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Toward the elimination of contamination from ancient DNA amplifications: use of 2'-Deoxyuridine-5'-triphosphate and Uracil DNA Glycosylase to eliminate carryover PCR products.

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Eliminating contamination from PCR amplifications of traditional and ancient DNA sources remains an elusive goal. Ongoing projects in our lab include intraspecific (c.f. Ellegren 1991, 1992; c.f. Thomas *et al*. 1990) as well as interspecific (e.g., Krajewski *et al*. 1992) comparisons of DNA fragments obtained from a variety of sources. Because many intraspecific comparisons of genes are expected to yield no observed differences, we do not always have the ability to recognize contaminants from sequence comparisons alone. Because of this inherent difficulty, we are exploring several strategies for minimizing and potentially eliminating contamination events (see Kwok and Higuchi 1989 for a review of the basics). In this note, we present our current overall strategy to minimize contamination and preliminary results of using Uracil DNA Glycosylase (UDG) and 2'-Deoxyuridine-5'-triphosphate (dUTP) to prevent previously amplified PCR products from becoming templates for subsequent amplifications (Longo *et al*. 1990).

Overall strategy for controlling contamination:

1. Subject all equipment and reaction tubes to UV-light, bake glassware, and use cotton-plugged pipette tips or positive displacement pipettes.
2. Minimize the number of steps in the DNA extraction protocol. Modifications of the Chelex protocols (Walsh *et al*. 1991) are the most promising.
3. Test negative extraction controls (with carrier DNA or RNA). Modified Chelex protocols allow for easy testing of solutions for contaminants prior to the addition of ancient tissue.
4. Use a master mix, and ensure the PCR mix negative control is the last tube manipulated and thus has the greatest opportunity for contamination.
5. All PCR reactions are set-up using dUTP rather than dTTP (=TTP). dUTP can compete efficiently with dTTP in the absence of enzymes which destroy dUTP or U-DNA (e.g. in *dut*- and *ung*- bacteria).
6. UDG is added to all reactions where amplification products from previous reactions (carry-over contamination) is likely to be problematic. UDG degrades U-DNA, but does not affect normal DNA or dUTP. Therefore, PCR products amplified using dUTP can be destroyed by UDG.
7. An additional primer (or primer pair) that would yield a product much longer than the target are added to all ancient DNA amplifications. The amplification of a large PCR product indicates high molecular weight DNA, which is uncommon in ancient DNA, but common in modern contaminants (see DeSalle *et al*. 1992).
8. Sequences are initially obtained by asymmetric sequencing.
9. Sequences are confirmed by cloning PCR products into plasmids from which single stranded DNA can be rescued.

We specifically address carry-over contamination, because we consider it to be the most likely source of contamination and the least likely to be prevented by other techniques in common use.

Basic dUTP and UDG protocol (cf. Longo *et al*. 1990):

1. Substitute dUTP for dTTP in all PCR reactions. All PCR products will be U-DNA.
2. Add UDG to the PCR reaction mix of all (or some reasonable subset of all) subsequent PCR reactions.
3. Incubate the reactions at 37°C for 15 min prior to "normal" thermal cycling. This allows the UDG to degrade all U-DNA in the reaction mix.
4. Ensure that the initial 94°C incubation (prior to the first standard cycle) is at least 15 min. This step will denature the UDG and reduce its activity to a nominal level.
5. Use standard thermal cycling parameters.
6. Terminate cycling by going to a 72°C soak file (rather than the usual 4°C or quit file). This is done to ensure that any residual UDG activity is inhibited (Thornton et al. 1992).

Special concerns for using UDG with ancient DNA: Uracil is the deamination product of cytosine. Some DNA will spontaneously undergo this mutation both in vitro and in vivo. It is possible that the use of UDG with some ancient DNA samples, which have been highly modified, may result in the total inability to amplify the modified DNA. Pääbo (1989) states that DNA obtained from museum study-skins was affected by UDG "... to only a limited and variable extent ...". Therefore, we completed the following preliminary experiment to determine the feasibility of switching to dUTP.

Experiment with UDG and dUTP: Samples - 1) DNA extracted from a one year old cowbird (*Molothrus ater*) study skin by the method of Pääbo (1990; DNA concentration = 10 ng/µl), 2) DNA extracted from red panda (*Ailurus fulgens*) tissue by standard phenol/chloroform extraction (DNA concentration = 100 ng/µl), and 3) ≈ 1 mg of red panda tissue placed in 500 µl of 5% Chelex and boiled for 15 minutes (c.f. Walsh et al. 1981; detailed protocol available upon request).

Protocol: We amplified a 308 bp segment of the cytochrome b gene using primers L14841 (5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3', c.f. Kocher et al. 1989), and H15149 (5'-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3', Pääbo 1989) which amplify a total product of 377 bp. 2 µl from each of the 3 samples were amplified, with "normal" dNTP's (dNTP Mix), and dNTP's substituting dUTP for dTTP (dUTP Mix). The cycling parameters were: 94°C x 3 min, 1 cycle; 94°C x 1 min, 50°C x 1 min, 72°C x 1 min, 25 cycles; 72°C x 7 min, 1 cycle; 4°C soak file.

In the second round of amplification, 5 µl of amplification product (= 100 ng) from sample 1 from each of the two PCR mixes was reamplified using UDG in the PCR Mix. Additionally, 5 µl of samples 1-3 (from both reaction mixes) was asymmetrically amplified using the dUTP Mix, except that only one primer was used. The cycling parameters were: 94°C x 15 min, 1 cycle; 94°C x 1 min, 55°C x 1 min, 72°C x 1 min, 20 cycles; 72°C soak file. Asymmetric amplifications using each primer were performed. The resulting single-stranded U-DNA (ssU-DNA) was then sequenced using the Sequenase Kit and protocol (U.S. Biochemical).

PCR Mixes: The PCR Reaction Mixes were as follows (per reaction):

Reagent	Amount dNTP Mix	Amount dUTP Mix
10x Taq Buffer	5 µl	5 µl
L14841 (10 uM)	5 µl	5 µl
H15149 (10 uM)	5 µl	5 µl
$MgCl_2$ (25 mM)	3 µl	3 µl
dNTP's (2.5 mM each)	4 µl	2 µl
Taq Polymerase (5 units/ul)	0.5 µl	0.5 µl
H_2O	25.5 µl	27.5 µl
Sample	2 µl	2 µl
Total	**50 µl**	**50 µl**

Taq polymerase and Taq buffer were from Promega (10X Buffer is 500 mM KCl, 100 mM Tris-HCl pH 9.0, 1.0% Triton X-100). dUTP and UDG were provided by Life Technologies. One unit of UDG was added to reactions where UDG was used.

Results: No amplification was observed in any of the negative controls. All samples were amplified in the first round of amplification. There was no discernable difference in the amount of product produced by the two PCR Mixes. Subsequent reactions in other experiments, have shown no obvious difference in the amount of PCR product produced when dUTP is substituted for dTTP.

In the second round of amplifications, no amplification was observed when the U-DNA PCR product from the first round of amplification was treated with UDG prior to PCR. Amplification was observed when "normal" PCR products (those amplified with dTTP) were treated with UDG

prior to PCR. No discernable differences in sequence quality due to the U-DNA templates were observed. Overall quality of asymmetric sequences using ssU-DNA was similar or slightly superior to the quality of "normal" ssDNA.

Additional information about UDG and dUTP:

Cloning U-DNA U-DNA PCR products can NOT be transformed into most common bacterial strains (most strains have UDG which will digest the U-DNA insert in the plasmid). We have successfully ligated U-DNA PCR products into Bluescript II KS+ (originally obtained from Stratagene). The resulting recombinant plasmids were successfully transformed into *dut*- and *ung*- bacterial strains CJ 236 and BW 313 (obtained from *E. coli*, Genetic Stock Center, Dr Barbara J Bachmann, Dept of Biology 255 OML, Yale University, P. O. Box 6666, New Haven, CT 06511, USA). We have also successfully rescued ssDNA from CJ 236 transformants, but sequencing has not yet been successful. Additional information on modifications of cloning techniques and restriction digestion of U-DNA is in Bebee *et al.* (1992).

Cost of dUTP - $70 per 25 umol for dUTP vs. - $36 per 25 umol for dTTP. Although dUTP is twice as much as dTTP (and potentially worse because you often get a big price break for purchasing dATP+dCTP+dGTP+dTTP all at once), the additional cost per reaction is nominal (much less than $0.01). Thus, the use of dUTP in all PCR reactions for specific genes (and probably all PCR reactions) is justified.

Cost of UDG - $0.60 per unit. UDG is similar in cost to (or more expensive than) most thermostable polymerases. Thus, UDG is probably not cost effective to include in all reactions, but its use is easily justified for reactions where carry-over contamination will be especially hard to detect. Currently, BRL provides UDG at the lowest cost among major U.S. Biotech firms. However, many other firms are now offering UDG, and we hope this will inspire a downward trend in its price.

Discussion: First we want to reiterate that one must amplify all PCR products (of a given DNA segment) using dUTP if UDG is going to be useful. Second, we found that 1 unit of UDG destroys a large amount of U-DNA template, but does not affect the amplification of normal PCR products. Experiments to directly test the hypothesis of UDG inhibiting amplification of ancient DNA are in progress. In general, there are only a few protocols that must be modified if one switches to U-DNA PCR products. Overall, these modifications have only been slightly painful for us to implement.

We recognize 4 major problems towards the general use of U-DNA. First, UDG is expensive. Our results suggest that the amount of UDG used per tube may be reduced. This titration, however, must be done with caution, because one must maintain a high enough amount of UDG that it will encounter and destroy ALL U-DNA in the tube (not just 90- 99%). (Results of our titration experiments will be available in early 1993.) Second, standard strains of bacteria cannot be used for cloning. The cloning strains that will accept U-DNA plasmids contain restriction modification systems, and sequencing of ssDNA derived from these strains has proven to be difficult. Third, some restriction enzymes do not work with U-DNA (Bebee *et al.* 1992). Therefore, RFLP analysis of U-DNA PCR products is less likely to yield informative results than RFLP analysis of normal PCR products (c.f. Karl *et al.* 1992). Fourth, the 94°C x 15 min pre-incubation needed to destroy the UDG will also have a negative effect on the activity of Taq polymerase. Although the decreased activity did not affect the results in our test, we have not completed any experiments where this may come into play. This may be particularly important for those who need to keep use of Taq polymerase to a minimum.

Overall we believe that the benefits of producing degradable PCR products outweigh the costs. Because the cost of producing U-PCR products is only slightly more than normal PCR products, we intend to amplify newly developed or adopted primers using dUTP. This will allow us to destroy previously amplified PCR products in selected amplifications where we are most concerned about carry-over contamination. This strategy should keep the additional costs to a

minimum. The second and third problems listed above are being addressed by several groups of researchers (principally at Life Technologies). And, the fourth can be addressed by adding more Taq polymerase to each UDG reaction or switching to a more thermostable polymerase.

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Ancient DNA Genotyping: HLA and CA Repeats by PCR.

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The analysis of DNA extracted from samples of bone from archaeological sites offers the prospect of gaining new kinds of information about the past. In this paper we report on the use of PCR amplification and fingerprinting techniques in the study of DNA from human remains from a Stone Age cave deposit. The results show that the genotyping of an individual who lived some 2000 years ago is possible. The archaeological significance of this approach is that it allows the degree of relationship to be determined, between individuals, in sites with multiple internments. Problems such as the marriage and residence rules practised in past communities may be investigated, given the availability of suitable samples. The approach may offer a means to investigate the composition, and continuity, of regional populations in the past.

The study of DNA from bone samples of any antiquity poses special problems because the DNA may be degraded to small fragments and the quantity of preserved DNA may be limited. In this study PCR was used to amplify an HLA sequence and a CA repeat region in the human genome. The CA repeats are genetically stable and transmissable, existing in many variations, usually differing