STUDY OF METHODS FOR DNA EXTRACTIONS FROM FEATHERS

By

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Abstract

Feathers have been used as a source of DNA for two decades. Feathers are made of keratinized cells which can make obtaining DNA from them difficult. This study compared three established methods of extracting DNA from feathers to ascertain if any produced a greater quantity of DNA. Each of the methods were conducted at five different time intervals (n=45). Each was a modification of the Qiagen DNeasy DNA extraction kit. A two way ANOVA determined if there was any differences among times or methods, and showed a significance difference among methods ($F= 45.67$, $P < 0.001$) but not times. A box-and-whisker plot was used to ascertain the most effective method. A second experiment employed the superior method to see if a trend of increasing DNA concentration with increasing time, suggested by first experiment's data, was real. This experiment was powered up to assure sufficient ability to detect differences among 3 sample sets if they existed (n=45). These results came back with no significance. This research found the extraction method used in Dr. Lisette Wait's laboratory superior and has resulted in a protocol change for all extractions done in Dr. Melanie Culver's ancient DNA laboratory at the University of Arizona.

Introduction

DNA extraction from feathers is not a new concept Feathers have been used as a source of DNA for almost twenty years (Olsen, Bengtsson, Bertelsen, Willerslev, & Gilbert, 2012). The advent of PCR made the minute amounts of DNA that can be obtained from feathers a viable source of DNA (Taberlet & Bouvet, 1991). Feathers as a source of DNA have already been used in a large scale study of eagle populations (Nadeau, 2012).

Feathers are made up of keratinized cells, much like hair and nails. Keratinization is the process where the cytoplasm of a cell is replaced with keratin and dies. Keratinization occurs in many species in many epithelium tissues including feathers, hair, claws, nails, hooves, and horns (Olsen et al, 2012). This process may degrade the DNA, but much like with the hair and nails of other animals DNA has successfully be extracted from feathers and used (Olsen et al, 2012)

One advantage of using naturally shed feathers as a source of DNA is that the process can be non-invasive; shed feathers can be collected around nests which would avoid the stress of handling. Plucked feathers have also been shown to be a viable source of DNA, while this is not non-invasive it can shorten the time birds are handled (Harvey, Bonter, Stensler, & Lovette, 2006). Another significant advantage of feathers as a source of DNA is they do not need to be refrigerated or frozen, making storing and transporting them easier (Harvey et al, 2006). In the case of rare or endangered birds feather can be the only source of DNA available (Horvath, Martinez-Cruz, Negro, Kalmar, & Godoy, 2005).

The objective of this study was to compare three established methods of extracting DNA from feathers to ascertain if any method produced a greater quantity of DNA. This study was

done in conjunction with an ongoing study of Arizona Bald Eagle populations. The use, possession and sale of bald eagle feathers is regulated by two separate laws The Bald and Golden eagle Protection Act and the Migratory Bird Treaty (Possession, 2009). On top of this many feathers are passed on to the Native American populations through the National Eagle Repository (Possession, 2009). Thus, finding a method of DNA extraction that needs only a small amount of feather or smaller less important feathers was also kept in mind.

Three different methods were chosen to compare. All three of the methods used the Qiagen DNeasy DNA extraction kit (Appendix 1). Each method was a modified version, developed by users, of the standard method suggested by Qiagen.

The first method tested was one which had previously been used in Dr. Melanie Culver's laboratory at the University of Arizona, where this study took place (Method One). The second method was from Dr. Lisette Waits' laboratory at the University of Idaho (Method Two). The last method was found in the web appendix of a methods paper (Method Three; Bush, Vinsky, Aldridge, & Paszkowski, 2005).

The yield of these three methods at five time intervals were compared, based on amount of DNA present in final extraction elution. After the most effective method was elucidated, a second experiment was conducted to investigate the effect of incubation time on DNA yield. This avenue of research was also important because if there is a method that can produce a high yield of DNA in a shorter time period this would be beneficial to know.

Methods

The modifications to the standard Qiagen kit method for Method One included an additional mix of chemicals and digestive enzymes not found in the Qiagen kits, while the rest

of the method follows the standard kit method (Isolation, 2001). This was a general method for extraction of DNA from hair and nails (Appendix Two).

The modifications to the standard Qiagen kit method for Method Two included heating of buffer AE to 70° C. Additionally the initial incubation of samples in buffer AL for 45 minutes was in buffer heated to 70° C. Subsequent incubations of samples in AL buffer after each centrifugation through filter columns also utilized the heated buffer. Another major difference was the running of the AE buffer through the filters twice (Appendix Three).

The modifications to the standard Qiagen kit for Method Three called for a single incubation of samples in AL buffer at 70° C for ten minutes and use of cooled ethanol (Appendix Four).

For the first experiment primary feathers from Harris's Hawks *(Parabuteo unicinctus)* were used. The samples used to run each of the three methods were taken from the calamus of the feathers, and were approximately one centimeter long.

The amount of DNA yielded was quantified at five different time intervals: four, eight, twelve, twenty four, and forty eight hours for each of the three methods (n = 45). Each time interval and method pairing had a sample size of three. The objective of this experiment was to determine if any method(s) were more efficient. The DNA extracted was quantified with Nanodrop DNA analysis.

Once the most effective method had been determined that method was run again at three different time intervals to investigate a possible trend of increasing DNA concentration with time. This experiment was powered up, with the number of samples extracted increased

to fifteen per time interval (n = 45). For this set of extractions the culmus of body feathers from a Harris's Hawk were used, with each sample being run roughly one centimeter in length.

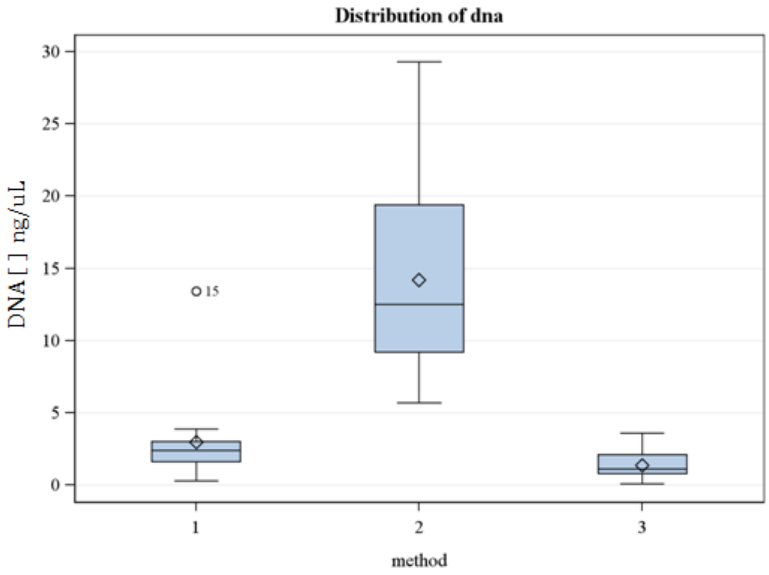
Statistical methods

For comparing the three methods a two way ANOVA was used to determine if there was a significant difference, either among the times or among the methods. The data were analyzed with two questions in mind; 1) Was there differences in concentration of DNA as a function of time, with methods pooled within times, and 2) Was there a difference in concentration of DNA as a function of method with times pooled within methods. After this analysis was completed a box and whisker plot was then used to visualize which method produced the highest concentration of DNA. For the second experiment a one way ANOVA was used to analyze the data.

Results & Discussion

For the first experiment DNA concentration varied among methods ($F= 45.67$, $P < 0.001$). The box and whisker plot demonstrated that the second method was the most effective (Figure 1).

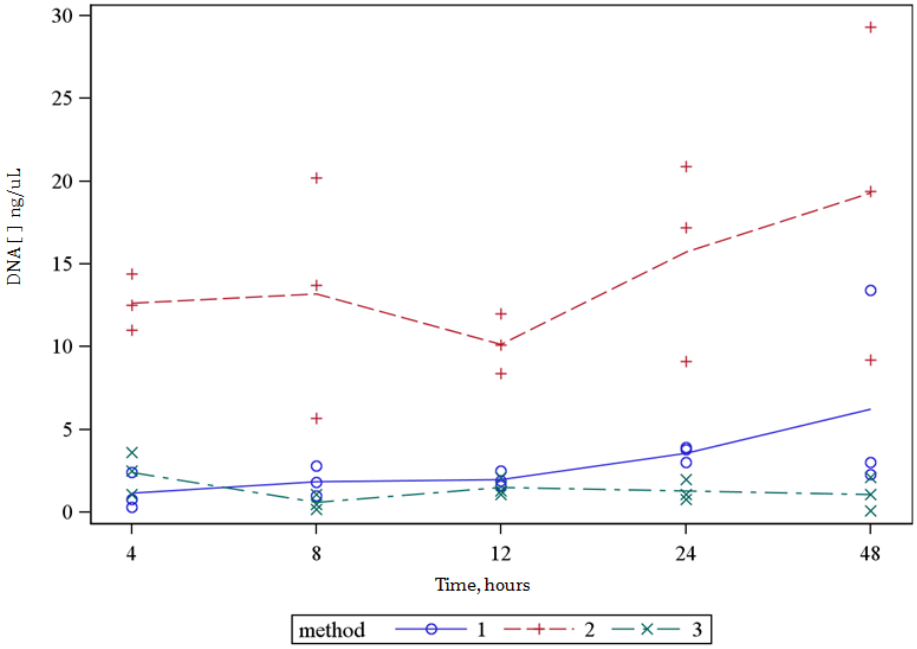
Figure 1: Box and whisker plot demonstrating that extraction method 2 produced significantly higher concentrations of extracted DNA.



The mean concentration of DNA extracted was 2.96 ng/uL, 14.21 ng/uL, and 1.38 ng/uL for Method One, Method Two, and Method Three, respectively.

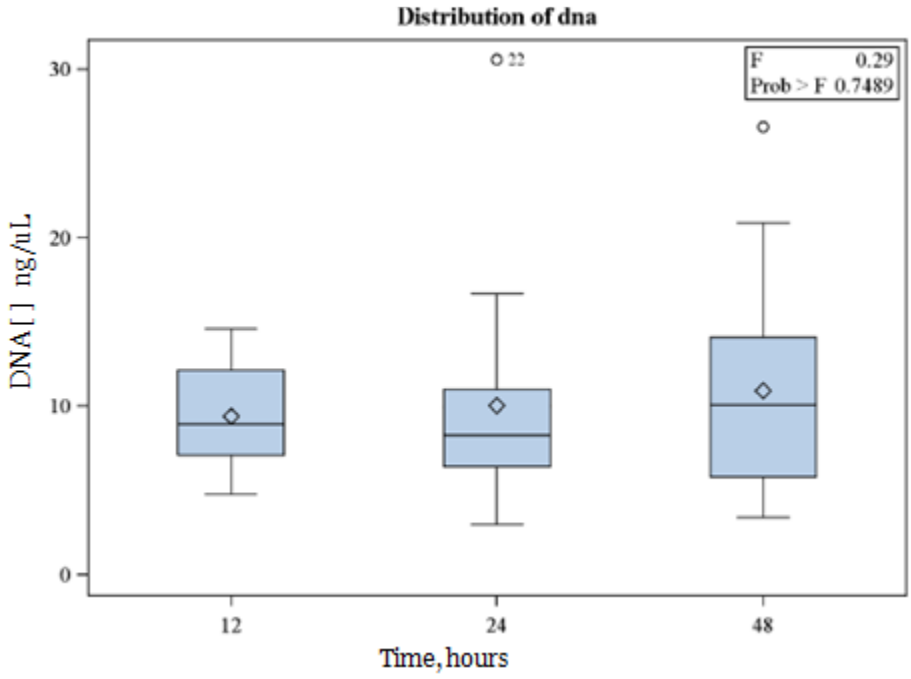
For the first experiment DNA concentration did not vary among times. However, the data suggested that there might have been a trend for an increase of DNA concentration over time (Figure 2).

Figure 2. DNA concentrations extracted from primary feathers by each of the 3 methods for each of the 5 time categories for all samples (n = 45).



On this evidence it was decided to run the second experiment at 12, 24, and 48 hours, the time interval where the increasing DNA concentration trend appeared to be. While the second experiment was powered up by running a larger number of samples no significant difference in DNA concentration extracted among times was demonstrated (Figure 3).

Figure 3: Box and whisker plot demonstrating average concentration of DNA extracted with Method 2 when a 12, 24, and 48 hour incubation was used.



Looking at the data from these experiments there are several things to note. Between the first and second experiment the feather type used for the extractions was changed. Much smaller body feathers were used for Experiment 2. Due to this the mass of feather used was likely less even though approximately the same length of feather culmus was used. Which may have contributed to the DNA concentrations obtained being less in Experiment 2.

With these smaller amounts of DNA the date from the Nanodrop becomes questionable. The negative controls for the second experiment came back with a reading for DNA concentration and this might have been background noise or actual contamination. Thus, the results from the second experiment are questionable.

Each sample for each method was incubated at the same temperature, despite each method calling for a different temperature, thus removing a possible additional variable in the experiments. The temperature decided for incubation was 54° C, this was chosen as an average

of the three different method specific temperatures. This average temperature was close enough to the original temperature of each method to likely have had minimal effect.

Conclusion

This study has concluded that the best method for DNA extraction was the method used in Dr. Waits' laboratory (Method 2). This method had significantly higher yields than the other methods. Future studies could further explore the effect of time on DNA concentration, especially due to the questionable nature of the results from the second experiment.

Other studies have found that the DNA extracted from feathers can be questionable in quality (McDonald & Griffith, 2011). This study did not test the quality of the DNA extracted. However, research is planned in the Culver Lab to investigate this. The quality of DNA extracted from Bald Eagle body feathers with Method 2 will be compared to DNA extractions from whole blood. If feathers are shown to be an effective and reliable source of quality DNA the Arizona Game and Fish Department plans to change their handling protocol. The possible changes would reduce handling time and possibly the stress incurred by study species.

One last point of interest was a trend related to DNA concentrations extracted with increased time. There was an increased variability in the concentration of DNA as incubation time increased noted in both experiments. The reasons behind this trend could be the focus of future research as well.

Appendix One

1. For blood with nonnucleated erythrocytes, follow step 1a; for blood with nucleated erythrocytes, follow step 1b; for cultured cells, follow step 1c.

Blood from mammals contains nonnucleated erythrocytes. Blood from animals such as birds, fish, or frogs contains nucleated erythrocytes.

1a. Nonnucleated: Pipet 20 µl proteinase K into a 1.5 ml or 2 ml microcentrifuge tube (not provided). Add 50–100 µl anticoagulated blood. Adjust the volume to 220 µl with PBS. Continue with step 2.

Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml) and incubate for 2 min at room temperature before continuing with step 2.10nimal Blood (Spin-Column Protocol)

1b. Nucleated: Pipet 20 µl proteinase K into a 1.5 ml or 2 ml microcentrifuge tube (not provided). Add 50-100 µl anticoagulated blood. Adjust the volume to 220 µl with PBS. Continue with step 2.

Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml) and incubate for 2 min at room temperature before continuing with step 2.

1c. Cultured cells: Centrifuge the appropriate number of cells (maximum 5 x 106) for 5 min at 300 x g. Resuspend the pellet in 200 µl PBS. Add 20 µl proteinase K. Continue with step 2.

When using a frozen cell pellet, allow cells to thaw before adding PBS until the pellet can be dislodged by gently flicking the tube. Ensure that an appropriate number of cells is used in the procedure. For cell lines with a high degree of ploidy (e.g., HeLa cells), it is recommended to use less than the maximum number of cells listed in Table 1, page 16.

Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml), mix by vortexing, and incubate for 2 min at room temperature before continuing with step 2.

2. Add 200 µl Buffer AL (without added ethanol). Mix thoroughly by vortexing, and incubate at 56°C for 10 min.

Ensure that ethanol has not been added to Buffer AL (see “Buffer AL”, page 18). Buffer AL can be purchased separately (see page 56 for ordering information).

It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution.

3. Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing. It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.

4. Pipet the mixture from step 3 into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.*

5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.*

6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

7. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 xg (8000 rpm) to elute.

Elution with 100 µl (instead of 200 µl) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield (see Figure 2, page 21).

8. Recommended: For maximum DNA yield, repeat elution once as described in step 7.

This step leads to increased overall DNA yield.

A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the microcentrifuge tube from step 7 can be reused for the second elution step.

Note: Do not elute more than 200 µl into a 1.5 ml microcentrifuge tube because

the DNeasy Mini spin column will come into contact with the eluate.

Appendix Two
User-Developed Protocol:
Isolation of genomic DNA from nails and hair using the QIAamp® DNA Mini Kit*
This procedure has been adapted by customers from the QIAamp® Tissue Protocol, and is for use with the QIAamp DNA Mini Kit. It has not been thoroughly tested and optimized by QIAGEN.
Please note that lysis time will vary depending on the size and density of the source material. Please be sure to read the QIAGEN® *QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook* and the detailed QIAamp Tissue Protocol carefully before beginning this procedure.
Procedure
1. Cut the sample into small pieces, place in a 1.5 ml microcentrifuge tube, and add 200 µl Buffer X1. Incubate at 55°C for at least 1 h until the sample is dissolved. Invert the tube occasionally to disperse the sample, or place on a rocking platform.
Buffer X1:
10 mM Tris·Cl pH 8.0
10 mM EDTA
100 mM NaCl
40 mM DTT
2% SDS
250 µg/ml Proteinase K
Just before use, add the appropriate volume of Proteinase K stock solution (20 mg/ml) supplied with the QIAamp DNA Mini Kit.
DTT oxidizes quickly in aqueous solutions and should also be added just before use. Store the DTT stock solution (1 M) at □–20°C.
2. Add 200 µl Buffer AL and 200 µl ethanol to the sample and mix by vortexing.
** This protocol has also been successfully used for the isolation of DNA from bird feathers. Note: Feather quills will remain undissolved during step 1; therefore, it will be necessary to transfer the tube supernatant to a new microcentrifuge tube at the end of step 1.*

2. Continue with step 5 of the Tissue Protocol in the *QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook.*
3. Elute the DNA in 50—100 µl Buffer AE or distilled water.
Note: Elution in 50 µl will yield more concentrated DNA, whereas elution in 100 µl will recover a greater amount of DNA. If the expected amount of DNA is not known, it is preferable to elute in several aliquots of 50 µl. These can then be combined if necessary.
Elution of the DNA in Buffer AE is recommended if the DNA is to be stored, since DNA stored in water is subject to acid hydrolysis.
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Appendix Three

Protocols for DNA Extraction from Feathers

Avian feathers using the QIAgen DNeasy Tissue Kit

1. Fill out the extraction sheet with the requested info (Lab ID and species ID).
2. Set up labeled 1.5mL tubes for each sample, plus an extraction negative.
3. Add 180ul Buffer ATL to each tube.
4. Add 20ul Proteinase K to each tube.
5. Vortex
6. For each sample, cut up ~1cm from tip of feather into tube (use scissors or clippers). If there is any crispy membrane inside the hollow quill, pull that out and place in tube also.
7. Be sure all of feather pieces are immersed in solution (centrifuge tubes briefly to be sure if you must).
8. Sterilize equipment between samples
9. Repeat steps 6-8 for all samples.
10. Incubate at 56°C overnight.

Next Day:

1. Preheat appropriate volume of Buffer AE to 70°C (100ul X # samples + a little extra), and also set up appropriate number of screw cap tubes under the UV light for sterilization.
2. Add 200ul Buffer AL to each tube, vortex, and incubate at 70°C for 45 minutes.
3. Remove carefully. If there is foaming at the opening, wipe each individual tube with part of a paper towel soaked with bleach. Wipe your gloves on a different towel that is soaked with bleach.
4. Add 200ul 100% EtOH, vortex and incubate for a few minutes at room temp.
5. Transfer to spin column/collection tube (make sure these are labeled with LabID correctly!) and centrifuge for 1 minute at 10,000rpms.
6. Discard collection tube and filtrate and place spin column in a clean collection tube.
7. Add 500ul of Buffer AW1 and centrifuge for 1 minute at 10,000rpms.
8. Discard filtrate and collection tube.
9. Place column in clean collection tube, add 500ul of Buffer AW2.
10. Centrifuge for 3 minutes at 12,000rpms to dry membrane. Discard filtrate and place spin column in clean 1.5mL microcentrifuge tube (with lid cut off).
11. Add 100ul of Buffer AE preheated to 70°C directly to the filter (not to sides of tube).
12. Incubate at 70°C for 5 minutes.
13. Centrifuge for 1 minute at 10,000rpms.
14. ONE AT A TIME – remove the filter (do not discard), extract the sample from the 1.5ml microcentrifuge tube, replace the filter into the now empty tube and pipette the 100 µl of solution onto the filter (this helps increase the concentration of DNA in the extraction)
15. Incubate at 70°C for 1 minute.
16. Centrifuge for 1 minutes at 10,000rpms.
17. Transfer filtrate to labeled (lab ID, date, and your initials) screw cap tube.
18. Store screw cap tubes in Styrofoam box in fridge.
19. Record all lot numbers for stock used and anything abnormal that should be noted.

Appendix Four

Qiagen DNeasy® Tissue Kit - Animal Tissue Protocol Modification

(Qiagen (2003) DNeasy® Tissue Handbook. Protocol for isolation of total DNA from animal tissues, pp. 18-20. QIAGEN (Valencia, California, U.S.A.).

Protocol for Molted Body Contour Feathers

DAY 1

(1) Cut the tip of the feather sample into a labeled 1.5ml microcentrifuge tube with sterile fine tipped scissors. For molted feathers, use one feather per DNA extraction, as not to cross contaminate samples. If the feather tip is very large (e.g. from a wing or tail feather), cut the tip into several pieces so that it can be fully immersed in the liquid for the overnight incubation step.Do not handle the tip of the feather, as not to remove the DNA containing cells.

(2) Add 200µl of Buffer ATL to each microcentrifuge tube.

(3) Add 20µl Proteinase K and vortex for 5-20 seconds. Incubate the samples at 50˚C for 24-48 hours. Extended incubations can lead to more effective lysis of the DNA containing cells and a higher end product DNA concentration.

DAY 2

(4) Add 200µl of Buffer AL and vortex for 5-20 seconds. Incubate the samples at 70˚C for 10 minutes.

(5) Add 200µl of cold 100% ethanol and vortex for 5-20 seconds.

(6) Carefully pipette the mixture from Step 5 into the labeled Qiagen spin column and centrifuge at 10,000 x gravity for 1 minute. If any of the samples still have visible liquid in the spin column due to debris in the sample, re-centrifuge at 14,000 x gravity for 2 minutes. Discard the tube containing the flow through.

(7) Add 500µl of Buffer AW1 to each spin column and centrifuge at 10,000 x gravity for 1 minute. Discard the tube containing the flow through.

(8) Add 500µl of Buffer AW2 to each spin column and centrifuge at 14,000 x gravity for 3 minutes. Discard the tube containing the flow through.

(9) Put each spin column in a labeled microcentrifuge tube and discard the tube containing the filtrate. Add 200µl of Buffer AE to the spin column and incubate at room temperature for 1 - 5 minutes. Centrifuge at 7000 x gravity for 1 minute and discard the spin columns. Freeze the extracted DNA samples at -20˚C for future use. DNA extracted from any kind of feather produces low quality DNA and 5-10µL of DNA is required per 15µL PCR reaction.

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