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DNA amplification from osmicated, plastic-embedded eye tissues

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Purpose: In the premolecular era, mammalian samples were embedded in epoxy resin blocks, such as Epon or Poly/Bed, for future evaluation by electron microscopy. However, use of these archival specimens for more modern mutation characterization studies can be challenging. The aim of this study was to determine if genomic DNA could be extracted from osmicated archival epoxy-embedded tissues to a quality suitable for short-amplicon PCR amplification.
Methods: We selected nine archived Epon, Araldite, or Poly/Bed embedded blocks of mammalian retinal and corneal tissue that were ~10 mm in length, embedded in the 1970s to 1990s, and had an extensive phenotypic description. Tissues were fixed in several combinations of glutaraldehyde and osmium before embedding. The blocks were shaved of excess resin, fragmented, and digested using an epoxy resin removal solution. The softened plastic was cut with a scalpel, washed, drained, and incubated at 56 °C overnight in a tissue lysis solution containing Proteinase K. Trizol was added to the samples, which were further mechanically homogenized. Chloroform was added, and the samples were centrifuged at 4 °C and 12,000 *g*. Upon phase separation, the upper clear phase was removed, 95% EtOH was added, the mix was filtered through a mini-genomic DNA extraction column and washed twice, and DNA was eluted with 10 mM Tris-HCL. Following final removal of phenol contamination using water-saturated ether, the purified DNA was quantified and used for PCR amplification.
Results: The extraction success was tested by targeted PCR amplification using primers that produced amplicons 90 to 260 bp in length and targeted genes relevant for inherited eye studies (progressive rod-cone degeneration-*PRCD*; rhodopsin-*RHO*; glucuronidase beta-*GUSB1*), plus an additional control gene receptor accessory protein 1 (*REEP1*). All but one of the epoxy-embedded eye samples were successfully amplified. Sanger sequencing confirmed the gene identity of amplified products.
Conclusions: By identifying methods to extract DNA from osmicated epoxy-embedded mammalian eye tissues, our results provide a valuable resource for determining the genetic basis of inherited diseases and for retroactively confirming molecular diagnoses based on microscopic analysis.

Acquisition of rare samples can be challenging for researchers, and the need for archival samples for diagnostic and genomic research may arise during the course of a study. While bioarchives with large collections of samples exist, the preservation techniques used and the lack of specific protocols to extract DNA from archived samples used for histopathologic studies often prevent their usage. Thus, although they are well characterized clinically and at the cellular level, these samples often are of an unknown molecular background and remain unused.

Biologic sample preservation for zoological, anatomic, forensic, and pathological purposes has been used since the 17th century [1]. The media used for preservation vary but include storage in ethanol [2], formalin fixation [3], paraffin [4] or epoxy-resin embedding, and cryopreservation, among others. Cryopreserving tissues is a relatively new way of preserving samples, and depending on the sample state before it was frozen—specifically, how fresh they were and how quickly they were frozen—DNA extraction from these specimens can easily be accomplished using standard DNA isolation kits designed for this purpose. However, cryopreservation requires specialized liquid nitrogen–filled vats or ultra-low-temperature freezers, which are costly to maintain, take up space, and fill fast.

Formalin tissue fixation is a much older preservation technique that was introduced as a fixative for biological samples in the 19th century [3]. Samples that have been fixed in this way can be stored in formalin-filled jars at room temperature or further embedded in paraffin blocks, which can then be used for sectioning and morphological analysis under the microscope. In the 1990s, protocols were developed to successfully amplify DNA from paraffin-embedded specimens [4], and today, the analysis of DNA from either wet formalin-fixed tissue or formalin-fixed, paraffin-embedded soft tissue samples is routine [5] by using commercially available kits for manual and automatic extraction of nucleic acids from the samples [6].

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In contrast to cryopreserved or formalin-fixed specimens, samples embedded in epoxy resins, as first described by Newman et al. [7], such as Epon 812, have been less amenable to molecular analysis for morphologic light or electron microscopy (EM) studies [8], despite their use in these studies [9-11]. Epoxy-embedded specimens are small and resilient, and they can be stored at room temperature without too much concern for temperature fluctuations, as the plastic embedding will not melt or crack unless under severe conditions. However, preserving samples for EM has a significant impact on DNA, as the tissue is fixed with harsh chemicals, such as glutaraldehyde and osmium tetroxide in sodium cacodylate or phosphate buffers. The samples are then dehydrated with an ascending series of alcohol solutions, followed by propylene oxide, before being fully infiltrated with the epoxy media [12].

Araldite and Epon epoxy resin (an aliphatic resin mixture of di- and triglycidyl ethers of glycerol) are standard embedding media that are still used today (now Araldite 502 and Epon 828) to preserve intracellular structures for EM analysis [12,13]. However, replacements for the now discontinued Epon 812, such as Poly/Bed 812 (also a polyglycidyl ether of a glycerol mixture), were developed to bypass some of the harsh chemicals used for tissue dehydration, which are used to reduce the loss of phospholipids and eliminate the epoxide anhydride to epoxide ratio variability seen with Epon 812 (thereby providing greater control in resin consistency from block to block) [14]. As a result, extracting DNA from tissue samples preserved in epoxy for EM has been challenging [9-11]. The aim of this study was to develop a technique for obtaining amplifiable DNA from samples embedded in Araldite, Epon 812, or Poly/Bed 812. For this study, we used glutaraldehyde and osmium fixed eye samples from two different mammalian species, with a focus on retinal and corneal samples. The goal was to develop a protocol that could be used to amplify short fragments from this extracted DNA, considering its inevitable fragmentation. Here, we demonstrate not only that DNA extraction from archival retinal and corneal tissues embedded in osmicated Araldite, Epon 812, or Poly/Bed 812 is possible but also that the DNA can be amplified and sequenced to confirm and detect the presence of disease-associated variants in the archival samples.

METHODS

Sample selection: Nine epoxy resin blocks containing archival mammalian Araldite, Epon 812, or Poly/Bed 812 embedded retinal or corneal samples of two different species, dog and nonhuman primate (rhesus macaque), were used for the DNA extraction. Plastic blocks with visible and intact tissues were selected. All blocks selected were ~1.5 cm in length, ~1.2 cm in width, and ~0.5 cm in height, with embedded tissues ranging from a few millimeters to ~1 cm in length, a few millimeters to 0.5 cm in width, and ~10 mm in height. Species, year of fixation, type of fixation, and embedding type are detailed in Table 1. In addition, for comparison, a cryogenically preserved archival blood sample from a dog collected in 1994 and buccal swabs collected in early 2024 from a cat were also selected for DNA extraction using standard established protocols.

DNA isolation from epoxy resin blocks—overview: Kits, buffers, and reagents and details of the protocol with pictures are in Appendix 1—Methods Protocol. Work was performed in a fume hood.

The embedded tissue was released by smashing and fragmentation, then sorted by prioritizing parts with less plastic. Epoxy Resin Removal Solution (Warrington, PA) was added, and the sample was briefly mixed; the fragments were kept in the digestion solution overnight at room temperature to expose the tissue. Digestion with Proteinase K was performed overnight, and after the incubation, fragmentation in Trizol with zirconium beads was performed. The sample was then incubated at room temperature, cold chloroform was added, and the mixture was vortexed. Centrifugation resulted in a lower pink-like organic phase and an upper clear phase that was discarded; 95% EtOH was added to the remaining organic phase, and the sample was mixed and incubated at room temperature. The precipitated DNA was resuspended in buffer and eluted through a DNA column. While using columns gave a cleaner DNA, in general, the DNA had a high phenol contamination content; this was resolved by using water-saturated ether. Since both the sample and the water-saturated ether are clear and hard to distinguish from one another, DNA loading dye was mixed with the extracted DNA sample before adding equal volumes of water-saturated ether. The less dense ether, including contaminants in the upper phase, was removed with a pipette and discarded, leaving the colored cleaned DNA behind. Next, the DNA sample was passed through the mini-genomic DNA extraction column once more to remove any remaining traces of ether and dye.

The concentration of genomic DNA was measured using NanoDrop (Thermo Fisher Scientific, Wilmington, DE), repeating the cleanup if needed (Table 2). A subset of samples was then submitted to a chip-based bioanalyzer to determine the extracted DNA fragment size and another subset to Qubit (Thermo Fisher Scientific) to assess the concentration of extracted double-stranded DNA (dsDNA).

DNA isolation from control samples: DNA extraction from one cryo-archived canine blood sample collected in 1994

Table 1. The samples were selected to represent an overall diverse array of fixation protocols, but all shared a variation of glutaraldehyde primary fixation, osmium tetroxide secondary and/or tertiary fixation and Araldite, EPON or Poly/Bed embedding.

Sample	Species	Tissue	Year fixated	Primary Fixation	Secondary fixation	Tertiary fixation	Infiltration/ Embedding
CLF1	Canine	Retina	1993	Glutaraldehyde, Formaldehyde, Sodium Cacodylate	Glutaraldehyde, Osmium Tetroxide in Sodium Cacodylate	Osmium Tetroxide in Sodium Cacodylate	EPON 812
CLF2	Canine	Retina	1996	Glutaraldehyde, Formaldehyde, Sodium Cacodylate	Glutaraldehyde, Osmium Tetroxide in Sodium Cacodylate	Osmium Tetroxide in Sodium Cacodylate	Poly/Bed 812
CLF3	Canine	Retina	1981	Glutaraldehyde, Sodium Cacodylate	Veronal acetate buffered Osmium Tetroxide	n/a	EPON 812
CLF4	Canine	Cornea	1986	Glutaraldehyde, Formaldehyde, Sodium Cacodylate	Glutaraldehyde, Osmium Tetroxide in Sodium Cacodylate	Osmium Tetroxide in Sodium Cacodylate	Poly/Bed 812
CLF5	Canine	Retina, Cornea	1985	Glutaraldehyde, Sodium Cacodylate	Osmium Tetroxide in Sodium Cacodylate	n/a	Poly/Bed 812
MR1	Non-human primate	Retina	1976	Glutaraldehyde, Sodium Cacodylate	Veronal acetate buffered Osmium Tetroxide	n/a	Araldite
MR2	Non-human primate	Retina	1976	Glutaraldehyde, Sodium Cacodylate	Veronal acetate buffered Osmium Tetroxide	n/a	Araldite
MR3	Non-human primate	Retina	1976	Glutaraldehyde, Sodium Cacodylate	Veronal acetate buffered Osmium Tetroxide	n/a	Araldite

Fixation and embedding were performed immediately after eye collection, following established protocols [20,21].

and two fresh feline buccal swabs collected in 2024 were performed using the Illustra DNA extraction kit BACC2 (GE Healthcare, Chicago, IL) and Puragene Buccal Cell Core Kit A (Qiagen, Venlo, Netherlands; operational HQ: Hilden, Germany), respectively, according to the manufacturer's instructions. The extracted DNA was diluted in the same elution buffer used to resuspend the DNA extracted from epoxy blocks.

Sample amplification and Sanger sequencing: After genomic DNA measurement using the NanoDrop, the quality of extracted DNA was further assessed by PCR amplification and subsequent gel electrophoresis. Genes relevant to vision research and/or expressed in the retina were targeted for analysis: rhodopsin-*RHO*; adenosine monophosphate deaminase 2-*AMPD2*; retinitis pigmentosa GTPase regulator-*RPGR*; glucuronidase beta-*GUSB1*; receptor accessory protein 1-*REEP1*; progressive rod-cone degeneration-*PRCD.* All the primers were designed with Primer 3 [15]. Targeted and aligned intronic mammalian regions are shown in Appendix 2. A primer list is detailed in Appendix 3.

A subgroup of primers (underscored in Appendix 3) was designed after verifying species specificity with BLAT as follows: first, a 500- to 1,000-bp intronic interval was selected from an annotated gene using the Integrative Genome Viewer. The interval was then run through BLAT (accessed February 2, 2024) against the other mammalian species described in Table 1, as well as against cat and human, as follows: rhesus against human, dog against cat, and all against dog. The primer pairs selected were then deemed acceptable only if at least one member of the pair fell within an interval with no matching alignments in BLAT with these other mammalian species. This would ensure that one cross-species matching primer would not have a paired match also able to hybridize cross-species, leading to the amplification of a contaminating fragment targeted at a different mammal. The reference alignments selected in BLAT were Canfam4 for dog, GRCh38 for human, felCat9 for cat, and rheMac10 for rhesus macaque. Primers selected in this way were marked as "exclusive" in the file.

For the PCR reaction, ATG360 MM polymerase (Applied Biosystems, Thermo Fisher Scientific) was used, and all primer pairs were diluted with DEPC-treated water (Ambion,

Table 2. Pre-and post-cleaning yield of the samples and the feline (Cat1 and Cat2) and canine blood (Cryo. Dog) DNA extraction (which had no cleanup).

Sample	Tissue	Pre-cleaning			Post- cleaning		
		Nucleic acid (ng/µl)	A260/A280	A260/A230	Nucleic acid (ng/µl)	A260/A280	A260/A230
CLF1	Retina	213.07 µl	1.480	1.150	16.12 µl	2.010	0.466
CLF2	Retina	87.97 µl	1.509	1.195	14.93 µl	1.923	0.435
CLF3	Retina	459.21 µl	1.721	1.25	13.29 µl	1.820	0.504
CLF4	Cornea	78.79 µl	1.392	1.288	13.23 µl	2.22	0.591
CLF5	Retina	130.16 µl	1.466	1.255	17.81 µl	1.961	0.503
	Cornea	76.79 µl	1.378	1.113	8.75 µl	2.188	0.590
MR1	Retina	97.76 µl	1.377	0.928	14.04 µl	1.988	0.400
MR2	Retina	186.82 µl	1.438	1.107	13.07 µl	1.832	0.601
MR3	Retina	61.05 µl	1.331	0.710	6.80 µl	1.454	0.579
Cat1	Buccal	113.03 µl	1.919	1.193	n/a	n/a	n/a
Cat2	Buccal	75.25 µl	1.850	1.181	n/a	n/a	n/a
Cryo. Dog	Blood	606.99 µl	1.934	2.152	n/a	n/a	n/a

Note the substantial yield loss after cleaning.

Thermo Fisher Scientific) to 10 µM each before use. The PCR conditions were 95 °C for 10 min; denaturation, 95 °C for 30 s; annealing, 58 °C for 30 s; and extension, 72 °C for 1 min), yielding results from 40 to 46 cycles (72 °C for 7 min, 4 °C indefinitely). Each species-specific PCR reaction was run separately in a thermal cycler that was sterilized before use to avoid amplicon contamination. Gel electrophoresis was done on a 1.7% agarose gel stained with EtBr (Apex Bioresearch Products, San Diego, CA). A 100-bp DNA ladder from GoldBio (Gold Biotechnology Inc., St. Louis, MO) was used as a marker. Bands of the correct amplicon size were enzymatically cleaned using ExoSAP-IT (Applied Biosystems) and were Sanger sequenced.

Microscopy: Archival corneal and retina epoxy sections obtained from CLF1, CLF4, and MR1 tissue blocks used for DNA extraction were examined and photographed with Zeiss Universal or Axioplan microscopes (Carl Zeiss AG, Oberkochen, Germany) under bright-field illumination. Sections were prepared and mounted between 1976 and 1993. The 1-µm sections (Figure 1) were stained with paraphenylenediamine (Figure 1A,C,D) or azure II/methylene blue (Figure 1B).

RESULTS AND DISCUSSION

Corneal and retinal preservation of the archival osmicated and epoxy-embedded tissue: The tissues showed excellent structural preservation. The MPSVII-affected canine cornea (from CLF4) had keratocytes and endothelial cells that were distended and accumulated vacuolated inclusions, representing the glycosaminoglycans that accumulate secondary to lysosomal beta-glucuronidase deficiency [16] (Figure 1A). The *prcd*-affected canine retina (from CLF1) showed the characteristic inner and outer segment disorganization, as well as loss of some outer nuclear layer nuclei characteristic of stage 2 disease [17] (Figure 1B). Similarly, the normal nonhuman primate (Figure 1C,D) showed excellent tissue preservation after fixation in glutaraldehyde and osmium, as well as embedding in Araldite.

DNA yield in pre- and post ether cleaning samples: Phenol can act as a PCR inhibitor, affecting primer binding and proper polymerase action [18], and needs to be removed from the extracted DNA sample. To determine if the ether-based cleaning step removed phenol and had any impact on DNA yield, NanoDrop measurements were taken before and after the procedure. A comparison of A260/280 and A260/A230 ratios indicated that samples had significant protein and phenol contamination before cleaning, which was eliminated following the cleaning procedure. However, a comparison of DNA yields (Table 2) before and after phenol cleaning indicated a large loss of DNA after cleanup. This might be due to ether not only removing phenol but also possibly trapping more DNA than expected due to its fragmented status, which was probably picked up by the microvolume spectrometer. The spectrometer measures the total amount of DNA present, not distinguishing between fragmented and nonfragmented. Thus, it is possible that the NanoDrop measurement before the cleanup reflects just that. Phenol cleanup using phase separator tubes and standard DNA cleanup columns was not successful.

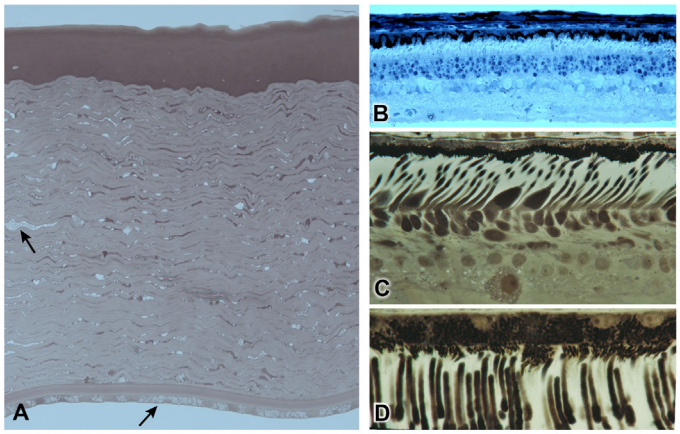

Figure 1. Structural appearance of the archival tissue sections from which the plastic-embedded tissue blocks were used for DNA extraction. Sections were stained with paraphenylenediamine (PPD; **A**, **C**, **D**) or azure II/methylene blue (**B**). **A**: A corneal section from dog CLF4 affected with mucopolysaccharidosis VII shows the typical vacuolated inclusions in the stromal keratocytes and corneal endothelial cells [16]. **B**: A mid-peripheral retinal section from *prcd*-affected dog CLF1 shows disorganization of the photoreceptor layer and ~50% reduction in outer nuclear layer thickness [17]. **C**, **D**: Panels show images of the far peripheral (**C**) retina of normal nonhuman primate MR1 and the photoreceptor outer segment–retinal pigment epithelial interface of the mid-peripheral retina (D).

A few randomly selected post-cleanup samples were picked for dsDNA quantification using Qubit. The Qubit results do show a presence of dsDNA in the extracted samples (Appendix 4). The yield results are comparable to the lower-concentration subset of the results obtained from a study extracting DNA from epoxy-embedded archival bone samples [11]; however, in that study, total DNA, not dsDNA, was measured using Qubit.

A chip-based bioanalyzer was used to further validate the DNA quality after the cleaning step and the fragment distribution (Appendix 4). The overall bioanalyzer results showed that indeed, the epoxy resin DNA is highly fragmented. However, this fragmentation is consistent with studies on extracting DNA from archival wet and dry museum specimens [19], suggesting that no matter the preservation type, DNA from archival samples similarly experiences a high degree of fragmentation and degradation.

DNA amplification and Sanger sequencing: Of the nine DNA samples extracted from epoxy-embedded retina and corneal tissue samples, eight could be amplified by PCR, with MR3, one of the three rhesus macaque samples preserved in 1976 and embedded in Araldite, consistently failing amplification. The two other rhesus samples similarly processed at the same time that year yielded appropriately sized PCR fragments that were validated by sequencing.

Of 15 attempted PCR amplification trials, 12 were successful. No amplification above a predicted amplicon size of 400 bp was attempted. Forty to 46 PCR cycles were successfully used in the amplification of DNA. The thermal cyclers used were sanitized before each amplification, and nontemplate controls were included with each PCR amplification to aid in detecting any contaminants that could have been introduced through the PCR reaction reagents or the thermocycler itself. Subsequent gel electrophoresis of the PCR reactions revealed a single clear band of the correct size for each sample, and no bands, indicating no contaminants, in the nontemplate controls. Regardless of the number of PCR cycles, no unspecific bands were detected.

RHO amplification was used as a standard for all extractions, as it is highly conserved among species. Thus, to ensure that the primers were species specific, canine epoxy samples were subject to PCR amplification with feline-specific *RHO* primers and vice versa. Negative results would also ensure that there was no contamination with DNA of other species in the extract (e.g., human DNA in the rhesus extract). Results are reported in Figure 2.

Since most of the epoxy-embedded samples were canine, DNA from a cryopreserved canine blood sample from 1994 was also extracted for comparison. At the time the blood was collected, it was mixed in a vial with EDTA solution (10% potassium EDTA in distilled water), with 1.5 to 1.8 mg of EDTA solution used per 1 ml of blood. Although the blood sample itself was old, it appeared in good condition after thawing and was not clotted. DNA extraction using a standard kit was performed, and no phenol contamination was detected in the DNA sample. The DNA yield from this blood sample was also significantly higher compared to what was extracted from Epon- or Poly/Bed-embedded tissue samples from the

same species, despite the CLF2 sample being embedded in Poly/Bed and having been obtained more recently.

We conclude that the described method allows for the extraction of amplifiable DNA from archival retina and cornea samples embedded in glutaraldehyde/osmium-fixed Araldite, Epon, or Poly/Bed. We validated this method using samples of varying archival ages and from different species, and confirmed the PCR amplification of target fragments by Sanger sequencing. Notably, little difference was noted when extracting DNA from samples embedded in Araldite, Epon, or Poly/Bed. However, the age of the sample appears to have an impact on the yield, as samples preserved for the longest time had smaller yields and more degraded DNA. A larger number of samples should be extracted to see if the time of embedding has a statistically significant impact.

Currently available protocols: Attempts at extracting DNA from tissue samples preserved in epoxy for EM were previously documented in 1970, 1990, and 2022 [9-11]. The earliest attempt to extract DNA from salivary glands of *Drosophila melanogaster* larvae samples preserved in an Epon-Araldite epoxy was reported by Douglas in 1970 [9]. In 1990, Grünewald and colleagues isolated DNA from Technovit 700–embedded human bone and bone marrow tissue, showing that it is suitable for PCR amplification [10]. However, in both studies, the samples themselves had only been embedded in plastic for at most a few years. Nonetheless, in 2022, DNA was recovered from bone archival samples embedded in Biodur epoxy resin, an Epon substitute [11].

To the authors' knowledge, there are no studies outlining the extraction of DNA from old archival osmicated soft tissue, especially of the eye, preserved in Epon or related epoxy resins. The authors acknowledge the limits of the methods. As the DNA is often highly fragmented and degraded, designing primers to create smaller-sized amplicons is desirable. Here, successful amplification of up to 260 bp was possible, which is suitable for amplicons aimed at a small number of known specific genetic variants or candidate genes (used, for example, for later diagnosis confirmation). There is variability in DNA quality and yield due to the age and quality of sample preservation, as well as the mechanical nature of the extraction process. So far, the application of this protocol to high-throughput sequencing would not be possible but could still be explored with better-preserved samples if these are able to support larger amplicons. Additionally, DNA cleanup steps must be optimized to improve the yield.

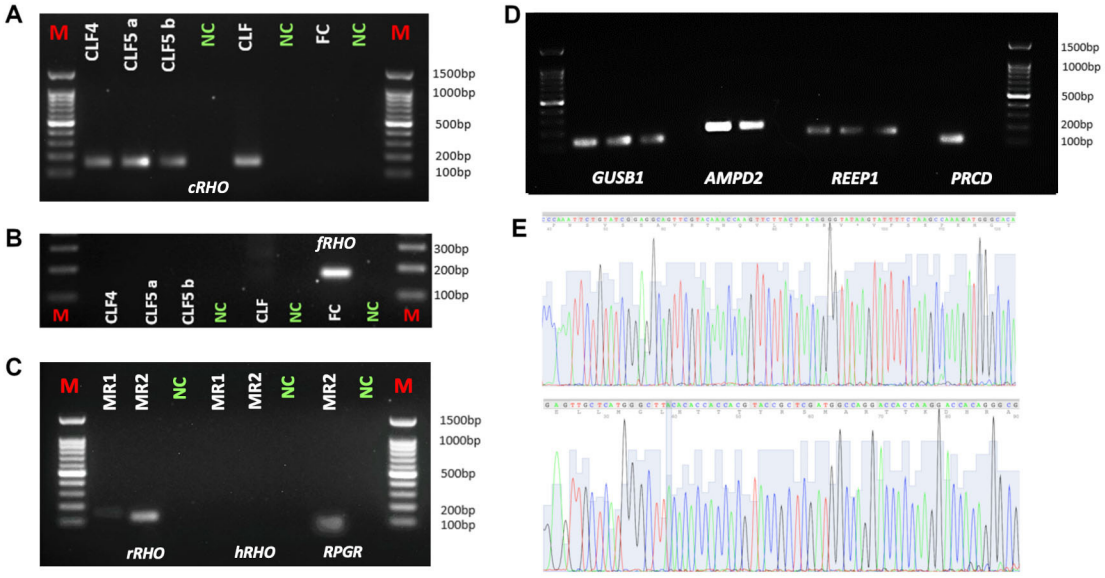

Figure 2. Targeted PCR amplification using DNA extracted from archival and fresh DNA samples is shown for comparison. Targeted PCR amplification of canine (**A**) and feline (**B**) *RHO* was performed. **C**: Targeted PCR amplification was carried out for rhesus *RHO* (*rRHO*) and RPGR, as well as human *RHO* (*hRHO*), using DNA extracted from archival epoxy resin-embedded rhesus samples (MR1–MR2). M-100 denotes the marker. **D**: Targeted PCR amplification of canine *GUSB1, AMPD2, REEP1*, and PRCD was performed using DNA extracted from archival epoxy resin-embedded canine samples (CLF1–CLF5). CLF4 represents canine cornea DNA (epoxy resin extraction). CLF5 represents canine DNA from epoxy resin extraction of (a) cornea and (b) retina. CLF, canine DNA from blood; FC, feline DNA from buccal swab; NC, non-template control. Note that the PCR aimed at non-species-specific targets for the primers failed. **E**: The electropherogram shows reads of PCR amplifications (RHO) from DNA extractions from dog cornea (top) and rhesus macaque retina (bottom).

Conclusions: As this method yielded amplicons, albeit small, for samples embedded in epoxy for different lengths of time, it will permit the usage of archival epoxy-embedded samples in forensic, diagnostic, and genomic applications. This technique will be especially useful for identifying disease-causing genes/mutations in well-characterized clinical diseases from which archival tissues exist from the heyday of the electron microscopy era.

APPENDIX 1. EXTENDED PROTOCOL

To access the data, click or select the words "Appendix 1."

APPENDIX 2. INTRONIC MAMMALIAN REGIONS ALIGNMENT

To access the data, click or select the words "Appendix 2."

APPENDIX 3. PRIMER LIST

To access the data, click or select the words "Appendix 3."

APPENDIX 4. BIOANALYZER AND QUBIT RESULTS

To access the data, click or select the words "Appendix 4."

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