Braz Dent J (2007) 18(2): 148-152 ISSN 0103-6440

A Simple and Cost-Effective Protocol for DNA Isolation from Buccal Epithelial Cells

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Buccal cells provide a convenient source of DNA for epidemiological studies. The goal of this study was to develop a convenient method to obtain buccal cells from mouthwash samples to be used as a source of DNA, and to evaluate the stability of the DNA in mouthwash solution over time. The procedures used in the method described in this paper avoid the use of any organic solvents. This is achieved by salting out the cellular proteins by dehydration and precipitation with a saturated ammonium acetate solution. The protocol described here is fast, simple to perform, sensitive, economical and several samples can be processed at the same time. The analyses provide consistent evidence that DNA extracted by this methodology is sufficient for several PCR amplifications. The total DNA yield ranged from 5 to 93 µg (median 15 µg, mean 20.71 µg). DNA can be extracted and PCR amplified after storage of mouthwash solution at room temperature for periods of up to 30 days.

Key Words: buccal cells, dna extraction, ammonium acetate.

INTRODUCTION

The sequencing of the human genome allied with the relatively low costs of DNA amplification by polymerase chain reaction (PCR) have made the analysis of DNA a common procedure in clinical medicine and basic sciences. In the past few years, thousands of scientific papers have been published on molecular epidemiology/anthropology studies and on the association of mutation/polymorphisms with human diseases. Many more will certainly be published in a near future.

The collection of samples for DNA extraction is a critical procedure as it is time-consuming and may involve ethical aspects. It is, therefore, desirable that this procedure becomes more simple and inexpensive. The analysis of DNA is usually made by PCR amplification of several markers or haplotypes. It is also desirable that the procedure yields fairly large amounts of DNA, as sampling cannot always be easily repeated. In most cases the preferred source of material is peripheral blood. Blood sampling, however, may be problematic in cases such as extreme illness or elderly people, babies and people that are unwilling to be submitted to this invasive procedure. For this reason several protocols have been developed to obtain DNA from buccal cells. Also, collection of buccal cells by mouthwash seems to give higher yields than many other methods (1). The diagnostic results obtained with DNA from buccal cells are compatible with whole blood (2). Several approaches have been developed to isolate DNA from mouthwashes. The more frequently used are: a) boiling lysis method, yielding poor-quality DNA (3,4); b) phenol-chloroform, which is laborious and uses toxic reagents (5,6); c) commercially available kits, which are non-toxic and simple to use (7-10). Although the use of kits is more straightforward they may be quite expensive. Commercially available DNA isolation kits are mainly produced in industrialized countries, and may not be readily available at affordable prices in developing and underdeveloped countries, where diagnosis and epidemiological surveys based on DNA analysis may be a key factor in planning and establishing disease treatment and community disease-prevention programs.

This study describes a simple and inexpensive

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protocol to obtain high-quality genomic DNA from buccal cells using a single mouthwash. DNA extracted by this methodology usually yields sufficient DNA for several rounds of PCR amplifications.

MATERIAL AND METHODS

Sampling

Ethical approval for the study was granted by the Faculdade de Odontologia de Piracicaba/UNICAMP's Ethical Committee for Human Research.

At least 1 h after tooth brushing, the consenting subjects of this experiment were asked to vigorously rinse their mouths with a 5-mL solution of sucrose (3 %) for 60 s. The individuals were oriented to rub their tongue on the oral mucosa and teeth. Each individual's mouthwash was collected in a 15 mL centrifuge tube. Three mL of TNE solution [17 mM Tris/HCl (pH 8.0), 50 mM NaCl and 7 mM EDTA] diluted in 66% ethanol, was added to the tube. In order to assess the DNA integrity over time, the mouthwash solution was divided into 4 tubes. One tube was used for immediate extraction and the three remaining were kept at room temperature for periods of 2, 15 and 30 days, respectively (Fig. 1) followed by DNA isolation.

DNA Purification

The tubes containing the epithelial cells were centrifuged for 10 min at 3000 rpm at room temperature to pellet the buccal cells and debris. The supernatant was poured off immediately to avoid pellet slippage. For the second washing, 1 mL of TNE was added to ressuspend the cells. The tubes were centrifuged at 2000 rpm for 5 min, and the supernatant was poured off. The cell pellet was vortexed vigorously for 5 s and a volume of 1.3 mL of lysis solution [10mM Tris (pH 8.0), 0.5% SDS, 5mM EDTA] and 10 µL of proteinase K (Sigma Chemical Co., St. Louis, MO, USA) (20 mg/ml) was added. The mixture was vortexed for 5 s at medium speed, followed by an overnight incubation at 55°C. After incubation, 1.4 mL of the mixture was transferred to a 2 mL micro-centrifuge tube. Proteins and other contaminants were removed by adding 500 µL of a solution containing 8 M ammonium acetate and 1 mM EDTA, followed by vortexing at high speed for 5 s, and centrifuging at 17000 g for 10 min. Nine hundred ìl of supernatant was poured carefully into two clean 1.75 mL micro-centrifuge tubes containing 540 µL of isopropanol (2-propanol). The solutions were mixed by inverting the tube gently 20 times and centrifuged at 17000 *g* for 5 min. The supernatant was poured off, and each tube was inverted and left to drain briefly on clean, absorbent paper. A volume of 2 mL of 70% ethanol was added, and each tube was inverted several times to wash the DNA pellet. After centrifugation at 17000 g for 5 min the ethanol was poured off carefully. Each tube was inverted and drained on clean, absorbent paper, then allowed to air dry during 45 to 60 min. The DNA was re-suspended in 100 [l of TE buffer [10 mM Tris (pH 7.8) and 1 mM EDTA].

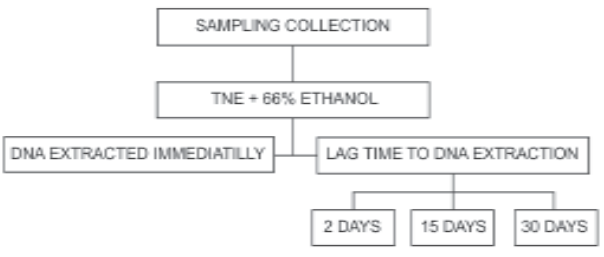

Figure 1. Scheme showing the sampling and storage of the mouthwashes.

Concentration Measurements

The amount and purity of the DNA was determined by spectrophotometry. The DNA concentration was obtained by readings at 260 nm. The ratio of readings at 260nm/280 nm was used to estimate the DNA purity.

Polymerase Chain Reaction (PCR)

Amplification reactions were performed with 500 ng DNA in a volume of 50 µL in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCL, 4 mM $MgCl_2$, deoxyribonucleotides (200 µM each), 1 µM each primers, 2 U *Taq* DNA polymerase (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Three pairs of primers were used (Table 1).

The samples were heated initially to 95°C for 5 min, each cycle comprising denaturation at 95°C for 50 s. Primer annealing was performed at the specific temperature for each set of primers (Table 1) for 1 min

and polymerization at 72°C for 2 min. The samples were subjected to 35 cycles of amplification followed by a final extension of 72°C for 7 min. Amplification was carried out in a *Perkin-Elmer GeneAmp 2400 thermal cycle.* Amplification products were visualized by electrophoresis on vertical 5% (KLK and MMP20) and 10% (PAX9) polyacrylamide gels in 1 X TBE (89 mM Tris-Borate, 89 mM boric acid, 2 mM EDTA), followed by silver staining (11). Electrophoretic analysis of the extracted DNA showed detectable levels of high molecular weight of genomic DNA in all samples.

Table 1. Primers used for PCR. F=forward, R=reverse.

Primer (5′-3′)	Annealing temperature	Product size (bp)
MMP-20		
(F) 5′ GTAAATCAATCATTGATCTTG	56°C	1432
(R) 5′ AAATAAAGATAGATAGTAAAAAGG		
KLK-4		
(F) 5′ TGCCACAAAACTGACCTGCC	58°C	555
(R) 5′ CCTCTTCAAGGAGGTCCTCT		
PAX-9		
(F) 5′ AGCCTGAATCCTGTGTGCAC	54°C	202
(R) 5′ CTAATCTAAAGTGTACCGTATGC		

RESULTS

The total DNA yield as measured by spectrophotometry, ranged from 5 to 93 µg (median 15 µg, mean 20.71 µg) (Fig. 2) and was compatible with results obtained in other studies using commercially available kits (12,13). The mean OD 260/280 ratio was 1.84 (range = 1.16-2.23), indicating that in most cases the bulk of proteins was removed by ammonium acetate precipitation. Figure 3 illustrates typical PCR products from DNA obtained from mouthwashes processed immediately after collection and also from mouthwashes stored at room temperature for periods of 2, 15 and 30 days. Large size products (1434 bp) can be successfully obtained by PCR amplification of DNA purified from mouthwashes stored for 30 days.

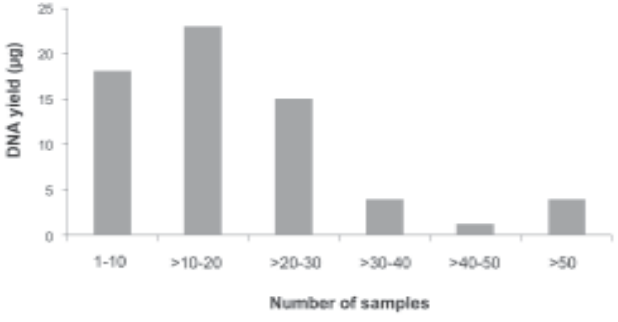

Figure 2. Distribution of DNA yield (µg) obtained from mouthwashes.

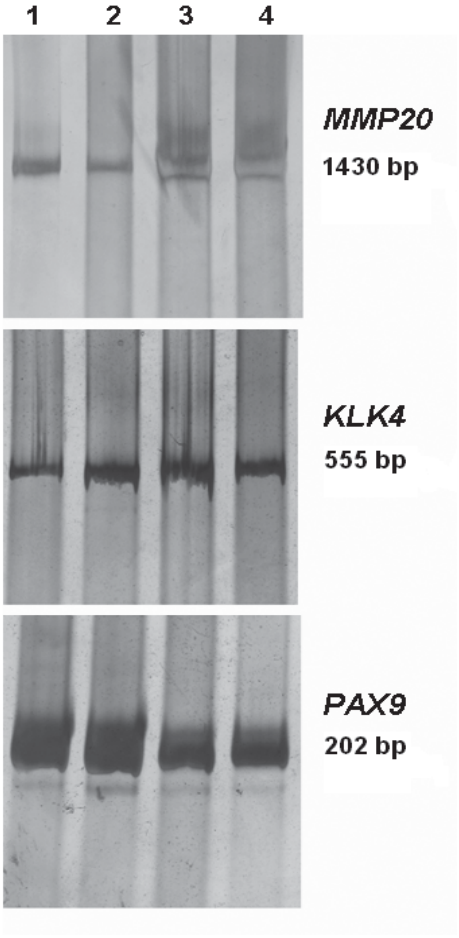

Figure 3. Polyacrylamide gel electrophoresis showing PCR products from total genomic DNA obtained from mouthwashes. 1: DNA extracted immediately after mouthwash. 2: DNA extracted 2 days after mouthwash. 3: DNA extracted 15 days after mouthwash. 4: DNA extracted 30 days after mouthwash.

DISCUSSION

Buccal cells are an excellent source of DNA for diagnosis and large-scale molecular epidemiological studies. Several protocols have been developed to obtain DNA from buccal cells, but cell collection by mouthwash seems to give higher yields than many other methods (1). In this study, it was noted that the rubbing of the tongue on the teeth and oral mucosa permitted a great increase in the amount of epithelial buccal cells collected. This procedure, however, increases the viscosity of the mouthwash solution, making it difficult, in some cases, to pellet the cells. This probably occurs due to high concentrations of salivary mucins in the mouthwash, which can hinder the collection of buccal cells after centrifugation. We have found that the addition of TNE reduces significantly the viscosity of the mouthwash. In fact, salivary viscosity is believed to occur due to the entanglement of long, high molecular weight oligomeric mucins (13).

The interaction of mucins seems to be mediated by calcium ions, and the removal of these ions by chelating agents can drastically reduce salivary viscosity (13). Therefore, the reduction of the viscosity of mouthwash after the addition of TNE-ethanol mixture can be imparted not only by the dilution of salivary mucins but also by the decrease of the interactions between mucins caused by the chelating action of EDTA. Additionally, EDTA also helps to preserve the integrity of DNA (14) as most enzymes that participate in the degradation of nucleic acids require divalent ion cofactors, usually magnesium, to promote activity (15). Due to these properties, EDTA has been added to all the solutions used for DNA purification in our protocol. The addition of ethanol in the mouthwash had two main purposes. It helped prevent bacterial growth during long-term room temperature storage and it also prevented mouthwash freezing when stored at low temperatures (0 to -20^{o}C). This procedure may prevent damage in the long strands of chromosomal DNA when the samples are frozen and thawed repeatedly. This stuyd have successfully extracted DNA for PCR analysis from mouthwashes stored at -20^{o}C for periods of up to 2 years (not shown).

The procedures used in the method described in this paper avoid the use of organic solvents. This was achieved by salting out the cellular proteins with 8 M ammonium acetate solution. Ammonium acetate precipitation of proteins has been used for DNA purification of seeds (16,17), bacteria, protozoarium, and white blood cells (18), buccal swabs (19) and formalin-fixed paraffin-embedded tissue sections (20). This reagent has also been used in commercially available kits (7,8,9).

In summary, the method described here is cheap and simple to be performed. Several samples can be processed at the same time, and the DNA extracted by this methodology yields sufficient DNA for many rounds of genotype analyses. Additionally, incubation of mouthwashes in TNE/ethanol prevents DNA degradation allowing safe storage and transport of field specimens collected in isolated communities, distant from the laboratory. DNA can be extracted and PCR amplified after storage in mouthwash solution at room temperature for periods of up to 30 days.

RESUMO

Células bucais são fontes convenientes de DNA para diagnóstico e estudos epidemiológicos. O objetivo deste trabalho foi desenvolver um método simples e prático para obter células epiteliais, através de bochechos, a fim de serem usadas como fonte de DNA e avaliar a estabilidade do DNA na solução de bochecho no decorrer do tempo. Os procedimentos usados neste estudo evitam o uso de solventes orgânicos permitindo uma pratica laboratorial mais segura. Isto é alcançado pela remoção das proteínas celulares por desidratação e precipitação com uma solução saturada de acetato de amônio. Este protocolo permite a extração de maneira rápida, simples, econômica e garante o processamento de várias amostras ao mesmo tempo, agilizando assim os procedimentos laboratoriais. Nossas análises forneceram evidências consistentes de que o DNA extraído por esta metodologia é suficiente para diversas amplificações por PCR (*polymerase chain reaction* - reação em cadeia pela polimerase). O produto total de DNA variou de 5 a 93 μg (mediana 15 μg; média 20,71 μg). Além disso, o DNA mostrou-se eficientemente preservado na solução de bochecho, a qual pode ser estocada em temperatura ambiente por até trinta dias.

ACKNOWLEDGEMENTS

This work was supported by FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

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Accepted March 26, 2007