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Optimal Storage Conditions for Highly Dilute DNA Samples: A Role for Trehalose as a Preserving Agent

ABSTRACT: DNA extraction from trace samples or noninvasively collected samples often results in the recovery of low concentration solutions of DNA that are prone to DNA degradation or other loss. Because of the difficulty in obtaining such samples, and their potentially high value in wildlife and forensic studies, it is critical that optimal methods are employed for their long-term storage. We assessed the amplification yield of samples kept under different storage conditions with the addition of potential preserving agents. We stored dilutions of known concentration human placental DNA, and gorilla fecal DNA, under four conditions (+4°C, −20°C, −80°C, dry at room temperature), and with three additives (Tris EDTA (TE) buffer, Hind III digested Lambda DNA, trehalose). The effectiveness of the treatment methods was tested at regular intervals using qPCR to assess the quantity of amplifiable DNA, and a PCR assay of a larger 757 bp fragment to evaluate the quality of that remaining DNA. The highest quantity of DNA remained in samples stored at −80°C, regardless of storage additives, and those dried at room temperature in the presence of trehalose. Surprisingly, DNA quality was best preserved in the presence of trehalose, either dried or at −80°C; significant quality loss occurred with −20°C and +4°C storage.

KEYWORDS: forensic science, DNA storage, DNA degradation, trehalose, noninvasive samples, qPCR

Technical advances in recent years have opened the scope of DNA studies to include all types of valuable samples. These include forensic samples, rare organisms, noninvasively collected samples, and very small sample types such as DNA from hair and sloughed skin. Noninvasive sample collection, in particular, has increasingly been used to provide access to genetic material for wildlife studies where access to tissue or blood is not possible (1,2–6). Recent improvements in extraction methods and genotyping protocols have allowed the use of these samples to provide important data for forensic and wildlife genetic studies (7,8–11). There remain, however, a number of key issues to be addressed to ensure optimal exploitation of these valuable but technically problematic samples. The quality and usefulness of extracts from non-traditional samples vary widely and are thought to be greatly influenced by such factors as: collection method and time prior to extraction (9,12,13); method employed for DNA isolation (9,10,14); and also storage and handling of DNA extracts prior to genotyping (8,14,15). While sample collection and DNA extraction have been studied to some extent, there is little more than "laboratory folklore" to guide researchers seeking information on optimal storage conditions for DNA extracts. The aim of this project was to investigate factors influencing degradation in the quality and quantity of DNA extracts over time and to formalize a preferred approach to the handling and storage of low concentration DNA extracts.

Two DNA types, high quality human placental DNA and gorilla fecal DNA, were used to assess the long-term storage outcomes on dilute DNA of different starting quality. These DNA preparations were deliberately chosen to represent the range of sample types that researchers may encounter in wildlife and forensic studies, from low concentration and highly degraded through to moderate concentrations of highly pure DNA. In line with common laboratory practice, the main storage conditions for testing were +4°C and frozen at both −20°C and −80°C. Additionally, dry storage was tested as an alternative for field laboratories or other situations where freezer space is limited (16,17). An earlier study by Gaillard and Strauss (18) reported a significant effect from tube type on the amount of DNA adsorption during storage so this was avoided as a confounding variable in this experiment by using only one tube type for all treatments.

Three storage buffers identified from previous research studies as solutions with high potential for storage enhancement (15,19,20) were selected for analysis in this study. Tris-EDTA buffer is widely used as a solution for the long-term storage of DNA aliquots due to the action of the EDTA to bind divalent cations that are necessary cofactors for DNA nucleases (21). HindIII digested Lambda DNA has been used as an additive to enhance the storage of standards for use in competitive PCR (15) and for improved transformation efficiency with fungal DNA (22). Lambda DNA acts as a carrier DNA such that it is preferentially adsorbed to the tube wall, making more of the target DNA freely available in solution for PCR or other applications. Trehalose is a compound found in high concentration in organisms that undergo periods of desiccation as part of their life cycle (cryptobionts). The exact mechanism of the protection conferred by this disaccharide at a molecular level is unclear, but it is thought to replace water molecules and make multiple external hydrogen bonds which are essential in the maintenance of tertiary structure within biological molecules (20). We examine the effect of each of these buffers in combination with storage temperature to assess the best conditions for the long-term storage of highly dilute DNA samples.

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Materials and Methods

DNA Preparation and Plate Set-up

Two types of genomic DNA were chosen as templates for this experiment: high quality human placental DNA (Sigma D-4642), and gorilla fecal DNA. To represent the range of variation in DNA concentration typical for extracts from trace samples, the human placental DNA was diluted from the supplier's concentration into working aliquots at four approximate concentrations: 100 pg/μL, 20 pg/μL, 5 pg/μL, and 2 pg/μL. Gorilla fecal DNA was extracted from four separate dried fecal samples of the same individual. Six extracts were made from 100 mg quantities from each of the four samples as per Morin et al. (7) and then pooled to provide sufficient volumes for all treatments. This resulted in four DNA extracts representing the four different fecal samples. All DNA samples were suspended in 0.1 X TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0).

Each DNA sample was divided into three equal volumes of 600 μL. Concentrated Lambda HindIII DNA (New England Biolabs #N3012S) in 0.1 X TE was added to one aliquot to a final concentration of 10 ng/μL. The second aliquot was combined with the disaccharide trehalose (Sigma T-0299) in 0.1 X TE to a final concentration of 0.2M. The final aliquot was also further diluted in 0.1 X TE buffer such that all of the 24 resultant samples (12 human and 12 gorilla) were of the same volume (700 μL) to maintain DNA concentrations.

Duplicate samples of each human DNA treatment were loaded into MicroAmp® optical 96 well plates (Applied Biosystems #N801-0560) in 5 μL volumes. For the duplicate samples of each gorilla fecal DNA, the template volume was restricted to 2 μL with a further 3 μL of water added to achieve the final volume of 5 μL. This precaution has been found to reduce the interference of inhibiting agents during the subsequent PCR (L. Vigilant, pers. comm). The plates were divided into the four storage groups: −80°C, −20°C, +4°C, and the final set was left to dry in a fume hood at room temperature overnight. All plates were sealed with PCR sealing film (Abgene #AB-1115).

Quantitative PCR

To monitor the amount of amplifiable DNA present in samples over time, the 5′ exonuclease assay as described in Morin et al. (7) was used. To avoid inconsistencies related to minor variations in PCR reagent quantities, a bulk qPCR mastermix was prepared to provide sufficient volume for all repetitions of this experiment. The mastermix was prepared using the Eurogentic "Taqman Core Reagents Kit" according to the standard conditions, except that 250 μM dNTP was replaced with 250 μM each of dATP, dCTP, dGTP, dTTP, and dUTP. The reaction mix also contained 300 nM of each primer, 200 nM probe, and 0.8 mg/mL BSA (Roche, Mannheim, Germany). This bulk mastermix was aliquoted into 6400 μL volumes (sufficient for an assay run of 4 plates and standards) and stored at −20°C (23).

The standard DNA dilution series consisted of a PCR amplified fragment of the c-myc proto-oncogene (24). The standard set was comprised of a twofold dilution series from 1010 copies down to 8 copies per PCR reaction, assayed in duplicate. One standard set was run as part of the first day's assays and was used to construct the standard curve from which all subsequent samples were quantified. This approach was employed to avoid an overestimation of sample quantity due to degradation of the standard itself. A standard set was run as a control with each additional assay repetition to monitor day-to-day variation in instrument performance. Sufficient quantity of each dilution was prepared prior to the experiment, aliquotted into volumes sufficient for each experimental replicate, and stored at −20°C.

Assay quantification was performed immediately after set-up to establish baseline concentrations and then after storage for 1, 2, 3 and 4 weeks, and after 2, 3, 4, 6, 9, and 12 months. For each repetition of the 5′ exonuclease assay, a standard set was run in duplicate together with 8 no template controls. Uracil-DNA glycosylase (Roche, Mannheim, Germany) was added to the PCR mixture to a concentration of 0.01 U/μL just before pipetting into the assay plates to eliminate PCR carry over contamination. The assay was performed in 20 μL reactions on an ABI PRISM™ 7700 Sequence Detector (Applied Biosystems, Foster City, CA), with initial incubations at 50°C for 2 min and at 95°C for 10 min, followed by 50 cycles of 95°C for 15 sec and 59°C for 30 sec. Analysis was performed using the ABI PRISM™ 7700 Sequence Detector software and starting template DNA quantities were calculated from standard curves and formulas in Microsoft Excel spreadsheets (7).

DNA Quality

A PCR assay of a 757 bp fragment of the 18s ribosomal gene was performed to give an indication of DNA quality, as the 5′ nuclease assay is unreliable for larger PCR products (17,24). Primers were synthesized by Eurogentec (Liege, Belgium). The oligonucleotide sequences are: forward primer (18SBC_938_F1U1): ATTCGTATTGCGCCGCTAGA, and reverse primer (18SC_1694_R1U1): ACAAAGGGCAGGGACTTAATCA. The assay was performed in 20 μL reaction volumes containing 1 X PCR Buffer (10 mM Tris/HCl, 50 mM KCl, pH 8.3), 4.5 mM $MgCl_2$, 0.8 mg/mL BSA, 250 μM each of dATP, dGTP, dCTP, dTTP, UTP, 0.025 U/μL Taq polymerase, 0.01 U/μL uracil-N-glycosylase (Roche, Mannheim, Germany), and 300 nM of each primer. Cycling was performed on a MJ Tetrad Thermal Cycler (MJ Research, Watertown, MA) and conditions were the same as for the qPCR assay with the addition of a 2 min annealing step at 72°C within the cycle. Due to the degraded nature and low concentration of some of the DNA extracts, 50 rounds of PCR were necessary to produce some amplification success across all treatments. Amplification products were visualized in 1% agarose gel stained with ethidium bromide. PCR success was scored as the presence or absence of a band at the corresponding product size. Data represent the number of successful PCRs out of a total of 8 reactions for each treatment. For gorilla fecal DNA this constituted duplicate reactions from each of the 4 individual extracts. For human placental DNA this constituted duplicate reactions from each of the 4 starting concentrations (10 pg, 25 pg, 100 pg, and 500 pg).

Trehalose PCR Enhancement Effect

After six months storage, one set of sample plates stored dry at room temperature was used to test the relative effect of trehalose on PCR amplification (19). For each DNA type (human and fecal), 0.2 M trehalose was added to 8 untreated samples in TE buffer just prior to PCR, 8 samples were left untreated in TE buffer and 8 samples were used that had been stored throughout in 0.2 M trehalose. All samples were subjected to the 5′ exonuclease assay as described previously and results were compared across treated and untreated samples.

Monitoring for Assay Consistency

To test for any increase in reflectivity of plastics over time, spare wells on PCR plates stored for 12 months at −20°C were used to

amplify new standard DNA via the 5′ exonuclease assay. These results were compared to those for new standard DNA amplified in new plates not subjected to long-term storage.

A further experiment was conducted using excess stored mastermix to identify any discrepancy against results achieved with a fresh mastermix cocktail. A new standard dilution series was created and amplified via the 5′ exonuclease assay using the stored mastermix (12 months old) and five independently prepared fresh mastermix cocktails. The standard curves for each replicate were calculated independently and plotted on the same graph for comparison.

Data Analysis

Statistical analysis was performed to determine the effects of buffer/temperature combinations (12 levels) on the final concentrations of each type of DNA. For each treatment and within each DNA type (gorilla fecal and human placental), the data were pooled across starting quantities such that there were 8 single data points for each treatment rather than just two. These pooled data sets were used as the input files for the statistical software package SAS (SAS Institute Inc., Cary, NC). Log transformation of the final DNA amounts was employed to standardize the variances associated with the large range of starting quantities. The program then calculated averages for each treatment and performed Kruskal-Wallis tests to search for statistically significant differences between treatments. As all treatments began with the same range of DNA samples and concentrations, pooling the data for each treatment was a way to increase the power of the statistical test whilst maintaining the validity of the relative comparisons.

Results

Fecal DNA

After 12 months storage, there were significant differences in the amount of amplifiable DNA remaining among the samples from different treatment methods ($P = 0.0001$). Samples stored dry in trehalose retained a significantly higher concentration of DNA than those stored at +4°C or dry in the other treatments (Fig. 1*a*). Likewise, samples stored frozen in any buffer retained significantly higher amounts of amplifiable DNA than those stored at +4°C or dry without the addition of trehalose. There was no significant difference in final DNA concentration among frozen samples, or between them and samples stored dry in trehalose. However, samples stored dry with trehalose or at −80°C with lambda or trehalose showed less variation than the other frozen samples indicating that, for fecal DNA, these conditions provide the most consistent amplification success after 12 months storage.

Small sample sizes and high standard deviations for these low concentration samples reduced the statistical support for the observed trends within individual extracts, however, pooling of the data resulted in strong statistical support. The large standard deviations recorded for these DNA samples from fecal extracts are typical of the range of variance encountered in qPCR when working with low copy number samples (24,25). This variance is related to the stochastic variation in the number of molecules pipetted from the highly dilute master solution into the wells during the experiment set up, and to the semi-logarithmic standard curve used for starting copy number calculation.

Human Placental DNA

Human DNA exhibited similar patterns of amplification success to the fecal DNA after 12 months storage (Fig. 1*b*). The level of variation across treatments increased inversely with starting DNA quantity, however, the same pattern of amplification success was reflected throughout the range of starting quantities. Samples treated with no more than TE buffer and stored dry had degraded to a level insufficient to yield PCR products for the 100 pg and 10 pg starting quantities. This was also true for the dried Lambda treated samples at the 25 pg and 10 pg starting concentrations (data not shown).

The Kruskal-Wallis test revealed significant differences between treatment combinations after 12 months storage ($P = 0.0013$). A significantly higher mean amplification yield was recorded for all frozen samples and dried or +4°C samples treated with trehalose when compared to samples stored dry without trehalose. Once again there was no significant difference in amplification yield between samples stored frozen and those stored dry in the presence of trehalose. For the human DNA, extracts stored at +4°C in the presence of trehalose were also not significantly different from frozen samples (Fig. 1*b*).

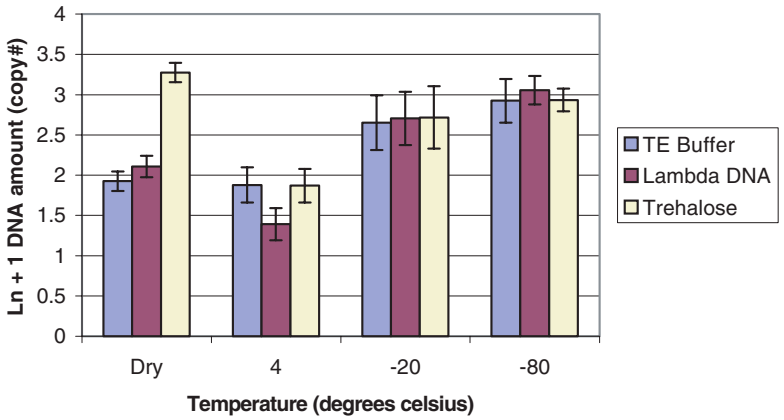

FIG. 1*a*—*Mean amplifiable DNA quantities for all starting concentrations in duplicate as calculated through qPCR of gorilla fecal DNA extracts stored for 12 months with different buffer additives (approximately 3.5 pg/DNA copy). DNA quantity is ln transformed, and values were all made >1 by addition of 1 for easier viewing.*

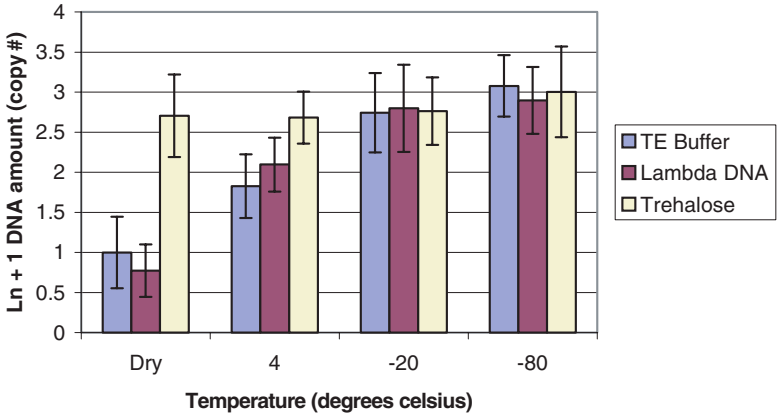

FIG. 1*b—Mean amplifiable DNA quantities for all starting concentrations in duplicate as calculated through qPCR of human placental DNA extracts stored for 12 months with different buffer additives (approximately 3.5 pg/DNA copy). DNA quantity is ln transformed, and values were all made >1 by addition of 1 for easier viewing.*

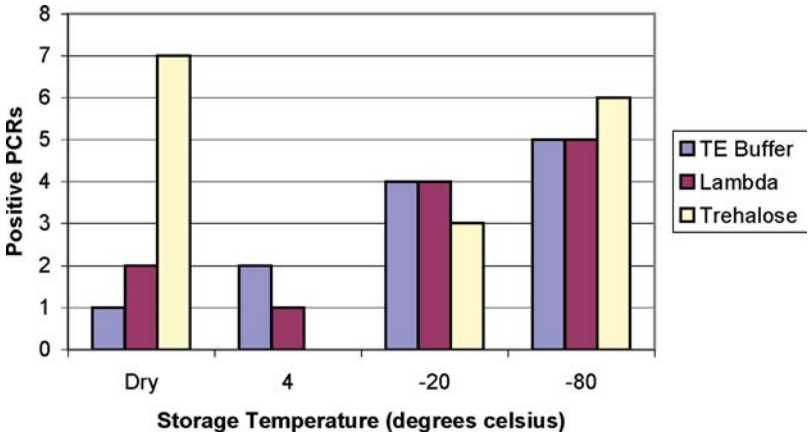

FIG. 2*a—PCR success for a 747 bp fragment of the 18s ribosomal gene amplified from gorilla fecal DNA stored for 12 months. Data represent number of successful PCRs from all 4 individual extracts in duplicate for each treatment.*

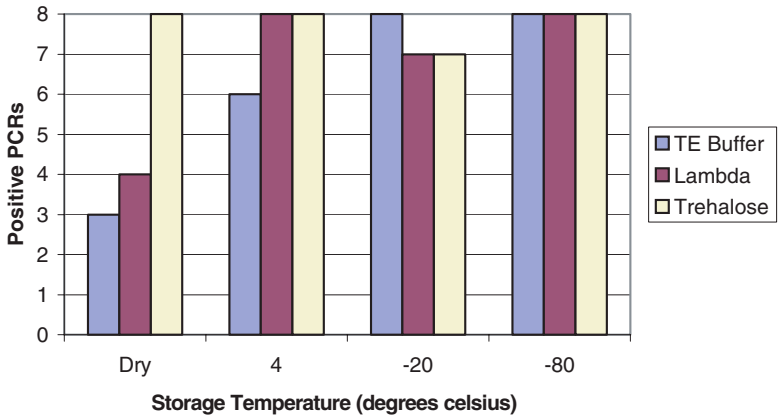

FIG. 2*b—PCR success for a 747 bp fragment of the 18s ribosomal gene amplified from human placental DNA stored for 12 months. Data represent number of successful PCRs from all 4 starting DNA concentrations in duplicate for each treatment.*

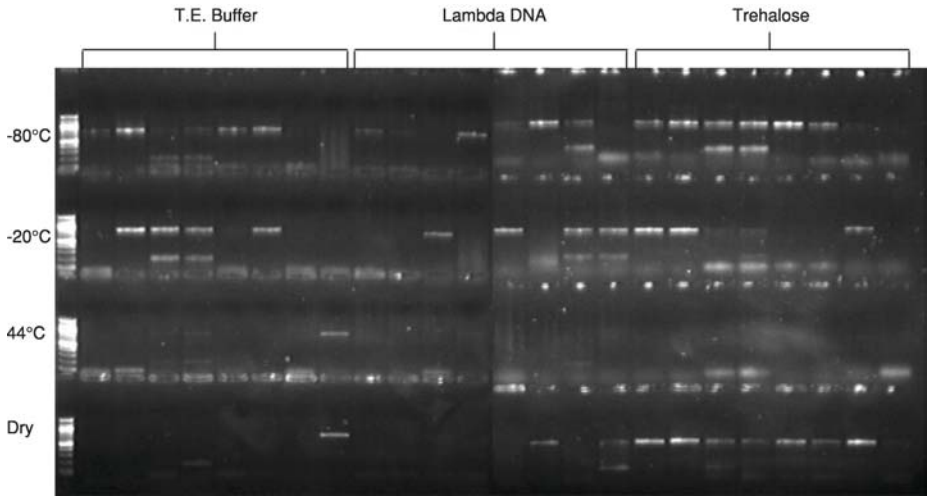

FIG. 3*a*—*Combined gel image of PCR products for a 747 bp fragment of the 18s ribosomal gene amplified from 4 individual gorilla fecal DNA extracts in duplicate stored for 12 months.*

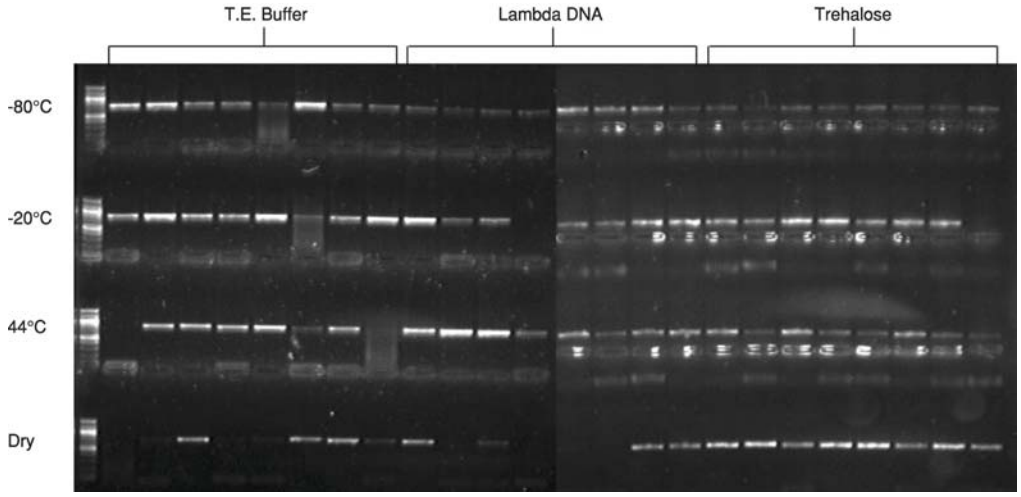

FIG. 3*b*—*Combined gel image of PCR products for a 747 bp fragment of the 18s ribosomal gene amplified from 4 different starting concentrations of human placental DNA in duplicate stored for 12 months. Duplicate starting concentrations within each treatment from left to right are 10 pg, 25 pg, 100 pg, and 500 pg.*

DNA Quality

The quality of the fecal DNA after 12 months storage, as measured by amplification of a 757 bp fragment, was best preserved by storage with trehalose at −80°C (6 out of 8 positive PCRs) or dried in trehalose (7 out of 8 positive PCRs) (Fig. 2*a*). Storage at −20°C produced surprisingly poor results in terms of DNA quality with a maximum value of 4 out of 8 positive PCRs (TE buffer and Lambda Hind III treated samples). Extracts stored at +4°C showed considerable degradation, with only three samples out of 24 resulting in positive amplification. Poor amplification success of the fecal DNA in comparison to the human DNA for this longer fragment highlights the problem of DNA shearing and other degradation encountered when using extracts from noninvasive samples (Fig. 3*a* & *b*).

For the human DNA (Fig. 2*b*), samples stored at −80°C, −20°C, or at +4°C produced high rates of positive PCR after 12 months regardless of buffer additive (8 out of 8, 7 out of 8, 7 out of 8 positive PCRs, respectively). The variation in band intensity, particularly for the +4°C samples, suggests that DNA quality is still reduced, however. This reduction in band intensity seems unrelated to starting concentration and more associated with treatment type. The addition of trehalose to dry stored samples resulted in 100% PCR success as well as producing bands of even intensities (Fig. 3*b*), and may provide a more consistent level of DNA quality preservation relative to other storage methods.

Trehalose PCR Enhancement Effect

The pooled data for samples treated by storage in trehalose across all DNA types exhibited a significantly higher mean amplification yield relative to the data pools of untreated and post-storage treated samples ($P = 0.004$, Fig. 4). In contrast there was no significant difference between mean amplification yield for samples treated with trehalose just prior to PCR and those left untreated, indicating that the observed improvement through the addition of trehalose is due to its role as a biomolecule protector (20) rather than its effect as a PCR enhancer (19).

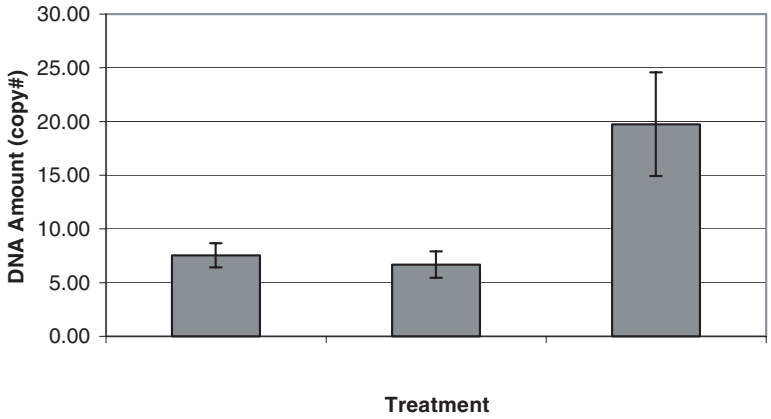

FIG. 4—*Comparison of means for amplification yield of dried DNA extracts (human and fecal combined) stored for 6 months. Treatment 1 = trehalose added just prior to amplification; treatment 2 = no trehalose added; treatment 3 = dried and stored in the presence of trehalose for entire 6 months.*

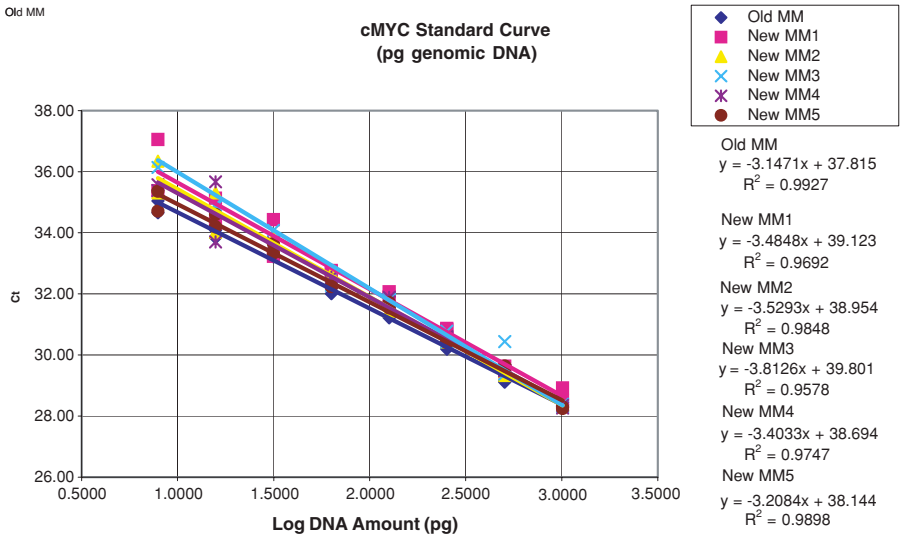

FIG. 5—*DNA standard curves generated from a mastermix stored frozen for 12 months (oldMM) and for 5 independently prepared fresh mastermixes (MM1-MM5). Ct is the cycle at which sample fluorescence reaches a given threshold value.*

Monitoring for Assay Consistency

The results of the comparison between frozen-stored 96-well plates and new plates indicated no observable difference in standard curves generated under these two conditions, suggesting that there has been no change in background reflectivity over time related to modifications to the plastic properties of the PCR plate during freezer storage (data not shown).

The results of the new versus old mastermix comparison (Fig. 5) show small differences between all replicates but importantly, show a slope difference between the standard curve generated from the old mastermix and all those generated from the fresh mastermix. These minor differences would result in an overestimation of DNA from one time period to the next but would not be reflected in any differences between treatments within a time point.

Discussion

The method employed for DNA storage is a critical issue in long-term genotyping studies (26). Results from this study clearly show that in some common storage situations, low concentration samples degrade and become otherwise unavailable for PCR amplification over time. The pattern of degradation showed similar trends across all starting concentrations and across both DNA types (human placental and gorilla fecal). Even samples stored in the conditions traditionally considered best (−80°C) are subject to some form of DNA loss and significant variation in final DNA quality (see Fig. 3*a* and 3*b*).

Trehalose PCR Enhancement Effect

Our data indicate a role for trehalose as a buffer additive prior to dry storage in situations where freezer space is limited. Further work is suggested to confirm this finding and to test the reliability of genotyping across multiple loci after such storage. Previous research has suggested that the observed improvement in PCR yield with the addition of trehalose to DNA samples may be due to either its role as a biomolecule stabilizer (20) or as a PCR enhancer (19). The results of this experiment indicate that it is the stabilization action of trehalose (20) that best explains the enhanced yield for samples dried in its presence. Trehalose forms a glass upon drying

and it is this process that has been shown to stabilize proteins in a dry state due to the action of the glass-like trehalose on the intra-molecular folding (20). It is likely that a similar process occurs in the case of DNA with the trehalose acting to replace the water molecules in the folded chain and keep the DNA as in a hydrated state.

Assay Consistency

The observed difference in qPCR standard curves between stored and freshly prepared mastermix cocktails, although small (Fig. 5) would be large enough to cause an overestimation of DNA quantity if plotted over time. Although this effect has no impact on the results reported here, it may be of concern for other projects interested in the rate of DNA loss over time. For such experiments, it would be important to rule out any run-to-run inconsistencies in assay performance no matter how small. The observed change in 5′ exonuclease assay efficiency detected in this experiment is likely to be due to a deterioration of the passive ROX reference fluorophore or some other mastermix component over time. In this experiment, core reagents with no added stabilizers were used to prepare the qPCR assay. Since then, Applied Biosystems (Foster City, CA) have advised that they have available a 2X qPCR mastermix, containing proprietary stabilizers within its formulation, which would be more appropriate for studies interested in tracking the rate of DNA loss over time.

This study has quantified the relative success of storage strategies for low concentration DNA extracts. Our results indicate that while the traditional method of storage in TE buffer at −80°C is appropriate for most situations, equally good results may be obtained by storage at room temperature of extracts that have been dried in the presence of 0.2 M trehalose. The qualitative data also suggests that the addition of trehalose may act as a biomolecule protector in most storage situations. Although difficult to quantify, our results suggest that substantial degradation of fecal DNA occurs at +4°C and −20°C regardless of additive, and even to some extent at −80°C except in the presence of trehalose (Fig. 3*a*). Dry storage with trehalose may provide both better preservation of valuable DNA samples, and a cost-effective opportunity to split them and store a portion under standard conditions (−80°C) and a portion at another site for safekeeping. This could be a good approach for long-term dry preservation of any type of sample and allow expanded future uses via whole genome amplification of small amounts of high quality DNA. This study has also highlighted the difficulty in obtaining unbiased estimates of the rate of DNA loss over time and suggests that careful consideration is needed when designing such experiments. The observation of high variability in PCR success between low concentration samples also suggests that much work is needed to improve collection and extraction methods to increase DNA yields (13).

Acknowledgments

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References

1. Constable JJ, Packer C, Collins DA, Pusey AE. Nuclear DNA from primate dung. Nature 1995;373–93. [PubMed]
2. Morin PA, Woodruff DS. Noninvasive sampling for vertebrate conservation. In: Wayne R, Smith T, editors. Molecular approaches in conservation. Oxford: Oxford University Press, 1996;298–313.
3. Gerloff U, Schlötterer C, Rassmann K, Rambold I, Hohmann G, Fruth B, Tautz D. Amplification of hypervariable simple sequence repeats (microsatellites) from excremental DNA of wild living bonobos (*Pan paniscus*). Mol Ecol 1995;4:515–8.
4. Kohn M, Knauer F, Stoffella A, Schröder W, Pääbo S. Conservation genetics of the european brown bear-a study using excremental PCR of nuclear and mitochondrial sequences. Mol Ecol 1995;4:95–103. [PubMed]
5. Morin PA, Woodruff DS. Paternity exclusion using multiple hypervariable microsatellite loci amplified from nuclear DNA of hair cells. In: Martin RD, Dixson AF, Wickings EJ, editors. Paternity in primates: genetic tests and theories. Basel: Karger, 1992;63–81.
6. Taberlet P, Mattock H, Dubois-Paganon C, Bouvet J. Sexing free-ranging brown bears *Ursus arctos* using hairs found in the field. Mol Ecol 1993;2:399–403. [PubMed]
7. Morin PA, Chambers KE, Boesch C, Vigilant L. Quantitative polymerase chain reaction analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*). Mol Ecol 2001;10(7):1835–44. [PubMed]
8. Dean MD, Ballard JWO. Factors affecting mitochondrial DNA quality from museum preserved *Drosophila simulans*. Entomol Exp Appl 2001;98(3):279–83.
9. Frantzen MAJ, Silk JB, Ferguson JWH, Wayne RK, Kohn MH. Empirical evaluation of preservation methods for faecal DNA. Mol Ecol 1998;7(10):1423–8. [PubMed]
10. Vigilant L. An evaluation of techniques for the extraction and amplification of DNA from naturally shed hairs. Biological Chemistry 1999;380:1329–31. [PubMed]
11. Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP, Bouvet J. Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Res 1996;24(16):3189–94. [PubMed]
12. Murphy MA, Waits LP, Kendall KC, Wasser SK, Higbee JA, Bogden R. An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples. Conservation Genetics 2002;3(4):435–40.
13. Roeder AD, Archer FI, Poinar HN, Morin PA. A novel method for collection and preservation of faeces for genetic studies. Mol Ecol Notes 2004;4(4):761–4.
14. Visvikis S, Schlenck A, Maurice M. DNA extraction and stability for epidemiological studies. Clin Chem Lab Med 1998;36(8):551–5. [PubMed]
15. Köhler T, Rost A-K, Remke H. Calibration and storage of DNA competitors used for contamination-protected competitive PCR. BioTechniques 1997;23:722–6. [PubMed]
16. Morin PA, Mahboubi P, Wedel S, Rogers J. Rapid screening and comparison of human microsatellite markers in baboons: Allele size is conserved, but allele number is not. Genomics 1998;53:12–20. [PubMed]
17. Morin PA, Saiz R, Monjazeb A. High throughput single nucleotide polymorphism genotyping by the 5′ exonuclease assay. BioTechniques 1999;27:538–552. [PubMed]
18. Gaillard C, Strauss F. Eliminating DNA loss and denaturation during storage in plastic microtubes. Amer Biotech Lab 2000;18(13):24.
19. Carninci P, Nishiyama Y, Westover A, Itoh M, Nagaoka S, Sasaki N, Okazaki Y, Muramatsu M, Hayashizaki Y. Thermostabilization and thermoactivation of thermolabile enzymes by trehalose and its application for the synthesis of full length cDNA. Proc Natl Acad Sci USA 1998;95:520–4. [PubMed]
20. Colaço C, Sen S, Thangavelu M, Pinder S, Roser B. Extraordinary stability of enzymes dried in trehalose: simplified molecular biology. Bio/Technology 1992;10:1007–10.
21. Micklos DA, Freyer GA. DNA science. In: Crotty D, editor. New York: Cold Spring Harbor Laboratory Press, 2003.
22. Irie T, Honda Y, Watanabe T, Kuwahara M. Efficient transformation of filamentous fungus *Pleurotus ostreatus* using single-strand carrier DNA. Appl Microbiol Biotechnol 2001;55(5):563–5. [PubMed]

23. Smetsers TFCM, Stevens EHP, van de Locht LTF, Mensink EJBM. Freezing of PCR master mixture retains full amplification activity and facilitates PCR standardisation for molecular diagnostics and real-time quantitative PCR. Leukemia 1998;12:1324–32. [PubMed]
24. Smith S, Vigilant L, Morin PA. The effects of sequence length and oligonucleotide mismatches on 5′ exonuclease assay efficiency. Nucleic Acids Res 2002;30(20):e111. [PubMed]
25. Raeymaekers L. General principles of quantitative PCR. In: Kochanowski B. RU, editor. Methods in molecular medicine. Totowa, N.: Humana Press Inc., 1999.
26. Vigilant L, Hofreiter M, Siedel H, Boesch C. Paternity and relatedness in wild chimpanzee communities. Proc Natl Acad Sci USA 2001;98(23):12890–5. [PubMed]

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