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SIMPLE TISSUE PRESERVATION METHODS THAT RESULT IN RELIABLE DNA ANALYSES

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Corinne Lindsey Michaud

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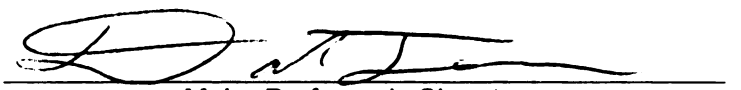

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SIMPLE TISSUE PRESERVATION METHODS THAT RESULT IN RELIABLE DNA ANALYSES

By

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ABSTRACT

SIMPLE TISSUE PRESERVATION METHODS THAT RESULT IN RELIABLE DNA ANALYSES

By

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In the event of a mass disaster, a large number of victims must be located and identified, and it is difficult to process the site in a timely and orderly manner. Many victims may only be identified through DNA analysis; therefore, obtaining viable tissue samples is of great importance. Likewise, tissue preservation of remains discovered in very remote areas can also be hindered. The goal of this study was to examine protocols for on-site preservation of tissues that would later undergo DNA analysis. Tissue samples were taken over the course of one week from recently killed pig carcasses that had been placed in a field during the summer. Six preservation methods were evaluated: storage of tissue in ethanol, isopropanol, RNA*later*, and silica, as well as oven drying (70°C) and freezer storage (-20°C). Muscle and skin samples were collected and preserved in 0.500g sections. DNA extractions were performed after two weeks and two months of storage, and DNA quality and quantity were assessed using a series of swine specific PCR assays. Samples from each of the methods evaluated were able to amplify nuclear DNA fragments of up to 642bp. Tissue type and level of decomposition significantly affected DNA quality. Ethanol and RNA*later* were shown to preserve DNA of the highest quality, though differences among preservation methods were not significant. Field practicality was considered in conjunction with the DNA evaluations, and the results of this study indicated that each technique could be implemented in the field and used to preserve DNA of a quality amenable to forensic analyses.

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INTRODUCTION

DNA as an investigative tool

DNA analysis has become an invaluable tool in the area of identification of human remains. Whether the case in question concerns a single missing person or a large-scale disaster, the ability to positively identify an individual's remains is an important service that forensic sciences are able to provide. The power of a DNA profile to identify a single individual makes DNA one of the most influential and unassailable types of biological evidence. Due to the accuracy and specificity of this type of testing, DNA analysis is regularly used as a tool in paternity cases, missing person's cases, rapes, murders, and virtually every other type of crime. Some cases, such as those involving paternity disputes or a recently murdered victim, are highly amenable to DNA profiling, when the sampled material is abundant and may be quickly analyzed.

Advances in technology have also allowed for very small amounts of biological material to result in successful DNA analysis. Samples such as a single drop of dried blood, a hair, or even the residue from a fingerprint have been used to generate DNA profiles. In a recent review of casework involving DNA analysis from hair, Melton et al. (2005) reported that full mitochondrial DNA (mtDNA) profiles were obtained from 570 out of 691 hairs. Samples ranging from head hair, pubic hair, and axillary hair have all been shown to provide enough genetic material for mtDNA sequencing (Pfeiffer et al., 1999). A quantification study by Andréasson et al. (2006) revealed that thousands of copies of nuclear DNA could be extracted from body hairs, fingerprints, and personal

jewelry items. These types of samples may be useful in some cases; however, there is a great amount of variability in the success of DNA typing using such limited or decayed samples. Even in instances where biological samples are relatively fresh or plentiful, DNA analysis is not always successful.

As recent tragedies such as 9/11 and Hurricane Katrina have illustrated, the sheer enormity of the task at hand may delay the identification process. The number of victims from the World Trade Center site stands at two-thousand seven hundred and forty-nine. As of September 11, 2005, positive identifications of 1594 victims had been reported, approximately 850 of which were based solely on DNA analysis (Biesecker et al., 2005). Even with advancements in forensic DNA analyses since 2001, such as the development of mini-STR analyses for degraded samples (Butler, et al., 2003; Coble and Butler, 2005), this means that 1155 of the World Trade Center victims still have not been identified. In April of 2005, New York City's Office of the Chief Medical Examiner announced that the identification efforts would be put on hold indefinitely (Lipton, 2005). These events demonstrated the great effort and cooperation that is necessary on the part of the government, scientists, the public, and various other agencies after a disaster of such magnitude. Unfortunately, they also underscored the need for improvements to the system, in the interest of providing timely and accurate identifications for the families and communities involved.

Obtaining DNA samples in the event of a disaster

From the case of a single unidentified body, to the scene of a plane crash or natural disaster, the forensic goal is the same: to efficiently and accurately process the scene and provide positive identifications of the persons involved. In order to achieve this goal, individuals from many different disciplines must work together to locate, acquire, and process samples from victims. Professionals from pathology, anthropology, and law enforcement are typically present at a scene, in addition to any special agencies necessary for a particular situation. Due to the diversity in the training backgrounds of disaster responders, the procedures that are followed at different locations vary greatly. Some agencies, such as the National Association of Medical Examiners (NAME) recommend cold storage for any remains that are found (NAME, 2005). In a recent special report on mass fatality incidents, the National Institute of Justice (NIJ) made the following statement concerning DNA sample collection: "Collect, place, and appropriately store samples of suitable size in separately labeled containers ... Store samples without preservatives (e.g., formaldehyde)." (NIJ, 2005). Secured freezer storage is mentioned as an aside, but not specifically included in the recommended protocol. The Federal Bureau of Investigation (FBI) also recommends that tissue samples be preserved only by freezer storage (FBI Forensic Services Handbook, 2003). The Interpol Handbook on DNA Data Exchange and Practice details the collection procedure with regards to sterile techniques and storage vessels, but does not outline any recommendations for subsequent storage of the samples (INTERPOL, 2001). Clearly,

consulting a variety of agencies' manuals for disaster response will yield an array of protocols for sample collection and processing.

Disaster response manuals are useful tools, but in reality a case must be managed according to the situation at hand. Biological samples are obtained and preserved to the best ability and knowledge of the responding team, and the procedures must be flexible to meet the demand of a particular event. Depending on the type of tissue available, the level of decomposition, and the preservation method (if any) used, subsequent DNA analysis may or may not be successful. Delays between the acquisition of a sample and the decision to attempt DNA analysis are also cause for concern, since the DNA will continue to degrade. Although these considerations become integral to cases that hinge on DNA results, preservation for potential DNA analysis is not the primary focus of a disaster response team. For example, the NAME disaster response manual places sample collection for subsequent DNA analysis as the tenth station in the progression through the morgue, after radiology, photography, anthropological examinations, and other administrative procedures have been completed (NAME, 2005).

As an investigation proceeds, unidentified remains pass through many stages of cataloguing and examinations. With large-scale disasters, this is normally done in temporary morgue facilities that are set up as close to the scene as possible. According to an NIJ flow chart, a set of remains passes through triage, admitting, radiology, photography, and cataloguing of personal effects before any anthropological or biological examinations are conducted (NIJ, 2005). This typical progression can be shortened at the discretion of scene supervisors, e.g., if it is apparent that a sample will not be useful for anthropological or other visual examinations (NAME, 2005). If a case involves a limited

number of victims, the remains are brought to the nearest morgue before any examinations are begun. When a positive identification can be made based on dental records or an anthropological examination, further decay of the remains is not an issue of concern. However, in situations where extensive injuries or decay impede a visual or anthropological identification, DNA analysis becomes the main tool of the investigation. If samples are not adequately preserved upon discovery of the remains, the potential for successful DNA analysis is reduced by the lag time during the initial examinations.

Preservation of tissue for DNA analysis

An effective tissue preservation method must have several attributes. The foremost concern is how effectively DNA is protected. Basic considerations affecting this include the pH of a solution, the temperature and moisture level of the storage conditions, and the ability to retard microbial activity. Once a methodology that is amenable to DNA protection is developed, the issue of storage time must be addressed. Will an extraction performed after one year of tissue storage yield the same quality and quantity of DNA as an extraction performed after two weeks of storage? If a preservation method is successful in maintaining a long-term, high quality sample of DNA, then various secondary factors can be taken into account, including portability of materials, toxicity of materials, ease of use in the field, and ease of subsequent DNA extractions.

Current preservation techniques can be classified into three broad categories: cold storage methods, desiccation methods, and storage in solution. Each of these routes has

advantages and disadvantages, depending on factors such as resources, equipment, and the future analyses that may need to be performed. When a technique is dependent on cooling tissue, the necessary electricity and equipment may present problems in remote locations or areas that have been flooded or lost power. Storing tissue in some type of solution may require that a large quantity of preservative be kept on hand; since manual preparation or commercial purchase would use valuable time in the event of a disaster. Such considerations are integral when implementing a preservation method in the field, however the first test of a technique's utility is how effectively it preserves tissue.

Cold storage techniques are widely accepted preservation methods, with storage at -20°C (a standard freezer) being one of the most commonly used and easily employed. This method is effective because degradative processes are slowed or functionally stopped by the low temperatures. Freezing limits the amount of chemical or physical modification that tissue is subjected to, and subsequent DNA extractions do not require any special considerations, the tissue can simply be thawed and processed. Freezer storage is also the main technique currently employed in the event of a mass disaster, as noted in the FBI and NIJ disaster response manuals (FBI Forensic Sciences Handbook, 2003; NIJ 2005).

Storage in liquid nitrogen (-196°C) is another temperature-based technique, common in medical and biological research. The long-term benefits of liquid nitrogen storage are similar to storage at -20°C, since degradation is prevented by the extremely low temperature. Liquid nitrogen is the industry standard for many medical research procedures, such as preserving tumor samples (Matsuo et al., 1999; Mutter et al., 2004). Although liquid nitrogen is a routine preservation method, the regulations and dangers of

transporting and maintaining liquid nitrogen tanks make this method a less than ideal choice for field applications. Likewise, tissues could be stored on dry ice (-78.5 °C) but this requires obtaining and transporting the rather bulky material to the scene.

Desiccation represents another common method of tissue preservation. Oven-drying is one such technique that exposes tissue to a hot dry environment, causing rapid desiccation. DNA preservation is aided by heat-killing potentially harmful bacteria as well as by drying out the sample and preventing natural cellular mechanisms from degrading the DNA. Oven-drying is a common practice in plant research as well as the food industry. Akpinar (2006) evaluated a range of air drying temperatures for the preservation of apple, pumpkin, and potato slices; and found that over 70% of the moisture was removed from the samples after oven-drying between 60 – 80°C for a period of 8 – 14h. Although this study was in the interest of food preservation, it shows the efficacy of oven-drying as a desiccation technique.

Storage in silica mesh also results in rapid moisture removal and preservation. Silica is a well-known desiccant, and has been included in numerous studies involving field preservation of potential DNA samples. Silica storage was used to preserve hair samples in the field during a 1997 study of carnivore populations in the western United States (Foran et al., 1997). The researchers were successful at amplifying mtDNA fragments of up to 600bp and nuclear DNA fragments of up to 440bp using the silica preserved hair samples. Studies involving DNA analysis from fecal samples have also utilized silica storage successfully. Roeder et al. (2004) compared the DNA yield and quality from three fecal sample preservation methods: 90% ethanol storage, silica storage, and a two-step ethanol and silica preservation technique. Results of their study

showed that the quality of DNA obtained through each method was comparable when DNA yields were low. When fecal samples contained a high amount of DNA, the silica storage was not as effective at preserving high quality DNA as the ethanol or two-step techniques, as measured by the successful amplification of a 757bp fragment. A more comprehensive study by Murphy et al. (2002) evaluated the performance of 90% ethanol storage, DETs buffer storage (a solution composed of dimethyl sulfoxide (DMSO), EDTA, and Tris), silica storage, and two oven-drying methods. Results showed that storage time significantly affected DNA yields from silica preserved samples, and also that silica storage yielded a lower quality of nuclear DNA than the other methods. Silica storage does effectively remove moisture from samples however, is safe and inert, inexpensive, and is easily transported and utilized in the field, giving it several positive attributes as a preservation technique.

Storage of tissue in solutions is a third preservation option; alcohols and formaldehyde are two common preservatives that fall under this category. The dehydrating and sterilizing properties of alcohols and aldehydes are beneficial to tissue preservation, by drawing the water out of a sample and killing off bacteria. Although such organic solvents are effective tissue preservatives, their usefulness for DNA preservation varies. Alcohol storage fixes tissue samples without cross-linking DNA, which is useful for future nucleic acid extractions. Aldehydes, including formaldehyde, permeate tissue and cause chemical changes in proteins and nucleic acids, making future DNA extractions both cumbersome and potentially unsuccessful (Srinivasan, 2002).

Fixation in alcohol has repeatedly been demonstrated to perform superiorly to aldehydes and comparably to freezing techniques with regards to DNA quality. In a

study using preserved human prostate samples, Gillespie et al. (2002) found that DNA from formalin-fixed tissues was highly degraded and achieved limited success with PCR experiments. Ethanol preserved samples from the same study consistently exhibited DNA yields of high molecular weight and had high success rates for amplification of microsatellite markers. Data from an assay of storage time periods by Kilpatrick (2002) also showed ethanol to be an effective long-term preservation method. The author analyzed relative DNA yields from murine livers that had been stored for up to two years in various preservatives. After this time the ethanol stored samples exhibited high molecular weight DNA when assayed on a yield gel, and a 400bp polymerase chain reaction (PCR) product could be amplified. These properties, combined with the fact that alcohols are inexpensive and easily obtained, make the two most safe and readily available, ethanol and isopropanol, attractive candidates for tissue preservation.

Buffered saline solutions represent another widely used liquid storage method. Various compositions of salts, chelating agents, buffering agents, and organic solvents have been documented as successful tissue and DNA preservatives. A study by Seutin et al. (1991) was one of the first evaluations of a DMSO-salt solution as a preservative, and found that a solution of 20% DMSO, 0.25M EDTA, and saturated NaCl preserved tissue samples as effectively as liquid nitrogen storage. In the comparison of ethanol, DMSO-salt solution, and lysis buffer (2M Tris-HCl, 0.5M EDTA, 5M NaCl, 20% SDS, pH 8.0) preservation by Kilpatrick (2002), it was shown that DNA quality and quantity was consistently greatest in the DMSO-salt solution over a time period of two years. DETs buffer (containing DMSO, EDTA, and Tris), another DMSO-salt solution, was used to preserve DNA from fecal samples by Murphy et al. (2002), and was shown to perform

comparatively to ethanol. Amplification of nuclear loci using PCR was successful in 75% of the DETs stored samples, as compared to 81% of the ethanol stored samples and 43% of the silica stored samples. All of these solutions have been shown to effectively preserve tissue and DNA samples, but perfecting the concentrations of the components and preparing the solutions can be time-consuming. Further, DMSO is slightly toxic.

A commercially prepared tissue preservative RNA*later* (Ambion, Austin, TX) is also available. RNA*later* is an aqueous sulfate salt solution that rapidly fixes fresh tissue and does not require refrigeration of the samples (Ambion). A recent study showed that storage in RNA*later* yielded RNA of a quality and quantity comparable to traditional fresh or flash-frozen tissue samples (Mutter, 2004). The commercial availability of RNA*later* has the potential to make it a convenient preservation method that is easily adapted to use in the field, since the solution does not need to be manually prepared. However this can also be considered a drawback, since it is only possible to obtain RNA*later* directly from the manufacturer and a stock would need to be maintained in the event of a disaster.

Practical applications of this study

The goal of this study was to evaluate simple, rapid methods for preserving tissue samples from unidentified remains, in the interest of aiding future DNA analysis. Based on a combination of efficacy and practicality, six preservation methods were chosen: storage in ethanol, isopropanol, and RNA*later* solutions, silica mesh, -20°C storage, and 70°C oven-drying. Tissue samples were obtained over the course of one week from three

pigs that were placed in a field and allowed to decompose. Samples were then preserved by the six different methods, and the relative success of each was evaluated by comparing subsequent DNA analysis results. PCR experiments were designed to evaluate the quality and quantity of DNA from the samples, by examining both the length of DNA fragments that could be recovered (DNA quality) and the relative DNA copy number (DNA quantity). Results were analyzed by looking at trends across preservative type, tissue type, decomposition level, and storage time. Recommendations for tissue preservation and further evaluations were developed based on these results. The molecular assays combined with field practicality considerations provide suggestions for simple and effective tissue preservation methods that are amenable to subsequent DNA analysis.

MATERIALS AND METHODS

Developing preservation techniques

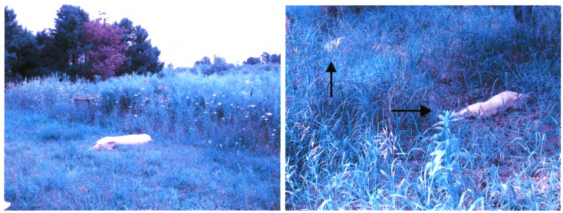
Six tissue preservation methods were selected for this study: Storage at -20°C, oven-drying at 70°C, silica desiccation, storage in 70% ethanol, storage in 70% isopropanol, and storage in RNA*later*. Screw-top, polyethylene 6mL sampule vials (Wheaton, Millville, NJ) were used for sample storage. Samples designated for -20°C storage were placed in vials and kept in a rack at -20°C. Samples preserved by 70°C oven-drying were placed on waxed paper in a 70°C incubator for ~72 hours, then transferred to vials and stored at room temperature. The remaining methods were storage in either 4mL of 70% ethanol, 4mL of 70% isopropanol, 2.5mL of RNA*later*, or 2.0 – 2.5g of silica (28 – 200 mesh, Fisher Scientific, Pittsburgh, PA). Aside from the samples stored at -20°C, all samples were kept at room temperature.

Obtaining and preserving tissue samples

The tissue samples for this project were taken from swine carcasses, obtained from the Swine Teaching and Research Center at Michigan State University. Three pigs, weighing approximately 30, 60, and 90lbs, were euthanized and placed in an agricultural field on the campus of Michigan State University during the month of August (Figure 1). Initial samples of skin and muscle tissue were taken within an hour of death, by excising

a tissue section from the shoulder of each pig with a disposable scalpel. The tissue sections measured approximately 10cm x 5cm and were approximately 3cm thick. Separate disposable plastic bags were used to transport the tissues from the field to the laboratory. Samples were preserved within approximately 24h of collection; storage of the entire tissue section at -20°C was used in the interim.

Figure 1 – Placement of the pigs in the agricultural field



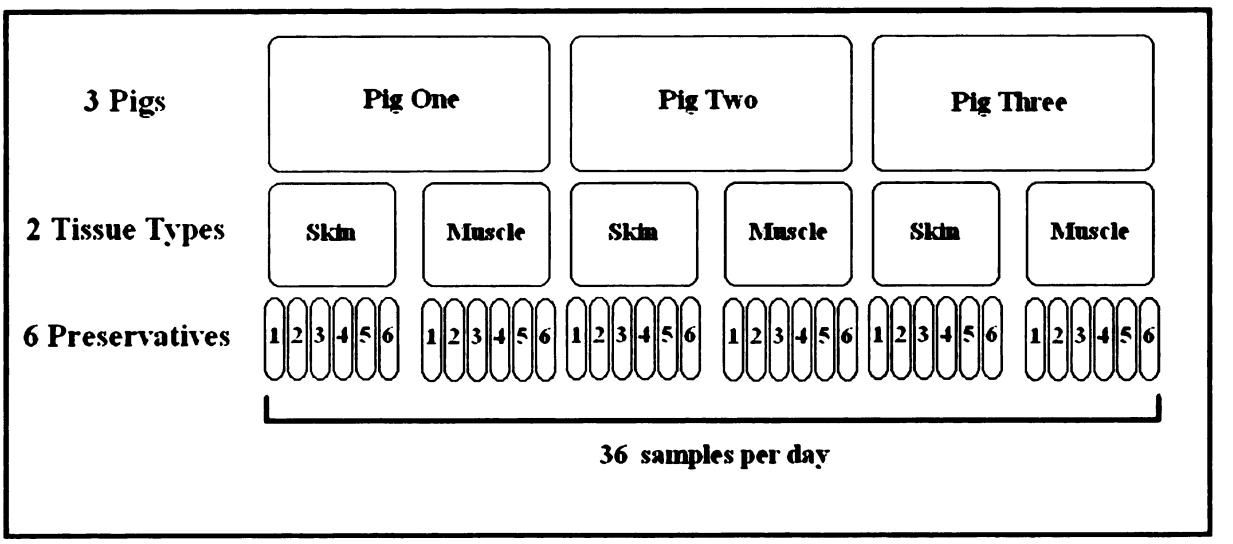
Pigs were placed directly on the ground within an area of approximately 25 square meters. The terrain varied slightly, from mowed grass to sparse tree cover. All of the pigs received partial sun and were exposed to the elements, aside from the partial cover that the trees provided over pigs two and three. From the time that the pigs were initially placed in the field, they were undisturbed except to obtain tissue samples. Pig one was placed on the edge of a tall grassy field (left) and pigs two and three were placed in brush under the cover of sparse trees (right).

Using sterile scalpels, forceps, and razor blades in the laboratory, control samples of 0.500g of tissue (+/- 0.002g) were excised from each tissue section, and DNA extractions were begun the same day (see below). Tissue surfaces were shaved with a disposable razor before taking samples, to minimize the amount of hair included in the

subsequent digestions. Scalpel blades were replaced often, due to the dulling that occurred while cutting through the pigs' bristles and skin. The tissues were then divided into 0.500g aliquots (+/- 0.002g) of skin and muscle to be individually preserved. This was accomplished by first separating the cutaneous tissue from the underlying muscle, and removing individual 0.500g samples of each, resulting in a total of six skin and six muscle samples per pig, on each day of collection (Figure 2). Samples that were to be preserved in silica or solution (ethanol, isopropanol, or RNA*later*) were placed directly into a vial of preservative after being weighed, and stored at room temperature. Samples to be stored at -20°C were placed directly into empty vials and frozen. Tissues for 70°C oven-drying were placed on squares of waxed paper and dried in an incubator for approximately 72h before being placed into individual vials and stored at room temperature. During the storage periods all of the samples remained undisturbed at their respective conditions.

The tissue collection and preservation procedure was repeated on days 3 and 5 post-mortem. By day 8, the 30lb and 60lb pigs had become mummfied, and the 90lb pig had been removed from the field, presumably by foraging animals, thus tissue sampling ended after the day 5 collection. Preserved samples were stored for time periods of 2 weeks and 2 months, at which point DNA extractions were performed.

Figure 2 – Schematic of daily tissue sample collection



A total of 36 tissue samples were preserved from each day of field collection. The collection procedure was repeated on days 1, 3, and 5 post-mortem, resulting in 108 preserved tissue samples. DNA extractions were performed on each of the tissue samples after 2 weeks of storage and after 2 months of storage, resulting in 216 DNA samples.

DNA extraction

DNA extractions were begun after two weeks by removing samples from storage, weighing, and processing half of the tissue. The remaining half of the tissue sample was returned to the corresponding preservative method until the two month storage time period, when a second DNA extraction was performed on the remaining tissue. Ethanol and isopropanol stored samples were removed from their storage vials and blotted on tissue paper before weighing. Preliminary experiments showed that rehydrating the tissues stored in silica or RNA*later* was necessary for tissue digestion; therefore, prior to the extraction procedure these samples were removed from preservative and rehydrated

in 2mL TE (10mM Tris, 1mM EDTA) for approximately 2 hours. RNA*later* samples were returned to storage in their original vials; however, silica stored samples remained in TE until the second DNA extraction. This was due to the fact that once the sample had been rehydrated and cut into small fragments, the remaining tissue would have been unrecoverable from the silica mesh.

Tissues for DNA extraction were placed in 1.5mL microfuge tubes with 500uL of digestion buffer (20mM Tris, pH 7.5, 100mM EDTA, 0.1% SDS) and 5uL of proteinase K (20mg/mL, Roche, Basel, Switzerland). Samples were macerated for ~10 seconds with a conical glass pestle, then placed at 55°C for 72 hours. Samples were vortexed for 10 – 15 seconds daily to aid in the digestion of the tissue.

The extractions proceeded by adding 500uL of phenol to the digested tissues, which were vortexed for 10 seconds and centrifuged at 14,000rpm for 5 minutes. The aqueous layers were removed and transferred to new 1.5mL microfuge tubes. Five-hundred microliters of chloroform was added to the extracts, and samples were vortexed for 10 seconds and centrifuged at 14,000rpm for 5 minutes. It was noted during the phenol and chloroform steps that the aqueous and organic layers of the RNA*later* samples were inverted after centrifugation. Therefore, samples were monitored to ensure that the appropriate layer was transferred for the subsequent steps. The aqueous layers of all samples were transferred to new 1.5mL microfuge tubes, to which two volumes of cold 95% ethanol and 1/10 volume of 3M sodium acetate were added and mixed by inversion. The samples were placed in -20°C storage to allow the DNA to precipitate. After 7 – 10 days of storage at -20°C, samples were centrifuged for 15 minutes at 14,000rpm, and supernatant was removed. Samples were vacuum-dried for 15 minutes,

and resuspended in 50uL of TE. DNA samples were returned to freezer storage. In the interest of treating each sample equally, no additional steps were taken to purify troublesome extracts, and all samples were resuspended in an equal volume of TE.

Evaluating the quality of DNA

Five microliters of each DNA sample were loaded on a 1.0% agarose gel. Presence of high molecular weight DNA indicated a sample of relatively high quality, whereas the presence of a wide range of DNA fragments (a smear) indicated a degraded sample. This technique was used to roughly estimate the differences in yield and degradation across the 216 samples.

DNA quality was evaluated by attempting to amplify DNA fragments of increasing length; successful amplification of a large fragment was used as an indicator of a high quality sample. Three sets of primers were designed for the porcine Insulin Growth Factor 1 gene (IGF-1), using the same forward primer but increasingly distant reverse primers (Table 1). The primer pairs resulted in amplificons of 257bp, 457bp, and 642bp. DNA samples were tested successively for amplification of each target, or until the sample failed to amplify. For example, if a sample was positive for amplification of the 257bp target but failed to amplify at 457bp, no further testing was performed.

Table 1 – Sequences of PCR primers for DNA quality testing experiments

Primer	Sequence (5' – 3')	Primer size (nt)	Amplicon size (bp)
IGF-1 Forward	AAT CAT TTG CCC CTC AAG TG	20	N/A
IGF-1 R257	TGA CCC CCT CAT CCT AGT TG	20	257
IGF-1 R457	GGC AGG AAG ACA CAC ACA TC	20	457
IGF-1 R642	TCT CTC CCT CTT CTG GCA AA	20	642

The sequences for the IGF-1 primers, which pair the same forward primer with various reverse primers to create amplicons of increasing lengths.

Primers were designed using porcine IGF-1 gene sequences obtained from the online National Center for Biotechnology (NCBI) database and the online primer design program Primer3 (Rozen and Skaletsky, 2000). The primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and were received as lyophilized pellets which were resuspended in TE to a concentration of 200umol/L.

PCR conditions were optimized using the DNA from day 1 unpreserved tissue samples as a control. The conditions were: 1U of Hot Master Taq Polymerase (Eppendorf, Hamburg, Germany), 1X Hot Master Taq PCR Buffer (Eppendorf), 20uM dNTPs (Promega, Madison, WI), 1uM forward IGF-1 primer, and 1uM reverse primer, per reaction. PCR reactions were set up in 10uL volumes, with 1uL of DNA sample per reaction. Unsuccessful PCR due to excess genomic DNA was observed in the majority of the IGF-1 257bp reactions. Products of these reactions were characterized by high molecular weight DNA smears and a lack of target amplification when observed by gel electrophoresis. DNAs were diluted 1/100 and re-tested for amplification of the IGF-1

257bp fragment. If a DNA extract successfully amplified using the 1/100 DNA dilution, all subsequent testing was performed using that dilution.

PCR experiments were divided into six groups, broken down by day of collection and time point of extraction (Table 2). The six groups were tested separately, including a positive control (Day 1 unpreserved DNA sample) and negative control (no DNA added) with each set of reactions. As testing progressed from the 257bp test through the 642bp test, the samples remained grouped in the same manner, so that six separate rounds of PCR were set up for each fragment size.

Table 2 – Grouping of samples for PCR experiments

Group	Title	Collection Day	Storage Time Point
1	D1/2MO	Day 1	2 Months
2	D1/2WK	Day 1	2 Weeks
3	D3/2MO	Day 3	2 Months
4	D3/2WK	Day 3	2 Weeks
5	D5/2MO	Day 5	2 Months
6	D5/2WK	Day 5	2 Weeks

In order to facilitate the progression of the PCR experiments, samples were broken into these 6 groupings, and reactions were run for one group at a time, including positive and negative controls with each set of reactions.

The same thermocycling program was used for the experiments with the 257bp, 457bp, and 642bp target amplicons (Table 3). Results were visualized by electrophoresing 5uL of each sample on a 1.0% agarose gel and staining with ethidium bromide. Samples were recorded as positive if the reaction produced a band of the targeted size, regardless of intensity.

Table 3 – Cycling parameters for IGF-1 amplification

Temperature (°C)	Time	
94	2m	
94	30s	35 Cycles
58	1m	
72	1m	
72	5m	
4	∞	

The same thermocycling program was used for the 257bp, 457bp, and 642bp amplification experiments.

Evaluating the quantity of DNA

DNA quantification was attempted using real time PCR analysis. The primer sequences used were located in the porcine hypoxanthine phosphoribosyltransferase (HPRT) and IGF-1 genes, the sequences of which were obtained from the NCBI online database. Sequences were entered into Primer Express software (Applied Biosystems, Foster City, CA), which provided primer pairs for small amplicons (50 – 100bp) suitable for real time PCR (Table 4). Primers were synthesized by Integrated DNA Technologies, Inc., and were received as lyophilized pellets which were resuspended in TE to a concentration of 200umol/L.

Table 4 – Primer sequences for real time PCR experiments

Primer	Sequence (5'-3')	Primer length (nt)	Amplicon (bp)
HPRT F93	GCT CGA GAT GTG ATG AAA GAG ATG	24	77
HPRT R170	AAA GAA TTT ATA GCC CCC CTT GA	23	
IGF-1 F679	TCG CCC ATC CTC CAC GTA T	19	69
IGF-1 R748	GGC AGG AAG ACA CAC ACA TCT G	22	

Pairs of primers used for the real time PCR experiments, resulting in the amplification of a 77bp fragment from the HPRT gene and a 69bp fragment from the IGF-1 gene.

The real time PCR primers were optimized by testing forward and reverse primer concentrations of 2uM, 1uM, and 0.2uM. The thermocycling program consisted of a 10 minute denaturation at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Once the conditions resulted in a clean, single-banded product, the experiment was tested under real time PCR conditions on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) at the Michigan State University Research Technology Support Facility. Multiple concentrations of primers were tested, again using combinations of 2uM, 1uM, and 0.2uM forward and reverse primers. Final conditions for the real time PCR experiments were: 1x *Power*SYBR Green Master Mix (Applied Biosystems), 1uL of DNA template (diluted as appropriate), and varying concentrations of primers (Table 5). The reaction volume was 10uL, with the remainder of the mix being made of water. The thermocycling parameters were optimized for amplification of the small target fragments (Table 6).

Table 5 – real time PCR Reaction mix concentrations

Ingredient	Final Concentration	Volume
*Power*SYBR	1x	5uL
DNA template	Unknown	1uL
IGF-1 Forward	0.2Um	1uL
IGF-1 Reverse	1Um	1uL
HPRT Forward	0.2uM	1uL
HPRT Reverse	2uM	1uL

The concentration of *Power*SYBR and volume of DNA template added were consistent across all of the real time PCR reactions. Results of optimization experiments indicated that the IGF-1 and HPRT primers were most effective at different concentrations, ranging from 0.2 – 2uM.

Table 6 – Cycling parameters for real time PCR

PCR Program

Temperature (°C)	Time	
50	2m	
95	10m	
95	15s	40 Cycles
60	30s	

The real time PCR thermocycling program was designed with short annealing and extension times to increase specificity and minimize non-specific amplification. The initial 50°C was required for optimum activity of the AmpErase enzyme (ABI), and the 95°C hold served to activate the room-temperature stable polymerase AmpliTaq Gold (ABI) contained in the real time PCR reagents.

Reaction mixes for the real time PCR experiments were prepared manually and aliquoted into 8-strip 0.2mL PCR tubes, and DNA templates were prepared in 96-well

plates. A Biomek 2000 Laboratory Automation Workstation (Beckman, Fullerton, CA) was used to mechanically transfer the master mix and DNA templates into 384-well plates. DNAs were tested in triplicate, including positive (Day 1 unpreserved DNA sample) and negative (no DNA) controls. All reactions for IGF-1 were prepared and amplified at one time, and all HPRT reactions were prepared and amplified on a separate day. The reactions were analyzed using the ABI Prism 7900HT Sequence Detection System and SDS software version 2.1 (Applied Biosystems). The dissociation curve and cycle threshold of each sample were obtained for analysis.

Results for the IGF-1 real time PCR experiment were ambiguous, so a subset of DNA samples were re-tested for amplification of the 642bp IGF-1 fragment to ensure that the dilutions being used still produced valid results. This test indicated that the DNA samples were still of high quality; therefore, the real time PCR testing proceeded with the HPRT primers.

Data analysis

Samples were scored as either positive (amplification occurred) or negative (no amplification) for the quality testing results. Positive results for the six time point groups and the six preservation methods were tabulated, and the mean success rate of each group for each fragment size was calculated. One-way ANOVA was performed to compare the differences in mean success rates among the collection time points and among the preservation methods at each of the IGF-1 fragment sizes. The mean success rates between storage times were also compared through ANOVA, to analyze preservation

method performance after two weeks of storage compared to two months of storage. Differences in the quality of DNA obtained from skin and muscle were analyzed only for the IGF-1 642bp fragment test results, due to clearly limited differences among success rates at the 257bp and 457bp sizes. A Bonferroni test was performed on all pair-wise comparisons within a group if ANOVA indicated a significant difference among means (α=0.05). All statistical analyses were performed using SPSS For Windows, version 11.5.2.1 (SPSS).

Results for real time PCR experiments were analyzed by determining the average Ct value for samples from each preservation method. Separate averages were calculated for the IGF-1 and HPRT results. Though DNA extracts were run in triplicate, there were few cases where all three reactions resulted in amplification. Because of this, three separate average Ct values were computed for each preservation method—an average including only samples that worked in triplicate, a second including samples that worked in duplicate, and a third including all samples that worked at least once. Due to the limited success of these tests, no further statistical analyses were undertaken. Results of the real time experiments were compared on the basis of mean Ct values for each preservation method as well as the number of DNA extracts that were successfully amplified within each preservation method.

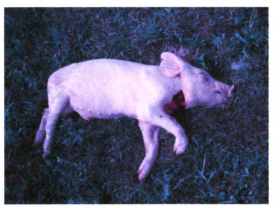
RESULTS

Tissue collection

Tissue collection from the pig carcasses was performed on days one, three, and five. Day one samples were taken from the cervical region of each pig within an hour of death, and no visible decay or insect colonization had occurred (Figure 3). By day three each of the pigs had begun to show signs of abdominal bloating and putrefaction in their limbs. Maggot infestation had occurred around the pigs' mouths, noses, eyes, and ears, as well as the cut site of the first tissue sampling (Figure 4). Tissue samples were obtained on day three by removing a section of tissue immediately posterior to the initial cut site, from an area free of maggot infestation.

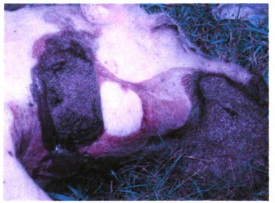
Mummification of the head and forelimbs had occurred on each of the pigs by day five, and maggot infestation had expanded to the forelimbs and shoulders of each pig (Figure 5). The abdominal cavity of pig one had ruptured by this time and flies had begun to congregate in the intestinal area (Figure 6). Tissue samples were again obtained from an area immediately posterior to the maggot infestation and previous cut sites. Tissue collection was halted after day five due to the extensive mummification of pigs two and three, and the disappearance of pig one (Figures 7, 8).

Figure 3 – Day one observations of pig carcasses



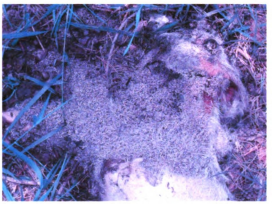
Tissue samples were obtained from the pig carcasses immediately after being placed in the field. Initial samples were removed from the cervical region of each pig, as illustrated on pig one (left) and pig three (right). No visible decay or insect colonization had occurred at this time point.

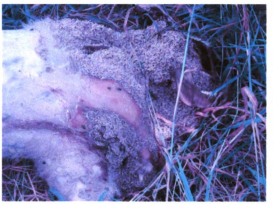
Figure 4 – Day three observations of pig carcasses



Substantial maggot infestation had occurred by day three, centered around the cranial orifices of each pig (left). Signs of bloating and putrefaction were also observed in the abdominal regions of each pig (right). Tissue samples were taken from the shoulder of each pig, immediately posterior to the initial cutting site.

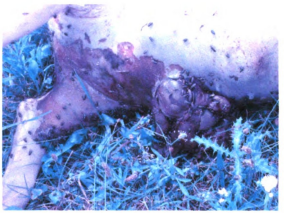
Figure 5 – Day five observations of pig carcasses





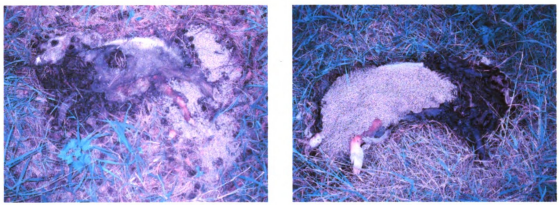
The head and forelimbs of each pig had begun to mummify by day five (Pig three, right; Pig two, left). Maggot infestation had continued to progress posteriorly, and further putrefaction was observed in the abdomen and limbs.

Figure 6 – Day five observations of decomposition in pig one



Pig one exhibited the most advanced state of decomposition among the three pigs. By day five, the abdominal cavity had ruptured, increasing the rate of tissue decay in the abdomen and hind limbs as compared to pigs two and three.

Figure 7 – Day eight observations of pigs two and three



The entire carcasses of pigs two and three had become extensively mummified by day eight. Maggots had completely transgressed the carcass of pig three and were migrating from the body (left). Pig two (right) had not yet reached that state of decomposition, but no tissue sections could be collected from either carcass by this date.

Figure 8 – Day eight observations of pig one

A mandible and partial cranium were all that remained of pig one after eight days in the field (left). It appeared that these bones were virtually undisturbed compared to the location of this carcass on day five (right). No further traces of the carcass could be located, nor could any determinations be made concerning how it had been removed. It was presumed that small carnivores such as coyotes had destroyed the remains.

Preparation of tissues for preservation

Tissue processing consisted of separating skin and muscle tissues and weighing individual samples for preservation. The separation of skin and muscle did not present any difficulties, since the sections obtained in the field greatly exceeded the amount of tissue needed and precise dissection was not paramount. As the carcasses decayed, this procedure became easier, with the skin separating readily and intact from the decomposing fat and muscle layers beneath.

Skin tissue maintained a rubbery and pliable consistency throughout the collection time period. Cutting through the epidermis continued to require the use of a razor blade and sharp scalpel. Muscle tissue became progressively more decomposed, to the extent that samples were slick, pulpy, and beginning to turn green by day five. Care was taken to avoid clearly rotten samples, by excising any discolored or putrefied sections. The odor of all samples strengthened as the level of decay increased, and this odor was still apparent when tissues were taken out of storage for digestion.

Digestion of tissue samples and extraction of DNA

Notable differences among preservation methods were observed throughout the course of the tissue digestions and subsequent DNA extractions. Oven-dried samples were difficult to halve without losing tissue, as the samples would crumble when attempting to cut them with a scalpel. This may have affected their yield when compared to other preservation methods. By two months of storage, both skin and muscle tissues

preserved in ethanol and isopropanol were beginning to fragment in the vials. The solutions had become cloudy with tissue debris, and samples often shredded when grasped with forceps. Digestions were prepared using all of the tissue that could be recovered from the solution, but the comparative DNA yields of these samples may have been affected by tissue loss. Silica stored samples had fragmented in the TE by the time the two month DNA extractions were begun. Again, as much tissue as possible was recovered from the solution, but due to the pulpy nature of the samples there was some tissue loss. RNA*later* samples and -20°C stored samples did not present any difficulties or disparities between tissue types in this phase of the procedure.

Digestions were continued for approximately 72h at 55°C, due to the difficulty in getting the oven-dried and RNA*later* preserved samples to fully lyse in the digestion buffer. In the interest of treating all groups uniformly, no samples were allowed to incubate longer than 72h even if the tissue was not completely digested.

Completion of DNA extractions

Phenol-chloroform DNA extractions were performed on all of the digested samples with few problems. Silica samples required extra care during transfer of the aqueous layer to ensure that no residual silica mesh was carried over into the DNA precipitation. Samples that had been stored in RNA*later* were consistently observed to have the aqueous and organic layers inverted upon addition of the phenol and chloroform. This required that the top, organic layer be discarded and the lower, aqueous layer be retained for the next step. Although relative yields may have been affected, these

extractions still resulted in DNA, as all extractions were observed to contain DNA when assayed on yield gels (see below). No further aberrations were observed through this phase of the extractions.

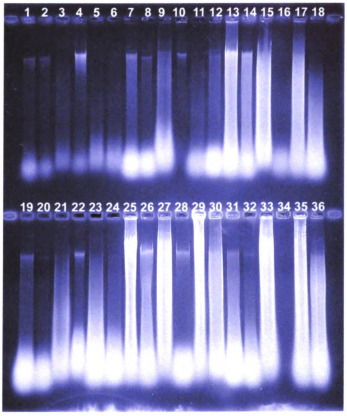
Following DNA precipitations and drying, the silica samples were found to contain a small viscous layer (~50uL) at the bottom of the microcentrifuge tube, along with an ill-defined DNA pellet. Care was taken to remove as much of this substance as possible without interfering with the DNA pellet; after vacuum-drying and resuspending these samples in TE there was no longer any visible trace of the viscous material. RNA*later* samples also exhibited atypical pellet formation. In some cases a white interface formed near the bottom of the tube, in other cases a small volume of opaque solution with no clear pellet was separated out at the bottom of the tube. The supernatant was removed as efficiently as possible without disturbing either the interface or opaque solution, which were presumed to contain the DNA. No further anomalies were observed after vacuum-drying and resuspension in TE.

Observations of DNA yield gels

Analysis of the extracts on yield gels indicated that each extraction had successfully isolated some quantity of DNA. The concentrations and the extent of degradation varied widely among all of the extracts (Figure 9). No consistent trends were observed between particular preservation methods and the apparent DNA yields. These results became most useful in determining which DNAs would be good candidates for

dilutions, in order to overcome the problems observed in subsequent PCR experiments caused by excess DNA.

Figure 9 – Yield gel of D5/2MO extracts



DNA yields varied greatly and without any clear trends across all extracts. Lane 13 is an example of an extract that exhibited a high concentration of high molecular weight DNA. Lane 16 is an example of an extract with no visible high quality DNA, and very little DNA overall. Extracts that contained DNA of a high molecular weight and concentration were diluted 1/100 for use in subsequent PCR experiments. The order of the samples on the gel is Ethanol, Isopropanol, RNA*later*, Silica, 70°C oven-drying, and -20°C, repeated six times from lanes 1 – 36.

Evaluating DNA quality by targeting IGF-1 amplicons of increasing sizes

Results of the quality testing experiments were analyzed by tabulating which DNA extracts successfully amplified using the primers for the three IGF-1 fragments. Results were analyzed separately for each of the IGF-1 experiments, beginning with amplification of the 257bp target (Table 7). The overall PCR success rate for this fragment was 89%, or 192/216 samples. Seventy degree oven-drying had the highest success rate, with amplification in 94% of the DNA extracts. Ethanol storage and RNA*later* had the second highest success rate, with amplification in 92% of each group. Isopropanol and -20°C storage had equal amplification success rates of 89%, and silica storage had amplification in 81% of DNAs. These results were not statistically different ($p=0.509$).

Amplification differences based on tissue collection time and length of storage showed that the D5/2MO and D5/2WK DNA extracts had the fewest positive results (Table 7). Seventy-five percent of the D5/2MO extracts successfully amplified and 69% of the D5/2WK DNAs amplified. The D3/2WK group had a success rate of 92%, and the other three time points had amplification in 100% of the samples. ANOVA indicated significant differences among the means of time points, and pair-wise comparisons showed that there were significant differences between seven pairs of time points. The significant differences all involved comparisons of the D5 time point groups (Table 8).

Table 7 – Success rates for amplification of the IGF-1 257bp fragment

% of Samples positive for IGF1 257bp amplification

	D5/2MO	D5/2WK	D3/2MO	D3/2WK	D1/2MO	D1/2WK	Pres. Avg.
Ethanol	0.83	0.83	1.00	0.83	1.00	1.00	0.92
Isopropanol	1.00	0.67	1.00	0.67	1.00	1.00	0.89
RNA*later*	0.83	0.67	1.00	1.00	1.00	1.00	0.92
Silica	0.33	0.50	1.00	1.00	1.00	1.00	0.81
70°C	0.83	0.83	1.00	1.00	1.00	1.00	0.94
-20°C	0.67	0.67	1.00	1.00	1.00	1.00	0.89
Time Pt. Avg.	0.75	0.69	1.00	0.92	1.00	1.00	

The results of the IGF-1 257bp test show that oven-drying had amplification in 94% of DNA extracts, the highest success rate among the preservation methods. Analysis of the time point groups show that both D5 groups had significantly lower amplification success rates than the other time points.

Table 8 – Significant differences among time points in amplification of the IGF-1 257bp fragment

	Time Point 1 (mean)	Time Point 2 (mean)	P value
1	D5/2MO (0.75)	D3/2MO (1.00)	0.004
2	D5/2MO (0.75)	D1/2MO (1.00)	0.004
3	D5/2MO (0.75)	D1/2WK (1.00)	0.004
4	D5/2WK (0.69)	D3/2MO (1.00)	<0.001
5	D5/2WK (0.69)	D3/2WK (0.92)	0.017
6	D5/2WK (0.69)	D1/2MO (1.00)	<0.001
7	D5/2WK (0.69)	D1/2WK (1.00)	<0.001

Seven pair-wise comparisons of mean success rates for amplification of the 257bp fragment were significantly different at the α=0.05 level. This was determined using a Bonferroni test on mean success rates for all time point groups. All significant differences involved comparisons between D5 time points and earlier sample collection time points.

The overall success rate for amplification of the IGF-1 457bp fragment was 86%. Ethanol, isopropanol, and RNA*later* had the highest mean success rate amongst preservation methods (Table 9). Isopropanol samples maintained the same amplification success rate as in the 257bp amplicon experiment; however, all other preservation methods exhibited a decrease in amplification success. Silica storage again had the lowest number of positive samples, with a 75% success rate. None of the differences among means were statistically significant.

Analysis of the IGF-1 457bp test by time point groups showed that the D5/2MO and D5/2WK sample sets were again the lowest ranked, with mean amplification success rates of 61% (Table 9). Statistical analysis indicated that the differences between the means of the two D5 groups and each of the other time points were significant (Table 10).

Table 9 – Success rates for amplification of the IGF-1 457bp fragment

% of Samples positive for IGF1 457bp amplification

	D5/2MO	D5/2WK	D3/2MO	D3/2WK	D1/2MO	D1/2WK	Pres. Avg.
Ethanol	0.67*	0.83*	1.00	0.83*	1.00	1.00	0.89*
Isopropanol	1.00	0.67*	1.00	0.67*	1.00	1.00	0.89*
RNA*later*	0.67*	0.67*	1.00	1.00	1.00	1.00	0.89*
Silica	0.17*	0.33*	1.00	1.00	1.00	1.00	0.75*
70°C	0.67*	0.50*	1.00	1.00	1.00	1.00	0.86*
-20°C	0.50*	0.67*	1.00	1.00	1.00	1.00	0.86*
Time Pt. Avg.	0.61*	0.61*	1.00	0.92*	1.00	1.00	

Mean success rates for amplification declined for all preservation methods except isopropanol storage. The only significant differences between means were again attributed to the low success rates of the D5 time point groups. *Samples that were negative for amplification of the IGF-1 257bp fragment are included as negative results in these calculations.

Table 10 – Significant differences among time points in amplification of the IGF-1 457bp fragment

	Time Point 1 (mean)	Time Point 2 (mean)	P values
1	D5/2MO (0.61)	D3/2MO (1.00)	<0.001
2	D5/2MO (0.61)	D3/2WK (0.92)	0.001
3	D5/2MO (0.61)	D1/2MO (1.00)	<0.001
4	D5/2MO (0.61)	D1/2WK (1.00)	<0.001
5	D5/2WK (0.61)	D3/2MO (1.00)	<0.001
6	D5/2WK (0.61)	D3/2WK (0.92)	0.001
7	D5/2WK (0.61)	D1/2MO (1.00)	<0.001
8	D5/2WK (0.61)	D1/2WK (1.00)	<0.001

Eight pair-wise comparisons of mean success rates for amplification of the 457bp fragment were significantly different at the α=0.05 level. All significant differences once again involved comparisons between D5 time points and earlier sample collection time points.

PCR amplification of the IGF-1 642bp fragment was positive in 81% of all samples. Ethanol and RNA*later* had the highest number of successful amplifications among the preservation methods (Table 11); silica storage had amplification in the fewest samples. Although the PCR experiments indicated that success continued to decline as target size was increased, there were no significant differences among the preservation methods.

The D5/2MO and D5/2WK sample sets ranked lowest among the time points, with mean success rates of 42% and 56% for amplification of the 642bp fragment (Table 11). The number of samples that were amplified in each of the other time point groups remained static. Once again, the D5 group means were found to be significantly different from the other four time point groups (Table 12).

Table 11 – Success rates for amplification of the IGF-1 642bp fragment

% of Samples positive for IGF1 642bp amplification

	D5/2MO	D5/2WK	D3/2MO	D3/2WK	D1/2MO	D1/2WK	Pres. Avg.
Ethanol	0.50*	0.83*	1.00	0.83*	1.00	1.00	0.86*
Isopropanol	0.50	0.67*	1.00	0.67*	1.00	1.00	0.81*
RNA*later*	0.50*	0.67*	1.00	1.00	1.00	1.00	0.86*
Silica	0.00*	0.33*	1.00	1.00	1.00	1.00	0.72*
70°C	0.50*	0.33*	1.00	1.00	1.00	1.00	0.81*
-20°C	0.50*	0.50*	1.00	1.00	1.00	1.00	0.83*
Time Pt. Avg.	0.42*	0.56*	1.00	0.92*	1.00	1.00	

Mean success rates continued to decline for each of the preservation methods in this experiment. Ethanol and RNA*later* had the greatest number of positive samples among the preservation methods, and silica storage had the least number of amplified DNAs. The mean success rates of the D5 time points continued to decline as well, with all earlier time points holding static. *Samples which failed to amplify the 257bp or 457bp fragments are included as negative results in these calculations.

Table 12 – Significant differences among time points in amplification of the IGF-1 642bp fragment

	Time Point 1 (mean)	Time Point 2 (mean)	P values
1	D5/2MO (0.42)	D3/2MO (1.00)	<0.001
2	D5/2MO (0.42)	D3/2WK (0.92)	<0.001
3	D5/2MO (0.42)	D1/2MO (1.00)	<0.001
4	D5/2MO (0.42)	D1/2WK (1.00)	<0.001
5	D5/2WK (0.56)	D3/2MO (1.00)	<0.001
6	D5/2WK (0.56)	D3/2WK (0.92)	<0.001
7	D5/2WK (0.56)	D1/2MO (1.00)	<0.001
8	D5/2WK (0.56)	D1/2WK (1.00)	<0.001

The statistically significant differences in the IGF-1 642bp test were attributed to the low mean success rates of the two D5 time point groups, which had significantly different means compared to all other time points.

Results for the 642bp IGF-1 test were also evaluated on the basis of tissue type. Skin samples had an average amplification success of 89%, and muscle samples had an average amplification success of 74% (Table 13). ANOVA indicated that the differences between these amplification results were significant (p=0.005). The effect of tissue type on DNA quality was also compared among time points (Table 13). The disparity in amplification success between tissue types was much greater in the samples from D5 time points. These individual comparisons corroborated the general trend of skin samples having more positive PCR results than muscle samples. Due to the low number of samples in each of these sets (n=18) statistical analyses were not attempted on the individual time point comparisons.

Table 13 – Differences in amplification of the IGF-1 642bp fragment due to tissue type and time of collection

% of samples positive for IGF-1 642bp amplification

	Skin	Muscle
D5/2MO	0.61	0.22
D5/2WK	0.72	0.39
D3/2MO	1.00	1.00
D3/2WK	1.00	0.83
D1/2MO	1.00	1.00
D1/2WK	1.00	1.00
Avg.	0.89	0.74

Overall, DNA from skin samples amplified with significantly more success than DNA from muscle samples (p=0.005). Differences between individual time points were more substantial, but due to small sample sizes no further descriptive statistics were calculated.

The results of the 642bp amplicon experiment were also analyzed with respect to each preservation method and tissue type (Table 14). Silica preserved samples amplified with equal success regardless of tissue type. Skin samples amplified with more consistency than muscle samples in each of the other preservation method sets. ANOVA indicated that isopropanol was the only preservation method found to have a significant difference between tissue type (p=0.002).

Table 14 – Differences in amplification of the IGF-1 642bp due to tissue type and preservation method

% of samples positive for IGF-1 642bp amplification

	Skin	Muscle	P value
Ethanol	0.94	0.78	0.157
Isopropanol	1.00	0.61	0.002
RNA*later*	0.89	0.83	0.641
Silica	0.72	0.72	1.000
70C	0.89	0.72	0.218
-20C	0.89	0.78	0.386

Tissue type affected the results of PCR amplification of the IGF-1 642bp fragment substantially. Silica preservation had the same number of PCR positive skin and muscle samples, but in every other method assayed the DNA from skin samples was amplified with a higher success rate than the DNA from muscle samples. Isopropanol was the only method found to have a significant difference between tissue types.

The affect of storage time on the DNA quality was also analyzed. The overall mean success rates for DNA extracted after 2 weeks and 2 months of storage were similar (Table 15). The 2 week extractions had a lower mean success rate for amplification of the 257bp fragment, but the success rate remained nearly static as amplicon size increased. The 2 month extractions initially performed better, but a more substantial

decline was observed in the mean success rate as amplicon size increased. The results of the PCR of the 642bp fragment were analyzed by ANOVA, which indicated no significant differences between storage time points (Table 17).

Table 15 – Overall success of IGF-1 amplification rates between storage time points

IGF-1 257bp

2 weeks	2 months
0.87	0.92

IGF-1 457bp

2 weeks	2 months
0.84	0.87

IGF-1 642bp

2 weeks	2 months
0.82	0.81

The mean amplification success rates for each fragment size were similar between the two storage times. The mean success rate for the 2 week storage time declined 5% when amplicon size was increased from 257bp to 642bp. The mean success rate for the 2 month storage time declined 9% as target size increased from 257bp to 642bp. The differences in performance between short and long storage times were not statistically significant.

Mean success rates for amplification of each IGF-1 amplicon were also calculated for the two storage time points within each preservation method. For all preservation methods except silica, DNAs from the two month storage time had higher amplification success with the 257bp target than the DNAs from two weeks of storage (Table 16). However, DNA extracts from two months of storage all exhibited a substantial decline in success as amplicon size increased. DNA from silica samples stored for two weeks was amplified with greater success than the DNA from samples stored for two months for each amplicon size. Analysis of the 642bp amplification results for individual preservation methods did not show any significant differences between the short and long storage periods (Table 17).

Table 16 – Differences among preservatives in IGF-1 amplification rates between storage time points

	IGF-1 257bp		IGF-1 457bp		IGF-1 642bp	
	2 weeks	2 months	2 weeks	2 months	2 weeks	2 months
Ethanol	0.89	0.94	0.89	0.89	0.89	0.83
Isopropanol	0.78	1.00	0.78	1.00	0.78	0.83
RNA*later*	0.89	0.94	0.89	0.89	0.89	0.83
Silica	0.83	0.78	0.78	0.72	0.78	0.67
70°C	0.94	0.94	0.83	0.89	0.78	0.83
-20°C	0.89	0.89	0.89	0.83	0.83	0.83

Comparisons between sample storage times indicated that the two month samples amplified more successfully than the two week samples for the 257bp fragment. As amplicon size was increased, the DNAs from two months of storage were amplified with less frequency. DNAs from two weeks of storage exhibited little or no decline in success rates as amplicon size increased.

Table 17 – Effects of storage time on amplification of the 642bp IGF-1 fragment

	2 weeks	2 months	P value
Ethanol	0.89	0.83	0.641
Isopropanol	0.78	0.83	0.684
RNA*later*	0.89	0.83	0.641
Silica	0.78	0.67	0.471
70°C	0.78	0.83	0.684
-20°C	0.83	0.83	1.000
Avg.	0.82	0.81	0.728

Differences in 642bp fragment amplification between the 2 week and 2 month storage times were not statistically significant. Silica exhibited the greatest disparity among preservation methods, while storage at -20°C had no change in amplification success between storage times.

Real time quantification of DNA yields using IGF-1 and HPRT

Results of the real time PCR experiments did not generate enough data to perform a comprehensive statistical analysis, since amplification occurred sporadically among all samples. Reactions were run in triplicate for each DNA extract; however, very few samples amplified in triplicate (4/216 for the IGF-1 assay and 34/216 for the HPRT assay). Comparisons of preservation methods were tabulated based on the number of DNAs within each group that successfully amplified in triplicate, the number of DNAs that successfully amplified in duplicate, and all DNAs that amplified at least once (Tables 18 and 19). Mean Ct values were calculated separately for each of these categories.

Ethanol storage performed well in both assays, achieving amplification rates and mean Ct values that consistently ranked among the top three methods. Storage in RNA*later* also performed well across all categories, although amplification of the HPRT locus was notably more successful. Storage at -20°C, and 70°C oven-drying had the greatest number of samples amplify in the IGF-1 assay; however, success rates for the HPRT assay dropped considerably. Sample sizes were highly variable across all of these categories, so no further statistical comparisons were attempted.

Table 18 – Real time PCR amplification of IGF-1

	# Singlets	Avg Ct	# Duplicates	Avg Ct	# Triplicates	Avg Ct
Ethanol	22	24	9	25.2	0	N/A
Isopropanol	21	25.8	6	24.7	0	N/A
RNA*later*	26	24.5	8	25.9	0	N/A
Silica	18	25.4	1	34	0	N/A
-20°C	26	24.8	8	24.3	2	22.7
70°C	26	23.3	6	24.6	2	22.2

Results of the real time PCR experiments were analyzed using data from all reactions that exhibited amplification in one replicate ("Singlets"), two replicates ("Duplicates") and in all three replicates ("Triplicates"). Amplification in the IGF-1 real time PCR was irregular, with only four DNAs amplifying in triplicate. Storage at -20°C and 70°C oven-drying were the highest ranked preservation methods, based on the number of reproducible amplifications and the average Ct values. Ethanol and RNA*later* had similar performances based on the singlet and duplicate categories but had no triplicates.

Table 19 – Real time PCR amplification of HPRT

	# Singlets	Avg Ct	# Duplicates	Avg Ct	# Triplicates	Avg Ct
Ethanol	30	24.9	20	25.1	6	22.4
Isopropanol	21	25.6	18	25.8	5	24.5
RNA*later*	32	25.7	22	25.4	9	25.4
Silica	25	29.1	17	29.1	7	27.9
-20°C	28	27.5	16	26.7	2	22.7
70°C	27	27.1	17	27.1	5	24.3

Amplification of the HPRT locus was notably more successful than the IGF-1 assay; however, only 34/216 DNAs were amplified in triplicate. DNA from ethanol samples was amplified with the most consistency in this experiment, followed closely by RNA*later* samples. The performances of -20°C and 70°C oven-drying dropped considerably compared to results from the IGF-1 experiment.

DISCUSSION

Evaluation of the field collection procedure

Tissue samples were obtained from the pig carcasses as proposed, without modification of the field collection procedure. Although the carcasses decayed rapidly, a wide array of samples were procured in the time period preceding the disappearance of pig one and the mummification of pigs two and three. Samples were therefore not diverse from a temporal perspective, but the entire range of decomposition from fresh through bloat and mummification had been observed. It appears that placing cages over the pigs is necessary to prevent interference from scavengers, and that placing pigs at different times of year would aid the collection process by slowing the rate of decomposition.

The amount of tissue in each preserved sample needed to be kept constant in order to generate relative comparisons across the preservative methods. This necessitated collecting large sections of tissue and meticulously cutting and weighing individual aliquots in the laboratory, adding time to the procedure that would not occur under normal (non-experimental) circumstances. There would be no such lag time between tissue collection and preservation in practical applications, as methods evaluated in this study could potentially all be employed in the field, and samples would be preserved on-site without regard for the exact weights or measurements that this study required.

Within 6 – 24h from the time of collection, all tissue samples had been processed and stored. As previously noted, tissue sections were temporarily kept at -20°C during

the delay between collection and processing, which could have influenced the quality of DNA before the experimental preservation procedures were implemented. This should not have affected storage comparisons as the tissues were frozen and subsequently thawed at the same time, ensuring that all samples were exposed to the same pre-processing conditions.

The actual collection of tissue would be identical for any of the storage methods tested, but the practicality of implementing a preservation technique must be weighed as well. Vials of a liquid or dry preservative could be transported to a crime scene or the site of a mass disaster, and tissue samples could then be stored as soon as they were acquired. The main consideration for on-site vial storage is maintaining readily available stocks of the selected preservative, whether that means manual preparation and dispensation of materials, or advanced ordering from a manufacturer. On the other hand, storage at -20°C or oven-drying at 70°C would require additional equipment such as freezers or incubators, as well as electricity. Unless either of these methods is far superior to all others, they are probably less than ideal for the outcome desired.

Optimization of the organic DNA extraction technique

The first phase of this study where marked variation among the preservation methods was observed was during the removal of tissues from storage for subsequent DNA extraction. Ethanol, isopropanol, and 2 month silica stored tissues (now in TE) had begun to fragment in solution, potentially affecting the amount of material available for DNA extraction. Dividing tissues in half proved difficult for the rubbery RNA*later*

samples and the extremely desiccated and friable oven-dried samples. Both of these did not always digest to completion within the 72h incubation. Oven drying was an effective preservation technique (with 81% of samples having DNA amplified up to 642bp) but the behavior of the tissues added substantially to the workload and efficiency of the extraction procedure. However, in casework it is unlikely that dividing and re-storing a sample would occur, thus these techniques should not be eliminated based solely on that factor.

To circumvent some of the difficulties with tissues after storage in alcohol, RNA*later*, silica, or by heating, samples could be divided initially and preserved, eliminating the need to isolate sections of tissue at a later date. By using an entire sample for the DNA extraction, no tissue would be lost and substantial time would be saved. Digestion of tissues might also be aided by increased maceration or more frequent vortexing. These options were not explored in this study as a bias in DNA yield would have been introduced when treating samples differently. None of the other preservation methods required special consideration during the digestion process.

Key differences among the preservation methods were also identified during the phenol and chloroform phases of the DNA extractions. Samples from ethanol, isopropanol, -20°C storage, and oven-drying methods did not exhibit any unusual behavior during the DNA extraction procedure. In contrast, the behavior of RNA*later* samples was atypical, due to the inversion of the organic and aqueous layers. It was presumed that a high salt content in the RNA*later* solution was affecting the density of the aqueous layer and causing it to settle below the organic layer. Although DNA was extracted successfully from these samples, it is possible potential yields were affected.

All other samples required that an upper aqueous layer be retained, but the RNA*later* samples had the modified treatment of trying to retain a lower aqueous layer while removing an upper, organic layer. This may have resulted in retention of unwanted materials in RNA*later* DNA extracts due to incomplete removal of the organic liquid. A potential remedy for this problem would be manipulating the rehydration of the sample prior to beginning the extraction. By increasing the rehydration time or using several changes of TE, the amount of RNA*later* carried into the subsequent extraction may be lessened, allowing the aqueous layer to behave normally.

RNA*later* samples were also problematic during pelleting and rehydration of the DNA. All other methods produced visible DNA pellets after centrifugation, as expected. A white interface or cloudy layer of solution formed during DNA precipitation in RNA*later* stored tissues, indicating that a solution more dense than ethanol was present in the tube, perhaps preventing the DNA from pelleting. This could be due to carry-over from the organic solvents or the effects of residual RNA*later*. As mentioned, an extended rehydration in TE would help determine if adequately removing the RNA*later* from the tissue prevented subsequent problems during DNA extraction.

Silica preserved samples also proved more difficult during DNA isolation, which was presumably due to carryover of silica mesh throughout the extraction, or from these samples generating an inefficient removal of the organic phase of the extraction. Instead of a distinct white pellet, the silica samples appeared to have a small volume (~50uL) of viscous solution collected at the bottom of the tube, resulting in malformed pellets that dried with a grainy appearance. A modified, multi-wash rehydration procedure would help identify the problem of silica mesh interference, since this would reduce the amount

of particles that were introduced into the digestion. Unlike RNA*later* samples, silica does not penetrate the tissue, so increased rehydration time would not necessarily affect the outcome.

Differences in DNA quality with regard to preservation method

Performances of each preservation method with regard to DNA quality were ranked based on overall results of the IGF-1 PCR experiments, including both the percentage of samples that amplified, as well as the maximum amplicon that could be obtained. Samples positive for amplification of the 642bp fragment were considered to contain "high quality" DNA; therefore, preservation methods that resulted in the greatest number of 642bp amplifications were deemed the most amenable to DNA analyses. There were DNA extracts from each of the preservation methods that were successfully amplified up to 642bp, validating the belief that each method helps preserve DNA. However, the sensitive nature of forensic casework requires extreme confidence and reproducibility in all procedures, thus the comparative amplification success and consistency of each preservation method are key factors in choosing a 'best' preservation protocol.

Ethanol and RNA*later* preserved tissues continually yielded high quality DNA extracts, as evidenced by the number of samples that were positive for amplification of the 257bp, 457bp, and 642bp fragments (92%, 89%, and 86%, respectively). The DNA extracts from these methods had more positive amplifications of the 457bp and 642bp fragments than any of the other preservation methods (Tables 9 and 11). These results

were not statistically different from the other storage techniques, but in terms of this study, ethanol and RNA*later* storage were shown to preserve the most "high quality" DNA.

Several factors likely contributed to the effectiveness of ethanol preservation. First, it is widely used as an antimicrobial agent, and it is logical to conclude that by killing the bacteria in or on the tissue, the degradation process would be slowed. Second, the small molecular size of ethanol allows it to rapidly permeate and fix a sample, which prevents bacterial mechanisms (putrefaction) and host pathways (autolysis) from functioning and degrading the DNA (Srinivasan, 2002). Since RNA*later* preservation resulted in DNA extracts of the same quality as ethanol, it seems plausible that RNA*later* is also effective at permeating tissue and halting degradation. Although the composition of RNA*later* is proprietary, it is known to be an aqueous salt solution, and prior research has indicated that such solutions are effective DNA preservatives (Seutin et al., 1991). Oddly, one area where ethanol and RNA*later* were found to have very different preservative capabilities was between tissue types (skin versus muscle; see below). The type of tissue available may therefore be an important factor when choosing between ethanol and RNA*later*.

Storage in isopropanol was the next most effective preservative method, based on the high number of samples that were amplified at each size. DNA extracts from isopropanol preserved tissues amplified with equal success for the 257bp and 457bp amplicons (89%), while all other methods experienced a decline in positive results (Tables 7 and 9). However, amplification of the 642bp fragment from isopropanol preserved tissues exhibited a decline in mean success rate to 81%. The major reason for

the lower overall success of isopropanol storage was the low 67% amplification rate in day 3, 2 week preserved samples (Table 11). One-hundred percent of the DNAs from day 3, two month preserved samples amplified in the same assay, which seems incongruous with the poor results of the earlier storage time. Due to the small number of samples in this portion of the study, the affects of outliers may have had a disproportionate impact on the overall amplification rates.

Storage in isopropanol likely had preservation benefits similar to those of ethanol. Both of these alcohols have been shown to be effective at killing a range of bacteria and fungi, which would be useful in preventing putrefaction of a sample (Penna et al., 2001). Despite similarities, the performance of isopropanol as a DNA preservative was not as successful as ethanol. A potential reason for this was that the increased molecular weight of isopropanol hindered its ability to permeate tissue. This could have caused an increase in lag time between when the tissue was placed in solution and when the tissue was effectively preserved. Another difference between ethanol and isopropanol is in the polarity of the molecules. Ethanol is somewhat polar, which has an impact on interactions with membranes and fluids, causing water to be displaced from the surface of the cell membrane (Chanda and Bandyopadhyay, 2006). Isopropanol is non-polar, thus some of the preservative benefits of this interaction may be reduced. Though the overall amplification success rates of isopropanol DNA extracts were inferior to those of DNAs from ethanol and RNA*later* preserved tissues, this was mostly due to the potential effect of outliers in the D3/2WK sample group, as previously mentioned. The cost, transportation, and preservation techniques related to ethanol and isopropanol are largely

identical, so further analyses are needed to identify specific advantages of one alcohol over the other.

The two temperature-based storage techniques were shown to have moderate numbers of high quality DNA extracts (Table 11). Oven-dried samples had the greatest success of all methods for the 257bp amplicon, with 94% of the DNAs being positive (Table 7). However, these samples experienced the largest decline in success among PCR assays, with only 81% of the overall DNA extracts being positive for the 642bp fragment (Table 11). This method also showed a large difference between tissue types; 89% of the oven-dried skin samples amplified in the 642bp test, while only 72% of the muscle samples amplified. These results indicate that small DNA fragments amplified consistently from oven-dried samples, but in other areas, including differences between storage times and between tissue types, the oven-dried samples amplified unpredictably.

It was clear from visual observations that oven-dried samples were well desiccated, to the point that tissues had to be essentially chipped into fragments before being digested during DNA extraction. The amount of material that was added to the digestion may have skewed the PCR results for this preservation method, since difficulty in dividing the tissue was encountered with all oven-dried samples. The temperature used in this study was sufficient for sterilization purposes, as per USDA food safety guidelines (USFSIS, 2003), so putrefaction was not a likely factor once tissues had been oven-dried. However, bacterial activity may have continued to occur before the tissues reached the temperature of 70°C and became sterilized.

DNA may begin to denature at a temperature of 70°C, which means that the two strands begin to separate from one another, reducing the stability of the molecule.

Bruskov et al. (2002) also found that the chemical structures of DNA bases were altered at increasing rates as temperatures were increased from 37°C to 95°C, and that reactive oxygen molecules were created, causing mutations in the sequence of DNA or loss of nucleotides. Without functioning cellular repair mechanisms, these molecular changes could lead to degradation of the DNA. Oven-drying also requires additional equipment, an electrical source, and lag time before samples are sufficiently preserved, which compounds the inefficiency of this preservation method.

Storage at -20°C resulted in a consistent number of DNA amplifications in each IGF-1 experiment, but as with isopropanol, the number of positive results never surpassed those of ethanol or RNA*later* (Table 16). Eighty-three percent of the DNA extracts from freezer stored samples were amplified in the 642bp PCR experiment. Freezing at -20°C was equally effective over the two storage periods, but again the quality of the DNA was not as high as other preservation methods (Table 12). This preservation method was poor at preserving the most decayed samples, with only 50% of all DNA samples from day 5 showing amplification of the 642bp fragment.

Freezer storage is a method that does not alter the permanent chemical or physical state of a sample. Storage at -20°C essentially slows or halts any metabolic pathways within the tissue, thereby slowing degradation. Since cold storage may not entirely kill bacteria as a chemical treatment would, low amounts of putrefaction may have occurred before tissues were completely frozen, or while they were thawed during the tissue digestion. One important characteristic of freezer storage was that it did not exhibit any substantial decreases in amplification success between storage times (Table 16). If

samples need be stored for a long time period, freezer storage would therefore be an effective preservation method.

Silica storage was the lowest-ranked preservation method in each of the IGF-1 fragment tests. This method was particularly unsuccessful with highly decayed samples; no extracts from D5/2MO samples were positive in the 642bp PCR experiment. However, silica was the only method that did not have any disparity between tissue types, with amplification in 72% of both skin and muscle tissues in the IGF-1 642bp assay (Table 14).

Falling under the preservation category of desiccation, silica storage was expected to preserve tissues in the same manner as oven-drying, by effectively dehydrating samples and preventing microbial or cellular activity. The samples were visibly withered following storage, but retained more pliability than the oven-dried tissues, indicating that there was some amount of moisture retained. DNA degradation can occur in a moist environment, so incomplete desiccation may explain the poor performance of the silica samples. Adjusting the amount of silica used for preservation may improve the quality of the DNA, since it is possible that the amount of silica used did not sufficiently desiccate the tissue.

The process of rehydration and TE storage of silica preserved tissues must also be considered, as the samples stored for two months were not kept in silica for the whole time course. Although TE is suitable for DNA storage, autolysis or bacterial activity could have been reinstated after the rehydration, causing a decline in DNA quality before the subsequent extractions. This could explain why none of the two month extractions were positive for the 642bp amplification; however, the two week silica extracts were

also the lowest ranked in amplification of the 642bp fragment, which does not involve the TE storage factor (Table 16).

Due to the exceptionally poor amplification results of the day 5 DNA extracts, silica samples had a low overall amplification success rate. Since tissues from this stage of collection were the most decayed, it is possible that the samples had higher moisture content or greater numbers of bacteria than tissues from earlier collection times. Silica preservation is routinely used for the preservation of feces for DNA analyses (Murphy et al., 2002; Roeder et al., 2004), which would preclude the notion that bacterial activity cannot be curtailed by silica storage. The poor performance of silica from the day 5 time points is most likely due to a combination of inefficient desiccation and the opportunity for degradation to occur while the samples were stored in TE.

In order to gauge the bias that was introduced by the TE storage, this phase of the experiment could be repeated without removing samples from storage until DNA extractions were begun. Further, varying the ratio of silica to tissue volume would show whether more efficient desiccation results in higher quality DNA samples. These variables would have to be addressed before a more complete view of silica performance could be developed.

Effects of tissue decomposition on DNA quality

The time of tissue collection (1 – 5 days) was found to be the most crucial factor in DNA quality, and in fact, the only area where significant differences were observed. Amplification of the 642bp fragment occurred in only 42% of the D5/2MO samples and

56% of the D5/2WK samples (Table 11). This is not surprising, since the extent of decay by day 5 was advanced to the point of mummification (Figures 5 and 6), and DNA would most likely have been more degraded before preservation was attempted. In addition to autolysis, putrefaction was likely a significant factor in the degradation of DNA by this time.

These observations support the notion that early collection and preservation of tissues is most likely to result in successful DNA analyses. A critical shift in the success of DNA amplification occurred between the samples from the first and last days of tissue collection. Amplification of the 642bp fragment decreased from 100% for the day 1 samples to an average of 49% for the day 5 samples, across all preservation methods (Table 11). This indicated that with relatively undecayed samples, all methods were equally effective in preserving DNA. Conversely, after a certain stage of decay the performance of the preservation method became integral to preserving the highest quality DNA.

In order to elucidate subtle differences in the preservative qualities of these methods, future studies could incorporate sampling over a longer time period, or obtaining more tissue samples within the same time frame. Repeating this procedure during a colder season would allow tissue to be sampled over a broader time period, which may reveal greater discrepancies among the preservation methods as the initial DNA quality continued to decline. Taking samples more frequently within a set time period could help to correlate the stages of decay with the effectiveness of the preservation methods. Environmental conditions would also have to be taken into account, since decomposition rate is largely a function of temperature, moisture, and

other seasonal factors. Based on these preliminary results it is clear that the extent of tissue decay plays a major role in the success of subsequent DNA analysis, and future investigations could strengthen this connection.

Evaluating the effects of storage time prior to DNA extraction

The "shelf-life" of a preserved sample is an important consideration, especially in situations where the number of victims prevents the timely analysis of samples, or when a specimen needs to be re-tested at a later date. A preservation method that results in high quality DNA samples over a wide range of storage times would be most beneficial for reliable and reproducible analyses. The data from the 642bp amplicon indicate that as storage time increased, the DNA quality decreased (Table 16). This conclusion is based on the fact that three out of the six preservation methods (ethanol, RNA*later*, and silica) had more "high quality" samples in the DNAs from two weeks of storage than the DNAs from two months of storage. Frozen samples had no difference in the number of positive PCR results between storage times. With isopropanol and oven-dried samples, amplification was more successful using extracts from two months of storage than extracts from two weeks of storage. The data from the IGF-1 642bp assay indicate that storage time decreased the quality of DNA; however, results from the 257bp PCR experiment indicate that DNA from the two storage times performed similarly when amplifying small targets (Table 16).

These aberrations can be explained by several factors. One of the major variables in this experiment was the investigator's prowess with the organic extraction procedure.

Several of the preservation methods required special attention during the DNA extraction; problems which were anticipated and addressed before performing the extractions for the two month storage time. In addition, the entire procedure went more smoothly during the later round of DNA extractions, due to the investigator's increased experience. Results of the IGF-1 257bp assay show that the isopropanol, oven-dried, and RNA*later* samples from two months of storage were amplified with greater success than the similarly preserved samples from two weeks of storage (Table 16). This corroborates the idea that the second round of extractions was performed more efficiently, although DNA was likely more degraded and only the amplification rates of small amplicons were affected.

The physical state of the tissues used for the two month DNA extractions was another factor that could have contributed to the unexpected 257bp test results. It was noted during the tissue digestions after two weeks of storage that many samples were difficult to cut and digest, and that after two months of storage the tissues were beginning to fragment and soften. It is possible that after the longer storage period the tissues were more amenable to digestion, and therefore more DNA was released from the tissue even though the time was kept constant. Observations of the ethanol, isopropanol, and silica samples corroborate this supposition, since it was noted that these samples were visibly pliable and fragmented when removed from preservative for the two month extractions. This may have resulted in more complete tissue digestion; but after the long storage time the DNA had likely been degraded into smaller fragments, so the quality of the DNA obtained was not as great as in extracts from the shorter storage time.

Further investigation of the affect of storage time on DNA quality could include more time points and a greater sample size. Assaying DNA quality over a longer storage time may support the theory that low quality DNA is more abundant as storage time is increased. This would be determined by keeping the tissue digestion procedure static, and comparing the quality of DNA extracts from samples that were digested after a range of storage times. It would be expected that increasing storage time would yield DNA that was successfully amplified for small targets. If degradation was occurring during storage, then these extracts should continue to show a decrease in quality, as assayed by amplification of larger amplicons. An increased sample size would benefit the storage time experiment by reducing the impact of problematic extractions on the overall performance of a preservation method. The small number of samples that had to be specially treated during extractions may have disproportionately affected the average for a particular method since the sample size was only eighteen per tissue type and preservation method for a particular time point. A larger sample size would also benefit the experiment in general by helping to reduce any random effects introduced by the limited number of pigs, tissue types, and samples used.

Effects of tissue type on DNA quality

The disparity between the PCR results of skin and muscle samples was substantial for nearly every preservation method (Table 14). Silica storage was the only technique that presented no difference in DNA quality between skin and muscle samples; DNA from skin was amplified with more success than DNA from muscle in all other cases.

The difference in amplification rates between tissue types ranged from 39% (isopropanol) to 6% (RNA*later*). The overall success rate for amplification of the 642bp fragment was 89% for skin samples, compared to 74% of muscle samples (Table 13), which was found to be statistically significant ($p=0.005$). These results indicate that tissue type may be an important consideration when choosing samples for subsequent DNA analyses.

One theory for the discrepancy between tissue types is that skin is naturally more resistant to decomposition, supported by the fact that skin acts as a physical barrier against pathogens and moisture loss (Elias and Friend, 1975). Even after death, these were likely factors in slowing the degradation process. Muscle tissue, in contrast, is located in the moist environment below the epidermis. Once metabolic by-products and bacteria began leaching throughout the body cavities, muscle tissue would have been affected before the skin tissue that was sampled. The universality of the tissue type discrepancy found in this study presents an interesting bias that warrants investigation.

A more comprehensive analysis of this trend could greatly influence decisions about which tissues to collect in the event of a disaster. The ideas presented previously may be tested by quantifying the DNA yield from fresh skin and muscle tissues, in order to determine if muscle is simply a poorer source of DNA. Second, samples of skin could be taken from areas of the carcass that were kept moist, such as the surface of the body that was in contact with the ground. By contrasting these samples with skin that had been exposed to the air, it would be possible to ascertain whether or not skin was being preserved to an extent by air-drying and exposure to sunlight. Although all preservation methods yielded higher quality DNA from skin samples, the disparity between tissue types for each method varied widely. Determining which methods are most versatile

across tissue types would be beneficial for situations where skin may not be available, and a different tissue type must be sampled.

Trends in real time quantification of DNA yields

The results of the real time PCR experiments were unreliable gauges of DNA quantity. The goal of this phase of the project was to determine which preservation method(s) yielded the highest amounts of DNA, and to correlate those results to the corresponding DNA quality. Unfortunately, the number of replicates that consistently amplified was very low; and the results of each preservation method varied between the two genes amplified as well. This made it difficult to place a high level of confidence in the assays. Each DNA extract was assayed in triplicate for the two real time experiments. Of the 432 triplicate reactions prepared, only 38 amplified in triplicate (Tables 18 and 19), and 130 replicates did not amplify at all. Samples which did amplify in duplicate or triplicate often did not have similar Ct values, which made interpretation of the results problematical. Due to the irreproducible nature of the real time PCR experiments, the results from this phase of the project were not factored into the evaluation of preservation methods.

All reaction mixes and DNA templates were aliquoted by robot for the real time experiments, which was intended to reduce measuring errors. A mechanical malfunction in the measurement or dispensation of DNA or reaction mix may explain why replicates were not exhibiting similar amplification results, although errors in the manual preparation of the reagents and DNA templates could also have occurred, resulting in

heterogeneity among the sources of input and causing inconsistent results. No amplification would have occurred if the correct reagents were not present, so the problem was not likely due to a simple error of omission. The data from real time PCR experiments may be useful for subsequent molecular analyses; however, further investigation is needed to identify the sources of error and determine if the assay could be optimized to achieve consistent amplification results.

Conclusions

The results of this study accent the importance of rapid sampling of tissue from crime scenes or sites of mass disaster. Regardless of tissue type, storage time, or preservation method, samples that were obtained a short time after death were more likely to yield high quality DNA than samples from several days after. Tissue type was also found to have a significant effect on DNA quality, with skin being the clear choice for reliable DNA analyses. The effect of storage time was less defined, but in general, the cause for rapid tissue collection and DNA analysis was supported.

The combination of these factors helped to distinguish the utility of each preservation method. Preservation in ethanol and RNA*later* resulted in the most high quality DNA extracts, based on amplification of the 642bp fragment (Table 12). Both of these methods also exhibited minimal decreases in efficacy over a storage time of two months (Table 16). RNA*later* was the most versatile preservation method for different tissue types, exhibiting a decrease in mean amplification rate of 6% between skin and muscle (Table 15). As previously discussed, the general availability of RNA*later* is one

of the main drawbacks with its use in the field, and the decision to use this method would have to be made in advance in order to maintain a supply on hand. Ethanol has an advantage over RNA*later* in the sense that it is available from a wide range of manufacturers and venues.

Oven-drying, freezer storage, and isopropanol storage were all found to have various levels of success; however, DNA extracts from these three methods never exceeded the quality of DNA from ethanol or RNA*later* samples. DNA from oven-dried samples was inconsistently amplified across amplicon sizes, storage time points, and tissue types. Coupled with the fact that this method would require extensive equipment, the field applications may be limited. Storage at -20°C was among the top three methods in performance for each factor evaluated, but like isopropanol, this method did not have any clear benefits over the quality of the ethanol and RNA*later* samples under these conditions. Since the transportation of cold storage equipment would be more cumbersome than vials of ethanol or RNA*later*, the utility of -20°C storage must be considered on a site by site basis. Isopropanol was similar to ethanol in practical considerations (composition, cost, etc.) yet did not perform as well with regard to DNA quality, although this may have been largely a factor of sample size. Silica storage was also one of the less effective preservation methods, but further optimization may increase the quality of DNA extracts.

The preservation methods evaluated in this study have been used in the field or the laboratory for years. Under the conditions tested in this study, each method was shown to have strengths and weaknesses in regard to preserving high quality DNA. The recommendations presented in this study provide useful information on field and

laboratory techniques associated with these methods; furthermore, many potential factors have been identified that may result in the preservation of higher quality DNA using each method. The trends identified can be investigated further and may be useful in any field applications that require tissue preservation for subsequent DNA analysis.

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