World Rabbit Science

World Rabbit Sci. 2012, 20: 117 - 124
doi:10.4995/wrs.2012.1077
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RELIABILITY OF NON-INVASIVE TISSUE SAMPLING METHODS FOR DNA EXTRACTION IN RABBITS (*ORYCTOLAGUS CUNICULUS*)

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Abstract: Deoxyribonucleic acid (DNA) can be extracted from different tissue sources. The most common is blood, but in some situations it can be easier to take a biopsy. In some cases when it is difficult to capture animals, especially in wild populations, faeces and hairs can be considered as a source of DNA. This paper presents a pilot study conducted to compare the applicability of invasive and non-invasive sampling methods for extracting DNA for use in genetic studies of rabbits (*Oryctolagus cuniculus*). The study included 24 rabbits from the INRA 1001 strain. Blood, hair, ear biopsies and faeces were collected and used as DNA sources. Our aim was to verify the quantity of DNA obtained from different tissues using 2 or 3 types of extraction. DNA was obtained for all tissue types and all extraction methods. DNA extraction was shown to be optimal with the LGC (Laboratory of Cellular Genetics) blood extraction method. With regard to non-invasive methods, DNA extraction for hair using the LGC protocol and QIAamp® DNA mini kit gave very low quantities of DNA that could not be used for PCR reactions. The Chelex extraction protocol gave good results for PCR but could not be quantified. DNA extracted from faeces is a viable source of DNA for determining individual genotypes. The use of such non-invasive samples as a source of genetic material is a recent and very promising technique, especially for the study of endangered species, but these techniques are still too unreliable and costly to altogether replace invasive techniques when the latter are possible.

Key Words: rabbit, DNA extraction, tissue sampling, faeces, hair.

INTRODUCTION

The use of molecular techniques for the management of endangered animal species has become an invaluable tool for the conservation biologist. Alternative approaches are therefore encouraged in wildlife studies. Non-invasive genetic analysis is a powerful tool that avoids over-handling stress-sensitive animals (Kohn *et al.*, 1997) while allowing studies on social organisation and mating systems (Archie *et al.*, 2008). Molecular genetic techniques have rapidly revolutionised the way natural populations are studied. The ease with which these techniques are applied to a wide variety of organisms and questions continues to improve steadily. However, the collection of genetic material from free-ranging animals can prove challenging. Difficulty in collecting genetic material can be further exacerbated when traditional invasive techniques such as biopsy darting or blood sampling are impractical or detrimental to ongoing studies (Green *et al.*, 2007).

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http://dx.doi.org/10.4995/wrs.2012.1077

Blood, hair, biopsy samples and intestinal epithelial cells recovered from faeces are the preferred biomaterial for genetic studies. The highest priority when obtaining biomaterials for genetic studies is the safety of the biologist and welfare of the animal.

Genetic studies in rabbits call for tissue samples for both genome analysis and population studies. Sampling procedures differ according to the livestock system (Ben Larbi *et al.*, 2008). However, the use of non-invasive collecting techniques is yet to be described in rabbits, and only Fontanesi *et al.* (2007) have to date reported their use of hair and buccal swab sampling for PCR analysis.

The purpose of this study was to determine DNA yields obtained from various rabbit (*Oryctolagus cuniculus*) tissue samples using different extraction methods.

MATERIAL AND METHODS

Animals

Twenty fourth young (approx. 90 d old) male or female rabbits, from the PECTOUL farm on the INRA facilities, were used in this study. A blood, faecal and hair sample as well as two ear biopsy samples were collected from each animal.

Sample collection and conservation

Blood was drawn from the marginal ear vein into a 5 mL tube containing EDTA as anticoagulant. It was then stored at +4°C for 1 wk.

For hair samples, 2 tufts of hair were plucked from each animal's back using surgical tweezers, which were cleaned with alcohol between samples. Hair samples were stored at –20°C in individual envelopes, each of which was packaged again in plastic waterproof bags (Ziploc type) as described by Roon *et al.* (2003).

Regarding ear biopsies, we used a standard Biopsitec ear punch (2 mm diameter) to take samples from the pinna. Biopsied ear samples were stored at –20°C in Biopsitec individual tubes containing silica gel beads.

For faecal samples, shavings were placed under the cages of the rabbits to avoid mixing of faeces between animals. Faeces were collected from distinct individuals, as fresh as possible, with a pair of disposable gloves. Faecal samples were placed in sealed jars and then frozen at –20°C. A scalpel blade was used to scrape and collect 2 g of material from the outer surface of the pellets of faeces which were considered to contain the intestinal epithelial cells (Frantzen *et al.,* 1998).

DNA extraction

All extractions were carried at the Laboratory of Cellular Genetics (LGC) at the INRA facilities. For blood, biopsy and hair samples we used 2 extraction protocols: the LGC extraction protocol used at LGC (unpublished, adapted from Miller *et al.*, 1988) and a commercial kit (QIAamp® DNA mini kit QIAGEN product). Faecal DNA was extracted with the QIAamp® DNA Stool mini kit specially developed for this type of material and following the manufacturer's instructions. In this case, we were unable to use the LGC extraction protocol because it is not appropriate for this type of samples. The Chelex® method was also used for hair samples (Gallan *et al.*, 2005).

In the case of blood, the LGC protocol consists of resuspending 2 mL of blood in 10 mL polypropylene centrifugation tubes with 3 mL of nuclei lysis buffer (NH_4Cl 150 mM, KCl 10 mM, EDTA 0.1 mM) followed by centrifugation at 4 000 rpm (4°C) for 15 min. The

pellet of white cells is resuspended in 5 mL of saline solution (NaCl 140 mM, KCl 0.5 mM and Tris HCl 0.25 mM, pH 7.4). The supernatant should be clear. Centrifuging is performed at 3200 rpm for 5 min after each wash and the supernatant removed by gently rotating the tube. The cell lysates are digested overnight at 37°C with 100 µL of 10% sodium dodecyl sulfate, 100 µL EDTA 0.5 mM pH 8 and 20 µL of a protease K. After digestion, 860 µL of saturated NaCl (approximately 6 M) are added to each tube and shaken vigorously for 15 s, followed by centrifugation at 8 000 rpm for 20 min. The precipitated protein pellet is left at the bottom of the tube and the supernatant containing the DNA is transferred to another 15 mL polypropylene tube. Exactly 2 volumes of room temperature absolute ethanol are added and the tubes are inverted several times until the DNA is precipitated. The precipitated DNA strands are resuspended using a pipette by transferring to a 1.5 mL microcentrifuge tube containing 200 µL TE buffer (10 mM Tris-HCl, 0.2 mM Na2EDTA, pH 7.5). The DNA is allowed to dissolve overnight at 37°C before quantitating. The same protocol is used in the case of biopsy except the nucleic lyse step.

The Chelex method extraction protocol for was as follows: sterile 1.5 microtubes containing the hair bulbs were filled with 200 µL resin solution of 10% Chelex-100. We then added 10 µL proteinase K at 10 mg/mL. The tubes were vortexed and incubated in a water bath at 56°C overnight. The next day, the tubes were again vortexed and then briefly centrifuged to collect the Chelex beads at the bottom of the tubes. We removed 2 µL from the supernatant containing the extracted DNA, which we then transferred to 200 µL PCR tube. We then immediately carried out PCR to avoid degradation of the DNA in view of the low concentrations obtained. We immediately froze the other tubes to avoid any degradation of the extracted DNA.

DNA quality: Nanodrop™

The Nanodrop™ system provides information concerning the purity of the DNA. The purity of nucleic acid samples was determined by measuring the absorbance at wavelengths of 260 and 280 nm for all samples except those for which DNA was extracted using the Chelex protocol. In the latter, DNA quality and quantity cannot be determined because Chelex is essentially a resin, which cannot be quantified with Nanodrop or visualised by gel electrophoresis (Figure 1). An A_{260}/A_{280} ratio greater than 1.8 indicated that samples contained only low levels of protein contaminants.

DNA quantity: Picogreen®

Eleven samples were selected randomly for each tissue type. The Quant-iT™ dsDNA Broad-Range Assay Kit makes DNA quantification easy and accurate. The kit contains concentrated assay reagent, dilution buffer, and pre-diluted DNA standards. The assay is highly selective for double-stranded DNA over RNA, and the fluorescence signal is linear for DNA in the range of 2-1 000 ng. The assay is performed at room temperature, and the signal is stable for 3 h. Common contaminants, such as salts, solvents, detergents or protein are well tolerated in the assay. In addition to the Quant-iT™ dsDNA Broad-Range Assay Kit described here, Molecular Probes offers the Quant-iT™ dsDNA High-Sensitivity Assay Kit (Q33120), designed for assaying samples containing 0.2-100 ng of DNA.

The Quant-iT™ dsDNA High-Sensitivity Assay Kit is used with a fluorescence microplate reader equipped with excitation and emission filters appropriate for fluorescein or Alexa Fluor® 488 dyes. The microplate contains 96 wells; we used 88 wells for the tested DNA samples (11 samples from each type of tissue and extraction) and 8 wells for the λ DNA standards to determine the DNA amounts with a Fluorescence Microplate Reader.

The Quant-iT™ dsDNA High-Sensitivity Assay Kit (Q33120) was used in this work to quantify DNA.

DNA characterisation

Gel electrophoresis: The quality of the genomic DNA extracted was analysed by gel electrophoresis for the same 11 samples as used for the Picogreen method. For this analysis, 10 µL of extracted DNA was loaded on a 0.8% agarose slab gel, stained with ethidium bromide and photographed under Sample collection and conservationtraviolet light.

PCR reaction: To confirm that the DNA extracted from faecal samples and using the Chelex protocol contained rabbit DNA, we amplified 2 rabbit microsatellites (Sat 2 and Sat 12, Mougel *et al.*, 1997) in 12 randomly selected samples from each tissue types.

Each 25 µL PCR reaction contained template DNA (concentration ranging from 5 to 50 ng/µL), forward and reverse primers (0.25 mM each), Taq polymerase (0.25 U), 1× buffer, $MgCl_2$ (1.5 mM) and dNTPs (0.2 mM). The PCR profile included 10 min at 94°C, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and an extension of 15 min at 72°C.

PCR products were then analysed by gel electrophoresis. For this analysis, 10 µL aliquots of PCR product were loaded on a 2% agarose slab gel, stained with ethidium bromide and photographed under ultraviolet light.

Statistical analysis

Data were analysed with SAS software (Statistical Analysis System). Because of the non-normal distribution of the trait, we used logarithmic transformation to analyse the DNA quantity. Log DNA and A_{260}/A_{280} ratio (quality) were analysed with the GLM procedure, given that the characteristics and distribution of transformed traits allowed the use of parametric tests. The model included the fixed effects of the tissue type and of the extraction method used for each tissue type. The significance of effects was determined with the F-Fisher test and least-square means were compared 2 by 2 using Student's test (Table 1). We used the parametric tests despite the small numbers of samples, due to the difficulty in finding a description of the tests and their tables of significant value using non-parametric tests.

RESULTS

DNA quantity and quality

The effects of the tissue type and the extraction method used for each tissue type were highly significant in both the DNA quantity and quality (Table 1). Even if the quantity of DNA was not comparable between blood (1 mL), biopsy (2 mm disk), faeces (2 g of the external wall) and hair (2 tufts) samples, we observed that the greatest amount of DNA could be obtained from blood using the LGC extraction method and that the least was obtained from hair, especially when the QIAamp DNA mini kit was used (low quantity and a A_{260}/A_{280} ratio 2.55±0.11). The contamination of DNA extracted from hair used kit is not protein contamination, since that would reduce the ratio. It is more likely due to traces of alcohols or even some salts still in the sample.

DNA quality, characterised by a A_{260}/A_{280} ratio greater than 1.8, was good for all samples, except for hair and biopsy samples with the LGC extraction method, which showed values significantly lower than the others, (1.09±0.11 and 1.64±0.07, respectively**)**.

Table 1: Results of the analysis of variance of DNA quantity and quality. Least square means ± standard error.

Tissue	Extraction method[1]	Log DNA quantity	DNA quantity (µg)[2]	A_{260}/A_{280} ratio
Biopsy	LGC	2.35^{c}±0.27	11.69	1.64^{ab}±0.07
	Commercial kit	1.88^{c}±0.27	7.43	1.9^{b}±0.07
Blood	LGC	2.27^{c}±0.27	12.58	2.12^{b}±0.08
	Commercial kit	1.71^{c}±0.27	6.52	1.88^{b}±0.08
Hair	LGC	1.10^{b}±0.28	2.27	1.09^{a}±0.11
	Commercial kit	0.07^{a}±0.28	1.82	2.55^{b}±0.11
Faeces	Commercial kit	1.81^{c}±0.27	6.89	2.05^{b}±0.07

[1] Method: LGC, extraction protocol of the Laboratory of Cellular Genetics (INRA); Commercial kit, QIAamp® DNA mini kit QIAGEN product. [2] No statistical analysis performed for DNA quantity (performed on Log DNA). [a,b,c] In a column, means with different letters were significantly different (P<0.05).

DNA characterisation

Gel electrophoresis: Gel electrophoresis provided information on the overall quality of the DNA. We observed that DNA was obtained from all tissue types, whatever the extraction method. DNA was shown to be partially degraded only in the case of DNA extracted from biopsy and hair samples with the LGC extraction protocol (Figure 1).

PCR reactions: The results of the PCR reactions proved that the DNA extracted was from rabbit cells and that its quantity and quality were sufficient to initiate amplification (Figure 2).

The object of our experiment is to confirm that DNA extract from faecal samples contained rabbit DNA; we did not check the absence of contamination by bacterial DNA, although it is common with this type of sample.

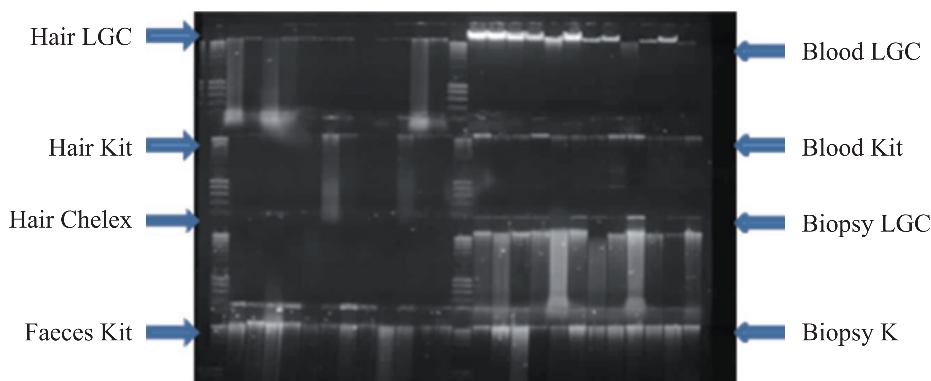

Figure 1: Agarose gel of DNA extracted from different tissues and using different methods. LGC, extraction protocol of the Laboratory of Cellular Genetics (INRA); Chelex, Chelex® protocol; Commercial kit, QIAamp® DNA mini kit QIAGEN product..

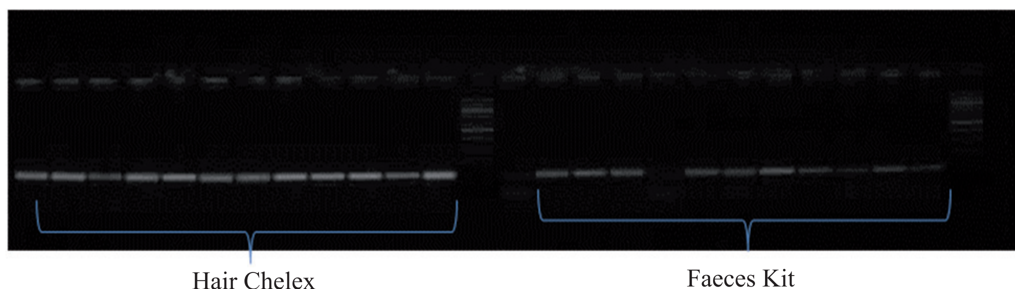

Figure 2: Amplification of DNA. Hair Chelex extracted from hair using the Chelex® protocol. Faeces kit extracted from faeces using a commercial kit (Commercial kit, QIAamp® DNA mini kit QIAGEN product).

DISCUSSION

Our aim was to determine the quantity of DNA obtained from different commonly used tissue samples using several different extraction methods. All tissue samples and extraction methods provided rather good quantities of DNA. So, non-invasive biological specimen collection methods can be readily implemented for DNA analysis when conducting molecular genetic studies in rabbits.

Blood

This experiment confirmed that extracting DNA from blood samples is a very efficient and valuable method in regards to DNA quantity and quality. However, collecting blood samples from the marginal vein of rabbit ears can be a difficult and long procedure.

Hairs

The DNA from hairs is issued solely from cells in the hair root (follicle). A hair that is cut off or falls out naturally without the hair follicle does not contain any DNA. Even when collected in ideal conditions, hair samples generally provide only a very small amount of DNA, a few picograms at the most (in bears, Taberlet *et al.,* 1996; and in chimpanzees, Morin *et al.,* 2001). Hair samples represent an extremely simple way of collecting biological material from rabbits and hundreds of samples can be obtained easily within a short time. Concerning extraction techniques, we found that the Chelex extraction method gave stronger PCR amplification products than both the QIAamp® DNA mini kit extraction procedure and the LGC protocol. The LGC protocol and QIAamp® DNA mini kit gave a very low quantity of DNA that could not be used for PCR reactions. This inability to amplify the samples was probably due to the small number and/or poor quality of the hair roots selected from very small tufts of hair (Fontanesi *et al.*, 2007).

We retained the Chelex extraction protocol for subsequent analyses, which not only provided good results in PCR but was also cheaper. However, the disadvantage of this protocol is that the DNA extracted by Chelex cannot be quantified, so the DNA obtained this way cannot be used for other analyses such as SNP genotyping.

The LGC protocol for DNA extraction from hair samples represents a good technique for future use, although it needs to be improved by: optimising the hair removal technique; identifying the most convenient region of the body for sampling, which is also the least painful for the animal

and provides the most hair roots; determining the optimal quantity of hair roots for a sufficient amount of DNA.

Biopsy

Biopsy is commonly used in swine. In our experiments, we obtained DNA of good quantity and quality as measured by the A_{260}/A_{280} ratio, but gel electrophoresis evidenced its degradation. The same results were obtained in pigs (Laval *et al.*, 2000). Further effort must be done to optimise this LGC protocol.

Faeces

During the passage of food in the gut, epithelial cells detach from the intestinal wall and agglutinate on the surface of the residues before they are ejected. Thus, the DNA from these cells can be used to determine the genetic fingerprint of an individual from faeces (Mainguy and Bernatchez, 2007).

DNA extracted from non-invasive samples, in particular faeces, is usually of poor quality (i.e. degraded DNA, presence of PCR inhibitors and DNA contamination) (Baldwin *et al.*, 2010). Herbivores potentially pose a faecal DNA challenge due to the inhibitory effects of plant secondary compounds on PCR reactions, and PCR success may depend on maximising the concentration of intact DNA from the target animal, while minimising secondary compounds derived from plants in the diet (Fernando *et al.,* 2003).

Obtaining satisfactory DNA extracts is the main difficulty when using faecal sampling for PCR. PCRs may fail because of the degradation of DNA and/or the presence of inhibitors if the extraction protocol used is inappropriate. Wehausen *et al.* (2004) proved that PCR amplifications were consistently excellent only when the very outer pellet material was used. In contrast, when any inner pellet material was included, PCR success declined, accompanied by increased variation among samples. Our study on *Oryctolagus cuniculus* shows that DNA extracted from faeces is a viable source of DNA when determining individual genotypes, and that it could improve population genetics studies, thus avoiding invasive and risky sampling of wild populations.

CONCLUSION

The use of non-invasive samples as a source of genetic material is a recent and very promising technique, especially for the study of endangered species. For animals whose capture is impossible or very risky, non-invasive sampling is the only method whereby this information may be obtained. Nevertheless, these techniques are still too unreliable and costly to altogether replace invasive techniques when the latter are possible. On the other hand, in most cases these techniques do not provide individual information and so may be irrelevant for some genetic studies.

REFERENCES

Archie E.A., Maldonado J.E., Hollister-Smith J.A. 2008. Fine-scale population genetic structure in a fission-fusion society. *Mol. Ecol., 17: 2666-2679. doi: 10.1111/j.1365-294X.2008.03797.x*

Baldwin H.J., Hoggard S.J., Snoyman S.T., Stow A.J., Brown C. 2010. Non-invasive genetic sampling of faecal materiel and hair from the grey-headed flying-fox (*Pteropus poliocephalus*). *Australian Mammalogy, 32: 56-61. doi: 10.1071/AM09015*

Ben Larbi M., Haddad B., Allalout S. 2008. Characterization of traditional rabbit breeding system used in the south of Tunisia. *In Proc.: 9th World Rabbit Congress, 10-13 June, 2008. Verona, Italy. 1505-1508.*

Fernando P.T., Vidya N.C., Pajapakse C., Dangolla A., Melnick D.J. 2003. Reliable noninvasive genotyping: fantasy or reality. *J. Hered., 94: 115-123. doi: 10.1093/jhered/esg022*

Fontanesi L., Tazzoli M., Russo V. 2007. Non-invasive and simple methods for sampling rabbit DNA for PCR analysis of melanocortin 1 receptor (MCR) gene mutations: a technical note. *World Rabbit Sci., 15: 121-126. doi: 10.4995/wrs.2007.598*

Frantzen M.A.J., Silk J.B., Ferguson J.W.H., Wayne R.K., Kohn M.H. 1998. Empirical evaluation of preservation methods for faecal DNA. *Mol. Ecol., 7: 1423-1428. doi: 10.1046/j.1365-294x.1998.00449.x*

Gallan M., Baltzinger C., Hewison A.J.M., Cosson J.F. 2005. Distinguishing red and roe deer using DNA extracted from hair samples and the polymerase chain reaction method. *Wildlife Soc. B., 33: 204-211. doi: 10.2193/0091-7648(2005)33[204:DRARDU]2.0.CO;2*

Green M.L., Herzing D.L., Balldwin J.D. 2007. Noninvasive methodology for the sampling and extraction of DNA from free-ranging Atlantic spotted dolphins (*Stenella frontalis*). *Mol. Ecol. Notes, 7: 1287-1292. doi: 10.1111/j.1471-8286.2007.01858.x*

Kohn M.K., Wayne R.K. 1997. Facts from feces revisited. *Trends Ecol. Evol., 12: 223-227. doi: 10.1016/S0169-5347(97)01050-1*

Laval G., Iannuccelli N., Legault C., Milan D., Groenen M.A., Giuffra E., Andersson L., Nissen P.H., Jørgensen C.B., Beeckmann P., Geldermann H., Foulley J.L., Chevalet C., Ollivier L. 2000. Genetic diversity of eleven European pig breeds. *Genet. Sel. Evol., 32: 187-203. doi: 10.1186/1297-9686-32-2-187*

Mainguy J., Bernatchez L. 2007. L'analyse de l'ADN sans manipulation des animaux: un outil incontournable pour la gestion et la conservation des espèces rares et élusives. *Nat. Can., 131: 51-58.*

Miller S.A., Dykes D.D., Polesky H.F. 1988. A sampling salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res., 16: 1215. doi: 10.1093/nar/16.3.1215*

Morin P.A., Chambers K.E., Boesch C.H., Vigilant L. 2001. Quantitative polymerase chain reaction analysis of DNA from non-invasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*). *Mol. Ecol., 10 : 1835-1844. doi: 10.1046/j.0962-1083.2001.01308.x*

Mougel F., Mounolou J-C., Monnerot M. 1997. Nine polymorphic microsatellite loci in the rabbit, *Oryctolagus cuniculus*. *Anim. Genet., 28: 58-71. doi: 10.1111/j.1365-2052.1997.00047.x*

Roon, D.A., Waits L.P., Kendall K.C. 2003. A quantitative evaluation of two methods for preserving hair samples. *Mol. Ecol. Notes, 3: 163-166. doi: 10.1046/j.1471-8286.2003.00358.x*

Taberlet, P., Griffin S., Goossens B., Questiau S., Manceau V., Escaravage N., Waits L.P., Bouvet J. 1996. Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Res., 24: 3189-3194. doi: 10.1093/nar/24.16.3189*

Wehausen J.D., Ramey II R.R., Epps C.W. 2004. Experiments in DNA extraction and PCR amplification from Bighorn sheep feces: The importance of DNA extraction methods. *J. Hered., 95: 503-509. doi: 10.1093/jhered/esh068*