# Nondestructive DNA extraction method for mitochondrial DNA analyses of museum specimens

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*Museum specimens have provided the material for a large proportion of ancient DNA studies conducted during the last 20 years. However, a major drawback of the genetic analyses is that the specimens investigated are usually damaged, as parts of skin, bone, or a tooth have to be removed for DNA extraction. To get around these limitations, we have developed a nondestructive extraction method for bone, tooth, and skin samples. We found that it is possible to amplify mitochondrial DNA (mtDNA) sequences up to at least 414 bp long from samples up to 164 years old. Using this method, almost 90% (35 of 40) of the investigated samples yielded amplifiable mtDNA. Moreover, we found that repeated extractions of the same samples allowed amplifications of the expected length for all samples at least three times and for some samples up to at least five times. Thus this method opens up the possibility to repeatedly use museum collections for mtDNA analyses without damaging the specimens and thus without reducing the value of irreplaceable collections for morphological analyses.*

## INTRODUCTION

Ancient DNA analyses are used to answer questions about the phylogeny, phylogeography, and population history of extinct and extant species (1–4). Museum specimens are a convenient source for species-wide sampling from many mammalian species that otherwise require extensive fieldwork to obtain noninvasive samples. Moreover, museum specimens are the only source for samples of extinct populations and also allow investigating whether the genetic diversity of animal populations has changed over time (5).

Methods for DNA extraction from bones and teeth are well established (6,7). However, in almost all extraction methods, a piece of bone or tooth is powdered before extraction (8,9), thus inflicting damage to the specimen. Therefore, most museums have understandably imposed restrictions on the use of their often irreplaceable collections. We have developed a protocol in which the samples are soaked in guanidinium-thiocyanate (GuSCN) without addition of substantial amounts of demineralizing reagents. Only the buffer is subsequently processed, thus preventing damage to the specimen.

## MATERIALS AND METHODS

### Samples

We used entire teeth from three western chimpanzees [*Pan troglodytes verus* (*P.t.v.*)] from Taï Nationalpark, Côte d'Ivoire, to test the efficacy of different buffers. To test the success rate of the best method, we used tooth, bone, coat, or soft tissue samples from 40 museum specimens, consisting of 17 spotted hyenas (*Crocuta crocuta*), 13 striped hyenas (*Hyaena hyaena*), and 10 brown hyenas (*Parahyaena brunnea*). Catalogue numbers for each specimen used are available in the supplementary material on the BioTechniques' web site at http://www.BioTechniques.com/May2004/RohlandSupplementary.html (see Supplementary Table S1).

### DNA Extraction and Purification

Chimpanzee teeth were incubated in different extraction buffers. We used a sodium phosphate buffer [50 mM $NaH_2$ $PO_4$, pH 7.5, 25 mM Tris, pH 8.0, 50 mM NaCl, 5% Triton® X-100, 50 mM dithiothreitol (DTT), 2.5 mM N-phenacylthiazolium bromide (PTB) (10), 0.25 mg/mL proteinase K (11)], a Tris/NaCl buffer [100 mM Tris, pH 8.0, 500 mM NaCl, 1% N-lauroyl sarcosine, 50 mM DTT, 2.5 mM PTB (10), 0.25 mg/mL proteinase K, 10 mM EDTA, pH 8.0 (12)], and a GuSCN-based buffer [5 M GuSCN, 50 mM Tris, pH 8.0, 25 mM NaCl, 1.3% Triton X-100, 2.5 mM PTB (10), 20 mM EDTA (13)]. All teeth were incubated with rotation in 40 mL buffer at 40°C in the dark. Half of the buffer was removed after 2 days and the remainder after 7 days. The teeth were rinsed with sterile water and air-dried at room temperature.

Two different methods were used to purify the DNA contained in the buffers. For the sodium phosphate and Tris/NaCl buffers, an isopropanol protocol previously described was used (14). The DNA contained in the GuSCN buffer was purified by binding to silica (8,13), using 50 µL silica suspension. Washing of the silica pellet and elution of the DNA were done as described (15), using an elution volume of 100 µL.

For the hyena specimens, DNA was extracted either from an entire tooth, a piece of coat, or a piece of dried soft tissue using 40 mL of buffer and the GuSCN/silica protocol described above or from a little piece of tooth or bone reducing the buffer volume to 1.5 mL and the amount of silica to 20 µL. The incubation was done for 5 days. All of the following steps were performed as described above.

### PCR and Analysis

Primers and expected product sizes are given in Table 1. Five microliters of the extract were used for the PCR using a hot-start protocol (16). The amplifications were performed in an MJ Research thermal cycler (MultiCycler PTC 200; Biozym, Oldendorf, Germany) with a 3-min activation step at 94°C, followed by 35–60 cycles at 93°C for 30 s, 50°–52°C (depending on the primer pair) for 60 s, and 72°C for 45 s. We used 40–60 PCR cycles for the chimpanzee teeth to make sure that we would obtain a product even in the presence of a single template molecule (17). Amplifications from hyena samples were done with only 35 cycles, as this was sufficient for successful amplification for most samples.

**Table 1. Primers Used in this Study**

| Primer | Species Specificity | Amplification Target | Sequence | Product Size Including Primers (bp) |
|---|---|---|---|---|
| L16214 | Chimpanzee | HVR1 | 5′-ATGCTTACAAGCACGCACAAC-3′ | |
| H16332 | Chimpanzee | HVR1 | 5′-GATTTGACTGTAATGTGCTATG-3′ | 161 |
| Crocuta Cyt b F1 | Spotted hyena | Cytochrome B | 5′-GAAAATCTCACCCACTCATTAAAA-3′ | |
| Crocuta Cyt b R1 | Spotted hyena | Cytochrome B | 5′-CCGTAGTTTACGTCTCGGC-3′ | 214 |
| Crocuta Cyt b F2 | Spotted hyena | Cytochrome B | 5′-CAACAACCGCCTTCTCATCAG-3′ | |
| Crocuta Cyt b R2 | Spotted hyena | Cytochrome B | 5′-CACCTCAGAATGATATTTGGCCTC-3′ | 252 |
| Crocuta Cyt b F1–R2 | Spotted hyena | Cytochrome B | Sequences as described above. | 414 |
| Hyena Cyt b F1 | Striped/Brown hyena | Cytochrome B | 5′-GAAAATCTCACCCGCTCATTAAAA-3′ | |
| Hyena Cyt b R1 | Striped/Brown hyena | Cytochrome B | 5′-CAGCCATAGTTGACGTCTCGGC-3′ | 217 |
| Hyena Cyt b F2 | Striped/Brown hyena | Cytochrome B | 5′-CAACCGCCTTTTCATCAGTA-3′ | |
| Hyena Cyt b R2 | Striped/Brown hyena | Cytochrome B | 5′-GTAAGACGTAACCTATGAATGCG-3′ | 222 |
| Hyena Cyt b F1–R2 | Striped/Brown hyena | Cytochrome B | Sequences as described above. | 387 |
| D2S1329F2 | Chimpanzee | Msat | 5′-ACCGTTCTCAAATACCAGGAATC-3′ | |
| D2S1329R2 | Chimpanzee | Msat | 5′-CCTGGGTTCTTAATTTAACCATAATTC-3′ | 154–198 |
| 12Sa′ | mammalian | 12S rDNA | 5′-CTGGGGATTAGATACCCCACTA-3′ | |
| 12So | mammalian | 12S rDNA | 5′-GTCGATTATAGGACAGGTTCCTCTA-3′ | 151 |

PCR products were visualized on 2.8% agarose gels using ethidium bromide. Amplification products of the expected length were isolated from the gel and melted in 100 µL double-distilled water. Amplification products of the three hyena species were sequenced directly after reamplification. In the case of the striped and brown hyenas, the longer fragment was reconstructed from the two short products by a jumping PCR procedure without primers (18). After that, the primers for the long fragment were added and 35 PCR cycles were done as described earlier. Reamplification for 30 cycles of the isolated products, either from first amplifications or from the reconstructed products, were carried out under the PCR conditions described above, except that the annealing temperature was raised by 2°C. The chimpanzee amplification products were cloned using the TOPO® TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). DNA sequences were obtained from individual clones as described (16).

Finally, we tested whether it would be possible to amplify nuclear templates using this method and investigated, using the chimpanzee teeth, whether repeated extractions from the same specimens would still yield DNA. After each extraction, the teeth were soaked in double-distilled water to remove any traces of GuSCN buffer and then dried to mimic the process of extraction by independent researchers as exactly as possible.

## RESULTS AND DISCUSSION

### Efficacy of the Different Buffers

To develop an efficient nondestructive DNA extraction method for museum specimens, we first used nonarchival material to compare three extraction/isolation procedures. To test the efficacy of the different buffers, we attempted DNA extractions on teeth from three chimpanzees, using two teeth from each of the three individuals (I, II, and III). All chimpanzee teeth were treated in buffer for 2 and 7 days. The two samples extracted using sodium phosphate buffer (individuals I and III) both yielded a distinct band of the expected size, both after 2 and 7 days, apart from one PCR failure (individual I) after 7 days (Figure 1). Of the two samples extracted using Tris/NaCl buffer (individuals I and II), none yielded a product after 2 days incubation, but individual I gave a distinct PCR product after 7 days (Figure 1). Finally, the extracts obtained using GuSCN buffer (individuals II and III) yielded a strong PCR product of the right size both after 2 and 7 days (Figure 1). Comparison of the obtained sequences using BlastSearch (19) shows that the products originated from chimpanzee DNA, with each sequence matching with 100% identity to chimpanzee control region sequences available in GenBank®. Furthermore, all of the amplification products obtained from a given individual were identical and differed from the other two individuals at several nucleotide positions (see supplementary Figure S1). As we used duplicate samples from each individual and also duplicate PCRs for each extraction, contamination is highly unlikely to explain these results.

The different yields of the three buffers tested allow some insights into the possible mechanisms of DNA release from the surfaces of the samples. Both the Tris/NaCl buffer and the sodium phosphate buffer contain proteinase K. Thus, in these two cases, proteins and protein fragments that contact the buffer should get digested, and thereby, DNA entangled between proteins should be released. However, DNA can also bind directly to hydroxyapatite, the mineral component of the bone matrix, which might explain the better performance of the sodium phosphate buffer compared to the Tris/NaCl buffer, as phosphate can displace DNA from hydroxyapatite. Interestingly, by far the best results are obtained using the GuSCN buffer. One possibility to explain the better performance of GuSCN could lie in the different purification methods, as silica purification was used with the GuSCN buffer while an isopropanol precipita-

tion was used with the other two methods. However, we found that isopropanol precipitation results in higher yields of DNA compared to binding to silica if the same extraction buffer is used before purification of the DNA. Thus, more efficient purification is unlikely to explain the difference in performance, and different abilities to release DNA from the bone/tooth matrix probably explains the higher DNA yields using the GuSCN buffer compared to the other two methods. GuSCN is a strong protein-denaturing agent (20) and has also been shown to bring cross-linked proteins back into solution, if used in combination with a reducing agent (20). It is possible that GuSCN as a chemical agent might be more efficient in denaturing proteins if these are bound to the hydroxyapatite matrix of the bone than proteinase K. Moreover, GuSCN may break certain chemical cross-links even in the absence of reducing agents. Finally, it is possible that GuSCN is even more effective than sodium phosphate in directly displacing DNA from the hydroxyapatite matrix.

**Success Rate**

Based on the efficacy of the GuSCN/silica method for DNA extraction from the chimpanzee teeth, we tested the success rate of this extraction method on 40 hyena specimens that had been stored in a museum for between 37 and 164 years. We were able to extract amplifiable DNA from 35 of these specimens, while from 5 no visible PCR product could be amplified. Of the 35 samples with a positive result, 17 gave the longer product (387–414 bp), and 18 gave only the short fragments (214–252 bp). The authenticity of the obtained sequences was confirmed using BlastSearch (19), which showed that the sequences are between 99% and 100% identical to sequences of the respective species in GenBank. Sequences identical to the obtained hyena sequences had not been obtained earlier in our laboratory, ruling out the possibility that the amplification products could represent contamination. As 88% of the samples yielded PCR products of 214–252 bp in length, and more than 40% of the samples yielded PCR products of 414 bp or more, the success rate and length of the amplifiable sequences is large enough to do both phylogeographic and phylogenetic studies using this extraction method.

We did not detect a strong difference in the likelihood of DNA retrieval or the length of the obtainable PCR products comparing the different tissue types (Table 2). However, this might be due to the overall small sample number in each category, as there is a tendency for teeth to have a higher success rate. Similarly, we did not find a clear correlation between the length of time a sample has been stored in the museum and either the likelihood of DNA retrieval or the quality of the retrieved DNA (see supplementary Table S2). This is interesting, as a recently published study detected a rapid decrease in the amount of amplifiable DNA that could be extracted from fox teeth within the first 30 years of storage (21). However, the samples we investigated in our study

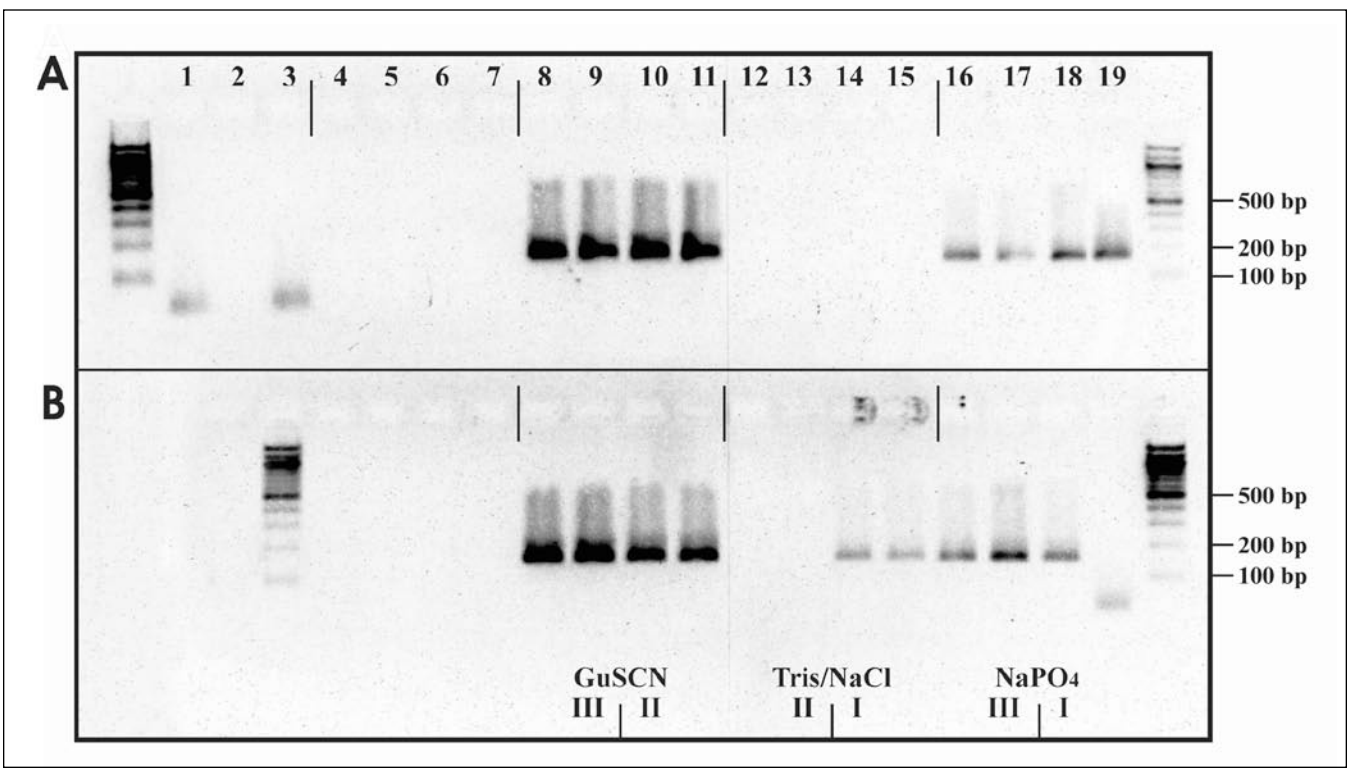


**Figure 1. PCR products of the extractions of chimpanzee teeth using different extraction buffers for 2 and 7 days incubation.** The PCR product (12.5%) was loaded, and the marker used was a 100-bp ladder (New England Biolabs, Beverly, MA, USA). (A) Gel electrophoresis of two amplifications from each DNA extraction using 2 days incubation. Lanes 1–3, negative PCR control; lanes 4–7, negative extraction control; lanes 8–19, six teeth from three individuals (I–III) extracted with the different buffers as indicated at the bottom. (B) Gel electrophoresis of two amplifications from each DNA extraction using 7 days incubation. All lane numbers as in panel A, except that a 100-bp ladder is in lane 3. GuSCN, guanidinium-thiocyanate.

**Table 2. DNA Extraction Success Rate for Various Hyena Tissue Samples**

| Hyena Tissue | Samples Tested (*n*) | PCR Product | | |
|---|---|---|---|---|
| | | Long Products | Short Products | No Products |
| Tooth | 23 | 11 (48%) | 10 (43%) | 2 (9%) |
| Piece of tooth | 6 | 3 (50%) | 3 (50%) | 0 |
| Piece of bone | 6 | 1 (17%) | 3 (50%) | 2 (33%) |
| Piece of coat | 4 | 2 (50%) | 1 (25%) | 1 (25%) |
| Piece of dried soft tissue | 1 | 0 | 1 (100%) | 0 |
| **Total** | **40** | **17 (42.5%)** | **18 (45%)** | **5 (12.5%)** |

The long PCR products are (including primers) 387 bp long for brown and striped hyena and 414 bp long for spotted hyena. The short PCR products are between 214 and 252 bp long.

probably experienced different kinds of chemical treatments during preparation of the museum specimens, which are likely to affect the DNA to a smaller or larger extent depending on the treatment and thus blurring any effects of DNA degradation over time. The fox samples on the other hand were collected only during the last 30 years, and all were treated the same way (21).

## Retrieval of Nuclear DNA

We tried to amplify a microsatellite locus (D2S1329F2R2) (22) that had been amplified earlier from different teeth of the chimpanzees using a different extraction method (14). In contrast to the amplification of mitochondrial DNA (mtDNA), the amplification of the microsatellite marker from the chimpanzee samples gave very poor results. Only individual II extracted with the GuSCN buffer yielded an amplification product. By comparison with published data (14), we found that the allele size obtained differs from what would be expected for this individual. The microsatellite primers also amplify human DNA, and the expected size range for human and chimpanzee is the same. Hence, the most likely explanation for this result is a slight contamination of the specimen with modern human DNA. Thus, the nondestructive extraction method is most likely less efficient than extraction methods that include a demineralization/digestion step using EDTA and proteinase K.

It is interesting that using a protocol that is very similar to the one that was least effective on the chimpanzee teeth (Tris/NaCl buffer), amplification of microsatellite markers from archived fish otoliths has been possible (23). The low DNA yield we obtained is therefore most likely explained by the high density of teeth and bones. Thus, if less dense tissues such as fish otoliths are used, the GuSCN/silica method should also increase the success rate of studies using microsatellite markers. Moreover, it remains possible that certain modifications further improve the yield of nondestructive DNA extraction methods.

## Repeated Use of Nondestructive DNA Extraction

To test whether mtDNA could be obtained repeatedly using nondestructive extractions, we did five consecutive extractions on the six chimpanzee teeth using the GuSCN/silica method. All teeth yielded amplification products in two independent PCRs after 60 cycles for the first three extractions. For the fourth extraction, one sample failed completely, whereas for another sample, only one out of two amplifications worked. Finally, for the fifth extraction, two samples yielded two products, two worked only for one of the two PCRs, and the other two failed for both PCRs. Sequencing of the PCR products from the last extractions that yielded a product confirmed that chimpanzee DNA of the respective individuals was amplified. However, mismatch incorporation patterns show that these amplifications started from 1 to 10 template molecules (17). Thus, successive researches could use the same collections for different projects, although the results indicate that the amount of extractable DNA decreases with increasing numbers of successive extractions.

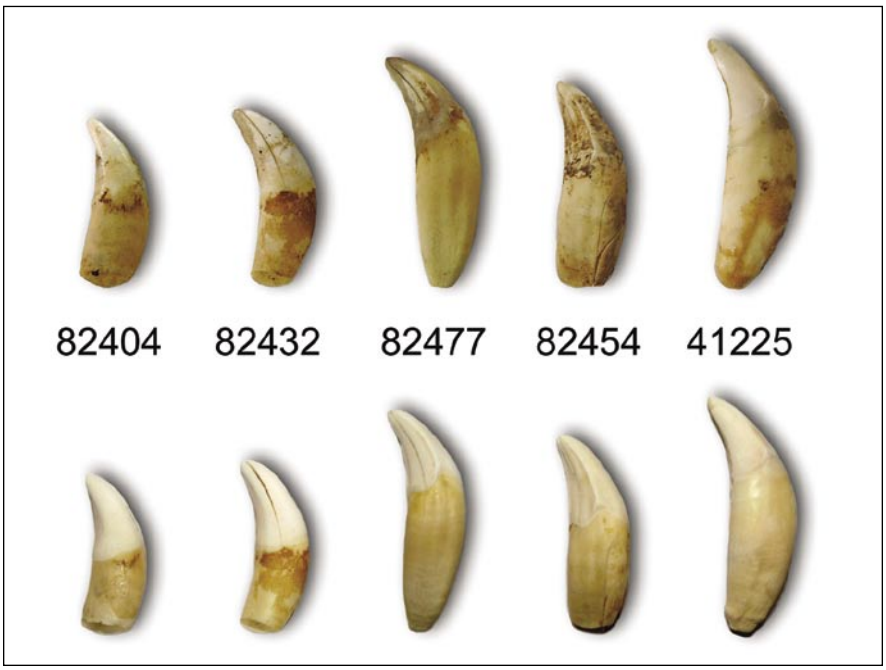


**Figure 2. Photograph of five hyena teeth before (upper row) and after (lower row) extraction using guanidinium-thiocyanate (GuSCN) buffer.** Museum catalogue numbers are indicated.

Since DNA yields for this nondestructive method are comparatively low, we tested, using general mammalian primers, whether the ratio between endogenous and contaminating DNA changes in favor of the former after the first extraction. We tested the first three extractions and sequenced between 46 and 60 clones for each extraction (5–12 per tooth). Besides chimpanzee mtDNA sequences, we found three other classes of sequences in the clones (see supplementary Table S3): (*i*) human mtDNA sequences, which is not surprising given the ubiquitous nature of human mtDNA (17); (*ii*) nuclear insertions (numts) of mtDNA, most likely of chimpanzee origin, have recently been identified as a potential source of incorrect mtDNA sequences (24); and (*iii*) sequences from a monkey and a turtle species. The Colobus and turtle sequences are likely caused by sample storage. While the prevalence of the latter sequence class drops from 18% of the clones in the first extraction to 0% in the second and third extraction, the former two together remain between 13% and 24% of the clones throughout all three extractions. It is therefore recommended to clone the products and sequence several clones if unspecific primers are used. If it is intended to directly sequence the products, species-specific primers are highly recommended.

## Damage to the Samples

We could not detect any change in the appearance of the teeth, except that they appear a little cleaner, after one extraction (Figure 2), and no sign of damage even after five extractions (see supplementary Figure S2). Although we cannot exclude chemical alterations of the extracted samples, there is no obvious damage to the specimens when this extraction method is used. Thus, the structure of the specimens, which is important for morphological studies, remains intact, opening up the possibility to use the enormous bone collections stored in natural history museums all over the world for both phylogenetic and phylogeographic investigations.

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# Isolation of high-quality RNA from gymnosperm and angiosperm trees

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*An improved protocol was developed for efficient and reliable extraction of high-quality total RNA and mRNA from various tissues of spruce (*Picea *spp.) and poplar (*Populus *spp.) trees, as well as other plant species. This method was specifically optimized for tissues with high content of polysaccharides, oleoresin terpenoids, and phenolic secondary metabolites, which often co-precipitate with RNA and inhibit subsequent reverse transcription. The improved protocol yielded up to 600 µg of total RNA per gram of tissue suitable for standard expressed sequence tags (ESTs), full-length cDNA library construction, and for microarray applications.*

## INTRODUCTION

Development of large-scale expressed sequence tags (ESTs) and full-length cDNA resources as well as microarray RNA expression profiling with woody plants require efficient methods for isolation of high-quality RNA from a diverse array of tissues that vary widely in contents of polysaccharides and secondary metabolites. However, working with trees, cDNA library construction and RNA microarray analysis are often difficult due to RNA degradation and contamination of RNA with polysaccharides or secondary metabolites (e.g., polyphenolics and oleoresin terpenoids) that may inhibit reverse transcription. Ideally, methods for comparative tree genome studies should be efficient with a range of different species including angiosperms and coniferous gymnosperms. Existing protocols did not fulfill our demands of working with a range of tissues from both conifers and poplars. We developed an efficient protocol for RNA isolation based on two existing protocols reported by Chang et al. (1) and Wang et al. (2). Combination and modifications of these protocols allowed us to obtain high yields of intact RNA from various tissues of poplar and spruce using one standard protocol. The method reported here is technically straightforward, requires minimum prior training in handling of plant RNA, and uses little glassware, which makes it a method of choice for high-throughput tree genome projects. RNA quality was confirmed by different methods (e.g., UV absorbance scans, gel electrophoresis, and Northern blot analysis), with reverse transcription being the most important quality control for RNA used for successful construction of full-length cDNA libraries and microarray hybridizations. The protocol described here consistently produces high yields and quality of RNA from different tissues of different tree species, which makes it particularly suitable for comparative plant genome research.

## MATERIALS AND METHODS

### Plant Materials

Sitka spruce (*Picea sitchensis*) and hybrid spruce (*Picea glauca* × *Picea engelmannii*) trees were provided by CellFor (Vancouver, Canada). White spruce (*P. glauca*) trees were provided by the BC Ministry of Forests (Victoria, Canada). Poplar (*Populus trichocarpa* and *P. trichocarpa* × *Populus deltoides*) tissues were collected at the University of British Columbia.

### Isolation of Total RNA and Purification of Poly(A) RNA

All solutions except Tris buffer were treated with 0.1% diethylpyrocarbonate (DEPC) and autoclaved. Autoclaved