ml Proteinase K (from Proteinase K powder with H2O)
· PTB
· Guanidine Isothiocyanate
· 1 M Tris buffer (pH 8.0)
· 5 M NaCl solution
· Silica (silicon dioxide, conc. or 30% Hydrochloric Acid)
· New Wash (New Wash Concentrate, Ethanol abs.)

equipment
· 1.5 ml tubes
· 15 ml tubes
· 50 ml tubes
· balance
· table centrifuge
· centrifuge for 15 ml tubes
· incubator
· rotator
· heatblock
· glass bottle (UV sterilized)
· pipettes (5 & 15 ml) + ball
· spatulas

making of buffers and solutions
· Silica
  o take 4.8 g silica in 50 ml tube
  o add H2O<sub>MQ</sub> to 40 ml and let sit for 24 h
  o take 35 ml off, add H2O<sub>MQ</sub> to 40 ml, vortex
  o let sit for 6 h, take 36 ml off
  o add 48 ml 30 % HCl (obtained by mixing 20 ml H2O & 76 ml conc. HCl)
  o resuspend, aliquote at 200 ml
Not to be quoted without author’s permission

- Store dark in fridge, resuspend before using

  - New Wash
    - 1 ml New Wash concentrate
    - 23 ml EtOH abs.
    - 20 ml H$_2$O$_{MQ}$
    - Mix, store at RT

  - L2 buffer (prepare fresh)
    - Prepare in sterilized glass bottle. **Per 3 extractions:**
      - 26 g GuSCN => final conc. 5.5 M
      - 1.65 ml 1 M Tris (pH 8.0) => 0.05 M
      - 180 ?l 5 M NaCl => 0.025 M
      - 14.25 ml H$_2$O$_{MQ}$
      (note: GuSCN dissolves endothermic. Put bottle into warm tap water and shake occasionally to quicken the process.)

  - Lysis buffer (prepare fresh)
    - Prepare in 50 ml tube. **Per 3 extractions:**
      - Sarcosyl 50 mg => 0.5 %
      - DTT 80 mg => 8 mg/ml
      - PVP 100 mg => 1 %
      - PTB 7.5 mg => 2.5 mM
      - 0.5 M EDTA (pH 8.0) 9.0 ml => 0.45 M
      - 10 mg/ml Proteinase K 250 ?l => 0.25 mg/ml
      - H$_2$O ad 10 ml
      (note: First add Sarcosyle, DTT, PVP. Mix salts. Add EDTA, H$_2$O and PTB. Dissolve salts. Finally add Proteinase K.)

**procedure (never handle more than 20 samples at one time)**

**LYSIS**
1. Start from max. 200 mg of bone/teeth material in a 15.0 ml tube with screw cap. Add 3.3 ml fresh prepared lysis buffer, with or without PTB.
2. Incubate samples rotating at 37 °C for 12-16 hours.

**BINDING, WASHING, ELUTING**
3. Pellet particulate by spinning at max. speed for 2 min. Add supernatant to 11.3 ml fresh prepared L2 buffer by decanting. Invert several times to mix solutions. Add 20 ?l silica and rotate at RT for 1 hour.
   (note: Silica sinks to the ground. Flick the tube several times to suspend silica properly. Keep remaining L2 buffer for washing step.)
4. Pellet silica by spinning at max. speed for 1 min. Discard supernatant by decanting.
   (note: After decanting spin remaining liquid down to reduce the risk of cross-contamination at the following step.)
5. Resuspend pellet in 1 ml L2 buffer and transfer to fresh 1.5 ml tube.
7. Resuspend pellet in 1ml New Wash.
9. Dry silica in heatblock at 56 °C for 5 min with open lids.
10. Add 100 ?l 1x TE and resuspend pellet. Elute at 56 °C for 8 min with closed lids.
11. Spin at 13,000 rpm for 1 min. Remove TE to a fresh tube.
12. Freeze at -20 °C.
13. Prior to PCR, spin at 13,000 rpm for 2 min to pellet traces of silica.

**10.2 QBiogene "Geneclean for aDNA" Kit**

**MODIFICATIONS:**
- Volume of Glassmilk reduced. Proteinase K predigestion strongly recommended.
- Protocol specified.

**reagents**
- QBIogene Geneclean For Ancient DNA Kit
- 10 mg/ml Proteinase K solution (from Proteinase K powder with H2O)
- 10 % SDS solution (w/v, pH not adjusted)
- 0.5 M EDTA solution pH 8.0

**equipment**
- 2.0 ml tubes with screw caps
- 1.5 ml tubes
- 15 ml tubes
- table centrifuge
- centrifuge for 15 ml tubes
- rotator for 15 ml tubes
- incubator
- heatblock (+thermometer) or thermomixer for 2.0 ml tubes

**procedure (never handle more than 20 samples at one time)**

**PROTEINASE K PREDIGESTION**
1. Start from max. 200 mg of bone/teeth material in a 2.0 ml tube with screwcap.
   - Add 5 ml 0.5 M EDTA pH 8.0
   - 200 ml 10 % SDS
   - 200 ml 10 mg/ml Proteinase K
2. Incubate samples rotating at 37 °C for 12-16 hours.

**LYSIS**
3. Add 1 ml Dehybernation Solution A to each sample and incubate for 4 hours at 60 °C on heatblock. Invert tubes occasionally.
   *note: By adding DehybA Proteinase K will floculate.
   Check the temperature is really 60 °C. 55°C might not work out.*
4. Spin samples (table centrifuge: 13,000 rpm, 2 min) to pellet particulate.
5. Take off supernatant with 1ml-pipet and transfer to 15 ml tube.
   *note: Flocculated Proteinase K centers in the bottom of the tube. Try carrying over as much supernatant as possible, including the Proteinase K part at the
BINDING, WASHING, ELUTING

6. Add 3.0 ml Dehybernation Solution A and 0.3 ml Ancient DNA Glassmilk.
[note: Shake Glassmilk very well prior to use. If you process many samples at one time, shake Glassmilk inbetween. Don't take Glassmilk right from the bottom.]
7. Rotate samples at 37 °C for 2 hours.
8. Pellet silica by spinning at max. speed for 1 min. Discard supernatant by decanting.
[note: After decanting spin remaining liquid down to reduce the risk of cross-contamination at the following step.]
9. Add 0.5 ml Salton Wash #1 to resuspend pellet and transfer to a Spin Filter. Spin 20" at 13,000 rpm. Empty catch tube.
[Don't process more than 20 samples at one time. If the washing takes too long, the Spin Filters are soaking and likely to break. It that happens, resuspend the silica in the catch tube and transfer to a new spin filter.]
10. Add 0.5 ml Salton Wash #2. Spin 20" at 13,000 rpm. Empty catch tube.
11. Add 0.5 ml Alcohol Wash. Spin 20" at 13,000 rpm. Empty catch tube.
   Repeat this step.
   [note: Be sure to add alcohol to Alcohol Wash prior to first use.]
12. Empty catch tube and spin 2 min at 13,000 rpm to dry the silica.
13. Place filter into new catch tube. Add (50-)100 ml DNA-free-Solution. Resuspend pellet completely by pipetting up and down.
   [note: Be careful not to destroy the Spin Filter.]
14. Spin 1 min at 13,000 rpm. Remove Spin Filter and discard.
15. Store extract at -20 °C.

10.3 Rosenbaum/Lifton's

MODIFICATIONS:
   · incubation temperature for lysis step lowered from 65 °C to 55 °C (Maximal incubator temperature. 65 °C denature Proteinase K ?)

reagents
   · Lifton's buffer (sucrose, 1 M Tris pH 8.0, SDS, 0.5 M EDTA pH 8.0)
   · 10 mg/ml Proteinase K solution (from Proteinase K powder with H2O)
   · Phenol (TE saturated, pH 8.0)
   · 1x TE buffer pH 8.0
   · Chloroform
   · Isoamyl-alcohol
   · EtOH abs.
   · 10 M ammonium acetate solution

equipment
   · fume hood
   · balance
· table centrifuge
· incubator
· rotator
· pipettes (5 & 25 ml) + ball
· spatulas

**making of Lifton's buffer, stock solution**

- Prepare in sterilized glass bottle

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>6.85 g</td>
<td>2.3% w/v</td>
</tr>
<tr>
<td>1 M Tris, pH 8.0</td>
<td>2 ml</td>
<td>6.7 mM</td>
</tr>
<tr>
<td>10% SDS</td>
<td>20 ml</td>
<td>0.67%</td>
</tr>
<tr>
<td>0.5 M EDTA, pH 8.0</td>
<td>20 ml</td>
<td>33 mM</td>
</tr>
<tr>
<td>H2O</td>
<td>ad 300 ml</td>
<td></td>
</tr>
</tbody>
</table>

**procedure**

**LYSIS**

1) Start from max. 200 mg of bone/teeth material in a 2.0 ml tube with screwcap. Add 500 µl Lifton's buffer and 80 µl 10 mg/ml Proteinase K.
2) Incubate samples rotating at 55 °C for 16 - 24 hours.

**ORGANIC EXTRACTION AND PRECIPITATION**

3) Pellet particulate by spinning at max. speed for 2 min. Transfer supernatant to a fresh 1.5 ml tube and add 500 µl buffer saturated phenol.  
   (note: Fume hood for working with Phenol and Chloroform!)
4) Rotate 30 minutes at room temperature under hood.
5) Spin for 5 min at 11,000 rpm. Transfer supernatant to new tube.
6) Add 500 µl Chloroform/Isoamyl-alcohol (24 : 1) and rotate 30 min at room temperature under hood.
7) Spin for 5 min at 11,000 rpm. Transfer supernatant to fresh tube.
8) Add 900 µl EtOH abs. and 33 µl 10 M ammonium acetate. Mix and incubate at -20°C for at least one night.
9) Defrost and spin for 20 min at 13,000 rpm. Pellet should be visible.
10) Remove supernatant by decanting. Be careful not to lose pellet.
11) Evaporate remaining ethanol in speedvac without heating.  
   (note: Takes 15 minutes or more.)
12) Dissolve pellet in 100 µl 1x TE.
13) Store at -20 °C.

11 **Sampling protocols**

11.1 **Grinding**

**equipment**

mortars & pestles
1.5 ml tubes and racks
15 ml tubes and rack
hammer, screwdriver (optional)

**preparation of equipment (contamination precautions)**

- mortars & pestles
  - After usage soak mortars and pestles in bleach over night. Rinse intensely with tap water to completely wash away bleach. Dry with fresh tissue papers. UV sterilize mortars in crosslinker 20 minutes upside down. Turn mortars around and repeat irradiation. Pestles are UV sterilized in the same way (turned after the first 20 minutes as well). Open the crosslinker door as well as the clean hood. Change gloves and place as many layers aluminum foil in the hood as needed (Aluminum foil must not be touched else than with new gloves). Take mortars and pestles out of the crosslinker and wrap them in aluminum foil. Unwrap them when they are needed.

- bonework hood
  - After usage wipe out hood with tissues soaked in 70 % ethanol. Close hood and irradiate with UV light over night.

- hammer and screwdriver (if needed)
  - Wipe with bleach and ethanol. Sterilize in crosslinker 2x 20 minutes. Store wrapped in aluminum foil until needed.

**procedure**

Open bonework hood. Put several open 1.5 ml-tubes into a rack and place the rack in a corner of the hood. Change gloves, remove two or three big pieces of aluminum foil and cover the ground of the hood. Pick another small piece of aluminum foil, fold it in the middle and unfold again. Put it into the hood as well. Unwrap a mortar and a pestle without touching them. Let the sample slide out of its package into the mortar. Change gloves and place mortar and pestle on top of the aluminum in the hood. The ground of the hood, mortar, pestle and gloves can be considered sterile.

Pound the sample with the pestle until it breaks into chunks of desired size. If some chunks are jumping out of the mortar, just put them back as long as they are landing on aluminum foil. Put several chunks into the open 1.5 ml tubes, but do not touch the rack. Grab the sample package with one hand, the rest of the sample with the other and let it slide in. Close lids of the 1.5 ml-tubes and weigh them after zeroing with an empty tube. Insert screw cap tubes for extraction into a rack and remove cap. Place the rack in a corner of the hood. Choose chunk(s) of desired weight for grinding. Let chunk slide into the mortar and change gloves. Grind or pound the chunk into powder. Decant powder onto the small piece of aluminum foil and let it slide into an open extraction tube.

For proceeding to the next sample just remove the aluminum foil.

If the sample needs to be broken with a hammer initially (or in combination with a screwdriver /chisel), put a plank on ground of the hood and cover it with aluminum foil.

Pieces of the pestle, sometimes breaking off during the grinding, are not affecting extraction
success.

11.2 Drilling

equipment
drill
drill bits
tube(s)

preparation of equipment (contamination precautions)
  · drill
    o Clean drill intensely with wet tissue.

  · bonework hood
    o After usage wipe out hood with tissues soaked in 70 % ethanol. Close hood and irradiate with UV light over night.

procedure
Open bonework hood. Put one or more open tubes into a rack and place the rack in a corner of the hood. Change gloves, remove two or three big pieces of aluminum foil and cover the ground of the hood. Take another piece of aluminum foil, fold it in the middle and unfold it. Put it on top as a second layer. Unwrap a new drill bit without touching it. Change gloves and adjust drill bit. Place drill in a corner of the hood without the drill bit touching anything. Let the sample slide out of its package onto the second aluminum foil layer. Change gloves. Use one hand to hold the sample on the second aluminum foil layer and the other one to drill. The ground of the hood, one glove and the drill bit can be considered sterile, not the drill itself. Therefore the drill should neither touch the sample nor the second aluminum foil layer. Drill at low speed and take breaks to prevent the sample from heating-up. When enough powder is collected, remove drill from the hood. Change gloves, lift the aluminum foil layer and let the powder slide into the open tube. Close the tube, discard aluminum foil and wipe out the hood with a wet tissue before proceeding to the next sample.

11.3 Cutting with an isomet saw

equipment
isomet saw and accessories
1x TE pH 8.0
funnel
filter paper
bottle
spatula

preparation of equipment (contamination precautions)
isomet saw
  o Clean the saw by wiping with a tissue soaked in 70 % Ethanol. Wipe all parts that may have contact with powder with a tissue soaked in bleach and afterwards alcohol. UV sterilize the parts 2 x 20 minutes from both sides. Store wrapped in aluminum foil until usage.

funnel und spatula
  o Rinse with bleach, water and ethanol. UV sterilize for 20 minutes in crosslinker. Store wrapped in aluminum foil until usage.

filter paper
  o UV sterilize in crosslinker for 20 minutes prior to usage.

**procedure**
For sample fixation and saw setup refer to Kelly Robertson. Fill tub of the isomet saw with 100 ml 1x TE. Insert filter paper into the funnel and place funnel into a glass bottle. After cutting decant powder suspension into filter paper. Place bottle with funnel in the closed hood and let the powder dry over night. When dried, collect powder into a tube using a spatula. Do not overdry the powder or it will not resuspend.

## 12 Amplification protocol

Amplifications were carried out under the following conditions: *insert table #...*

<table>
<thead>
<tr>
<th>PCR mix</th>
<th>supplier</th>
<th>concentration</th>
<th>cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR buffer</td>
<td>Invitrogen</td>
<td>1x</td>
<td>init. denat. 94 °C 2' 30&quot;</td>
</tr>
<tr>
<td>50 mM MgCl2</td>
<td>Invitrogen</td>
<td>2.5 mM</td>
<td>denaturation 94 °C 0' 30&quot;</td>
</tr>
<tr>
<td>25 mM e. dNTPs primer</td>
<td>Invitrogen</td>
<td>250 mM each</td>
<td>annealing 48 °C 0' 45&quot;</td>
</tr>
<tr>
<td>Taq</td>
<td>Invitrogen</td>
<td>0.2 mM each</td>
<td>elongation 72 °C 0' 45&quot; - 1' 30&quot; *</td>
</tr>
<tr>
<td>H2O template</td>
<td>MilliQ</td>
<td>1.25 U. (0.25 ml) ad 25 ml</td>
<td>final elong. 72 °C 10' 00&quot;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 ml</td>
<td></td>
</tr>
</tbody>
</table>

e | H2O | | |

Elongation time was extended to 1' 30" for all fragments longer than 500 bp. PCR conditions were selected for high amplification yield (high MgCl₂ concentration, low annealing temperature, many cycles) and are therefore quite unspecific. This is necessary, because most primers are universal for cetacean and do not fit 100 %. Furthermore, the PCR should be capable of catching few molecules.