Short Communication

A Simple Mouthwash Method for Obtaining Genomic DNA in Molecular Epidemiological Studies

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Abstract
Genomic DNA for genetic analyses has traditionally been derived from blood samples. With the availability of PCR techniques requiring only minute amounts of DNA and the current demand for high-volume testing, a less invasive, simpler to perform, and cheaper method to obtain DNA is desirable. We developed a method to obtain high-quality genomic DNA from buccal cells that has high acceptability and allows for a large number of PCR assays from a single sample. Sixty subjects vigorously swished 10 ml of undiluted commercial mouthwash in the mouth for 60 s and expelled the liquid into a collection container. DNA was isolated from the buccal cells with a rapid method using proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. Electrophoretic analysis of the extracted DNA showed detectable levels of high molecular weight genomic DNA in all samples. The DNA yields ranged from 0.2 to 134.0 µg, for an average of 49.7 µg. Using these samples, all 60 subjects were successfully genotyped by PCR-based assays for polymorphisms in the CYP1A1 (MspI and exon 7), CYP2E1 (RsaI), GSTM1, GSTT1, and NQO1 genes, confirming that the quality of DNA isolated from mouthwash samples was sufficient to reliably support PCR amplification. Storage of the (unprocessed) specimens at room temperature or at 37°C for 1 week (temperature conditions that may be encountered when mailing samples) or at −20°C for at least 6 months did not affect the DNA yield or ability to PCR amplify the samples. The results suggest that this mouthwash procedure may be suitable for large community-based studies of genetic susceptibility to disease in which samples can be collected by the participants themselves, mailed back to the study center, and stored for months prior to DNA analysis.

Introduction
Almost invariably, genomic DNA for PCR-based genetic analyses has been derived from leukocytes prepared from whole blood. Although this method yields large amounts of DNA (~100 µg from a 10-ml blood sample), an alternative source of DNA is desirable because venipuncture is invasive and uncomfortable for the subject and involves risk of exposure to blood-borne pathogens. It is also unacceptable in some individuals for cultural or religious reasons. As a consequence, it is not uncommon in molecular epidemiological studies for a substantial proportion (20–40%) of the participants to refuse the blood collection. Furthermore, obtaining blood samples becomes prohibitively expensive and logistically arduous when the study size is large or when the participants reside across a large geographical area, as is typical in family studies, in association studies examining gene-gene or gene-environment interactions, and in prospective studies of rare diseases.

DNA for genetic analysis has also been derived from finger-stick blood, paraffin-embedded tissue, urine, hair roots, saliva, cheek scrapings, buccal brushes, buccal swabs, and oral saline rinse samples (1–8). However, these methods are somewhat invasive (finger stick, cheek scrapings or brushes, and saline rinse) or do not yield an adequate amount (urine, hair roots, and saliva) or quality (paraffin blocks) of DNA. Also, some of these methods require the samples to be stored in a preservative solution that is toxic, which makes it problematic for use by mail (buccal brushes and swabs). This work was aimed at developing a method to obtain high-quality genomic DNA from buccal cells that would have high applicability and acceptability and allow for a large number of PCR assays from a single sample. Our requirements were that, with this method, samples could be collected by the participants themselves, mailed back to the study center, and stored for months prior to DNA extraction.

Materials and Methods
Subjects for this feasibility study were recruited among the employees of the Cancer Research Center of Hawaii and their acquaintances. A total of 64 individuals were given a 30-ml wide-mouth, screw-capped jar that contained 10 ml of undiluted mouthwash (FreshBurst Listerine), along with written instructions for collecting the sample at home. About 1 h after they brushed their teeth, the subjects swished the mouthwash vigorously throughout the mouth for 1 min and expelled it back into the jar. On the same day or on the following day, the participants returned the specimens to the laboratory. The mouthwash samples were either processed within 1 week or stored at −20°C for later extraction. Processing consisted of transferring the sample to a 50-ml conical tube for centrifugation at 2700 rpm for 15 min. The supernatant was decanted, and the pellet was washed in 25 ml of TE buffer [10 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0)]. The suspension was centrifuged at 2700 rpm for 15 min, and the supernatant was discarded. We modified the protocol published by Walsh et al. (9) to extract DNA. Briefly, the pellet was resuspended in 700 µl of lysis buffer [10 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0), 0.1 M NaCl, and 2% SDS] and transferred to a 2-ml microcentrifuge tube.

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Fig. 1. Distribution of amounts of DNA in µg obtained by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation from single mouthwash samples collected by 60 subjects. The DNA concentration of each sample was determined by spectrophotometer (GeneQuant II; Pharmacia Biotech) and ranged from 0.2 to 134 µg.

Fig. 2. Five µl of each buccal cell DNA sample were resolved by electrophoresis in a 1.8% agarose gel and visualized with ethidium bromide, as shown here for the first 17 samples extracted. Arrow, high molecular weight DNA.

tube containing 35 µl of 20 mg/ml proteinase K. The samples were mixed and digested at 58°C for 2 h. The DNA was then extracted from each sample with equal volumes of phenol-chloroform (1:1) and with an equal volume of chloroform alone, each time vortexing for 10 s and centrifuging at 14,000 rpm for 2 min. The DNA was removed from the supernatant with 3 M NaOAc (pH 6.0; 1/10 volume of supernatant) and 2 volumes of cold 100% ethanol and precipitated at -20°C for 2 h. The DNA was pelleted at 10,000 rpm for 10 min, washed with 70% ethanol, and dried in a SpeedVac (Savant) for 15 min. The pellet was resuspended in 200 µl of TE, and the concentration of DNA was calculated on a GeneQuant (Pharmacia Biotech). The integrity of the genomic DNA was determined by resolving 5 µl of the buccal DNA extract on a 1.5% agarose gel followed by visualization with ethidium bromide staining. The DNA samples were then subjected to genotyping following the same PCR-based protocols used in our laboratory for blood-derived DNA samples.

The first of the two CYP1A1 polymorphisms studied was a T-to-C transition 264 bp downstream from the poly(A) signal, which creates an MspI restriction site (m2 allele). Genotyping of the CYP1A1 alleles associated with the presence or absence of this MspI site was carried out by PCR amplification using primers 5'-TAGGAGTCTTGTCATGCTC-3' and 5'-CAG-TGAAAGAGGTGTAGCCGCT-3' (10). Amplification was performed in a thermal cycler (Perkin-Elmer) using 300 ng of DNA template with initial denaturation at 95°C for 4 min, followed by 30 cycles with denaturation at 95°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 1 min and a final annealing and extension step at 65°C for 1 min and 72°C for 8 min, respectively. The PCR product was then digested with MspI and subjected to electrophoresis on an agarose gel.

The second CYP1A1 polymorphism studied was an A-to-G transition, which results in the substitution of valine for isoleucine at residue 462 in the heme-binding region of the CYP1A1 protein. We assessed this polymorphism by the allele-specific PCR method described by Hirvonen et al. (11). For this purpose, each of the primers 5'-AAGACCTCCAGCG-GCAAT-3' (for the detection of the A allele) and 5'-AAGACCTCCAGCGGCAAC-3' (for the detection of the G allele) were used in subsequent PCRs, together with the opposite strand primer 5'-GAAAGGGCTGGTCCACCTCT-3', the 5' end of which is located 303 bp upstream of the A/G polymorphic site. Three hundred ng of DNA were used in each reaction. The PCR conditions consisted of an initial denaturation step at 94°C for 1 min 30 s, followed by 25 cycles with denaturation at 94°C for 1 min and annealing at 94°C for 1 min and extension at 70°C for 1 min 30 s. The PCR products were then subjected to electrophoresis on an agarose gel.

We also genotyped subjects for a polymorphism in the 5' flanking region of CYP2E1, consisting of two distinct base
substitutions that are in genetic disequilibrium with each other and create RsaI and PstI restriction sites (12). We used the primers 5'-TTCCATAGCTCTCTTCTTACCTG-3' and 5'-CCAGTGCAGTCTACATTGCTCA-3' to amplify a region containing the two distinct base substitutions. Five hundred ng of DNA were used for each PCR. Cycling conditions included an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min and a final annealing and extension at 72°C for 1 mm, respectively. RsaI digestion of the PCR products followed by resolution on an agarose gel helped identify the genotypes.

To detect deletion of the GSTJ1 and/or GSTT1 gene loci, we used the multiplex PCR method described by Deakin et al. (13), using primers 5'-GAACCTCTGAAAAGCTAAAGC-3' and 5'-GTGAGGTCCAAATATAGCTGC-3' for GSTM1 and 5'-TTCGCTAGTTGCATCCTC-3' and 5'-GCCGATCTGGCCAGCA-3' for GSTT1. We amplified a 268-bp fragment of the β-globin gene as an internal standard using the primers 5'-CAACTTCATCCAGTTCACC-3' and 5'-GAAGAGGCGAAGAGAAGTAC-3'. Three hundred ng of DNA template were used for each reaction. PCR conditions included an initial denaturation step at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 1 min and a final annealing and extension step at 72°C for 10 min.

Genotyping for the NQ01 polymorphism followed a PCR method modified from Traver et al. (14), using primers 5'-TTCCTCAGAGGTGCGATCCTGC-3' and 5'-TTCCTCAGAGGTGCGATCCTGC-3'. One hundred ng of DNA were used in each reaction. The PCR conditions included an initial denaturation at 94°C for 1 min, followed by: 2 cycles of denaturation at 94°C for 15 s, annealing at 69°C for 15 s, and extension at 72°C for 30 s; 2 cycles of denaturation at 94°C for 15 s, annealing at 67°C for 15 s, and extension at 72°C for 30 s; 31 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 5 min.

In a separate experiment, multiple mouthwash samples were collected from four individuals 1 day apart and either stored at room temperature or 37°C for 1 week to mimic conditions that may be encountered when samples are mailed or stored at -20°C for 3 or 6 months to test the effect of long-term storage. PCR assays were performed following the same protocols on these stored samples.

Results
Sixty subjects returned a mouthwash sample, giving a participation rate of 94%. These included 23 males and 37 females, ages 26–68 years, and they were of various ethnic backgrounds (19 Caucasian, 18 Japanese, 8 Chinese, 6 Hawaiian/part-Hawaiian, 3 Asian Indian, 2 Koreans, 2 Filipinos, and 2 African-American subjects). Sixteen subjects were smokers.

Fig. 1 represents the distribution of the amounts of DNA extracted from the mouthwash samples. The DNA yields ranged from 0.2 to 134.0 μg, for an average of 49.7 μg (SD = 31.7 μg). Electrophoretic analysis of the extracted buccal cell DNA showed detectable levels of high molecular weight genomic DNA in each sample (Fig. 2). The size of the gene regions amplified with the PCR methods used here ranged from 215 bp (for GSTM1) to 720 bp (for GSTT1). Ethidium bromide gels of PCR products after electrophoresis or after digestion with restriction enzymes followed by electrophoresis are shown.
Obtaining DNA with a Mouthwash Method

**Discussion**

With the availability of PCR techniques that require much less DNA than traditional Southern blotting, the notion that blood samples are the specimens of choice for molecular genetic epidemiology may need reevaluation. Blood collection is invasive and expensive and is not always accepted or practical. It also requires special handling and storage. By contrast, buccal cell collection is noninvasive and simple to perform and requires no special equipment or training. The use of such a source of genomic DNA in lieu of, or as an alternative to, blood collection is likely to increase participation and reduce costs in molecular epidemiological studies.

The validity of using DNA isolated from buccal cells has been demonstrated in previous studies. Richards et al. (16), collected buccal cells on cytology brushes or swabs from 533 individuals for the multiplex amplification of five exons within the CFTR gene. The success rate of PCR multiplex amplification in this study was 99%. In a blind comparison of the frequency of 12 mutations responsible for cystic fibrosis in products amplified with DNA from both blood and buccal cell samples collected from 464 individuals, there was 100% agreement in the results for the two types of DNA source (16).

Buccal brushes or swabs may be perceived as invasive, especially outside a clinical setting. Additionally, if not analyzed...
shortly after collection, the brushes or swabs should be placed in a preservative solution to optimize DNA yield and PCR amplification (5, 8). The toxicity of this storage solution does not make the method appropriate for unsupervised collection and transport by mail.

In contrast to using brushes or swabs, several authors have collected buccal cells by asking subjects to rinse their mouth with isotonic saline. This simpler and noninvasive collection method requires no supervision by trained personnel, no use of toxic reagents, and the samples can be obtained through the mail. The method has been validated in mass screening with PCR amplifications for specific mutations with specificity and sensitivity of 100% (17, 18). In an investigation of the AF508 mutations of the cystic fibrosis gene in mouth rinse samples collected in sputum containers from over 11,000 blood donors, the PCR failure rate was only 5.6% (17). These failures were thought to be due to insufficient rinsing of the mouth, containers leaking during transportation, or residual food contamination.

In another study testing the stability of the mouth rinse specimens when exposed to a variety of temperature conditions (7 days at -20°C, 4°C, 25°C, or 37°C) possibly encountered when samples are obtained by mail, it was noted that the specimens stored at 25°C and 37°C were more likely to yield increased amount of high molecular weight DNA, possibly of bacterial origin (19). Foreign DNA is unlikely to interfere with PCR amplification of specific alleles, and the subsequent visualization of the amplification products on the agarose gel. However, it was also noted in this study that "samples stored at higher temperatures resulted in slightly less robust PCR reactions" (19). The method we propose here minimizes the chance of substantial bacterial contamination by collecting samples after subjects brushed their teeth and by using an alcoholic solution. The alcohol content of the mouthwash brand that we used was 21.6%. Indeed, we did not observe greater DNA yields in samples stored at room temperature or at 37°C for 1 week, and the PCRs worked well. Moreover, mint-flavored mouthwash presented in a store-bought, sealed bottle of a familiar brand is likely to be more acceptable to study subjects, as part of a sample collection kit, than the saline solution used in previous studies. Possibility of leakage during transport can be minimized with the use of an appropriate container.

Our studies thus far have also shown that the suitability for PCR amplification of the DNA obtained by this method is not affected by freezing the mouthwash samples at -20°C for 6 months before DNA isolation. Although we have not yet tested longer storage durations, the samples are likely to remain stable for a longer period of time.

In previous studies, samples were apparently collected for a single genetic test. Thus, these previous reports focused on the successful amplification of a particular sequence and did not include DNA yields. This is an issue in studies of disease susceptibility genes in which a large number of PCR amplifications are typically conducted on the same samples. However, a few studies reported DNA yields that can be used for comparison with this study. Meulenberg et al. (5) reported a DNA yield of 1.3 ± 0.05 µg per buccal swab. Similarly, in the study by Freeman et al. (8), in which 10 buccal swabs were collected per subject, the average total DNA yield was 32 µg, with a range of 3.2–110.8 µg. Thus, these results suggest that a single mouthwash sample yields an amount of DNA that is comparable to that obtained from 16 to 38 buccal swabs. This amount is sufficient to run several hundred PCR assays and can be increased by collecting multiple samples.

We believe that this noninvasive method of buccal cell collection is likely to have a high acceptability, at least in populations where mouthwash is commonly used in oral hygiene, such as in the United States. The DNA extraction method used is sufficiently short to be practical for the processing of a large number of samples and yields human genomic DNA in a form that is easily amplified by PCR. Thus, this mouthwash procedure appears suitable for large community-based studies of genetic susceptibility to disease in which samples can be collected by the participants themselves, kept at room temperature for several days during transportation back to the study center, and stored for months prior to DNA extraction.

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References


Fig. 8. GSTM1 polymorphism for samples from four subjects (A, B, C, and D) using freshly collected samples (Lane 1) and samples that have been stored at room temperature (Lane 2) or at 37°C (Lane 3) for 1 week or at −20°C for 3 months (Lane 4) or 6 months (Lane 5). Lane M, HindIII-digested X174 DNA molecular weight marker; Lane (-), negative control.


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