

*Short Communication*Collection of Buccal Cell DNA in Seventh-Grade Children Using Water and a Toothbrush¹

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Abstract

We developed a simple and effective method for collecting a large quantity of buccal cell DNA in school-based studies of seventh-grade and older children. Seventh-grade students at schools in Wuhan, China brushed each buccal surface with a soft toothbrush and then rinsed with 10 ml of water. We added 5 ml of 99% ethanol to preserve the sample. Among 1563 samples transported at room temperature over 1 week and then stored for 13–14 months at -70°C before extraction, using a modified Gentra Puregene protocol, the median total DNA yield was 108 μg , range of 14 to 416 μg . We assayed every 20th sample ($n = 77$) for *NAT2* by the PCR, and all samples gave a 1093-bp product. From the 1563 samples, we obtained a result for single nucleotide polymorphisms in the *interleukin-13* gene (at +2044) by RFLP-PCR on 98.8% and in the promoter of the myeloperoxidase gene (at -463) by real-time PCR on 99.7%. A water-rinse method, that we used among 12th-grade students in Southern California, gave a lower total DNA yield than the toothbrush rinse (median of 17 μg) and a slightly reduced ability to generate a PCR product. However, 26 of 27 water-rinse samples gave a result for two genes, *albumin* and *CYP1A1*, using real-time PCR methods. We did not quantify human, versus bacterial, DNA in our samples. However, given the amounts of total DNA required for genotyping, a sample with the median yield of 108 μg should suffice for ~ 2160 genotypes by RFLP-PCR methods or five times as many by real-time PCR. We recommend the toothbrush-rinse method,

combined with a modified Gentra Puregene DNA extraction protocol, for large-scale, in-person collections of buccal cell DNA in children. The method requires only inexpensive, readily available materials and produces a large quantity of high-quality DNA for PCR analyses.

Introduction

Buccal cells provide a noninvasive source of DNA for epidemiological studies. A number of methods have been described for collection of DNA from buccal cells (1–19); fewer have been tested in children (1, 3, 7, 13, 14, 19). The published methods vary in DNA quantity and quality for PCR assays and their suitability for archival collection. In adults, rinsing with alcohol-containing mouthwash gives a relatively large quantity of DNA of sufficient quality for PCR-based genotyping (6, 10, 12). However, alcohol-containing mouthwashes are not recommended for use in young children, and older children might object to their taste. Furthermore, some parents, school officials, and human subject committees might raise concerns about the use of mouthwash in school children because of product warnings regarding dangers of accidental ingestion and because of the alcohol content. A method using a relatively expensive modified “Guthrie card” typically gives lower yields, and the card, like specific brands of mouthwash, may be difficult to obtain in international studies (8). Yields from cotton swabs and cytobrushes are lower than those from the mouthwash rinse.

We developed a buccal cell collection method suitable for use in large-scale, school-based studies of children in the United States and abroad. We required a protocol with the following characteristics: large yield of high-quality DNA for future PCR assays, readily acceptable to children and parents, quick to collect, uses readily obtainable materials, requires no abstinence from food or drink, and produces a stable sample under transport at room temperature. We report on a protocol that we developed for a school-based study in China using gentle abrasion of the buccal mucosa with a toothbrush followed by a water rinse. We also report on a water-rinse method that we used earlier in a school-based study in the United States.

Materials and Methods

The institutional review boards at the Wuhan Public Health and Anti-Epidemic Station and the NIEHS³ approved the protocol for Wuhan, China. The institutional review boards at the Keck School of Medicine at the University of Southern California and NIEHS approved the protocol for Southern California. Informed consent was obtained from the parents of all subjects.

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³ The abbreviations used are: NIEHS, National Institute of Environmental Health Science; β -hCG, β subunit of human chorionic gonadotropin hormone; *NAT2*, *N*-acetyl transferase 2; *IL-13*, interleukin-13; MPO, myeloperoxidase.

Methods for Buccal Cell Sample Collection

Water-rinse Method. Technicians collected samples individually on 100 12th-grade students from four Southern California schools in February 1998. First, to clean the mouth, students gently brushed the teeth with a new soft toothbrush and then rinsed with 30 ml of water. We discarded this cleaning rinse. Subjects returned 20 min later to swish 10 ml of water vigorously in the mouth for 60 s and then expectorate into a 50-ml collection tube. A technician then added 10 ml of 70% isopropanol to the sample. These samples were kept at room temperature for ≤ 2 days and then stored at 4°C until extraction 4–6 weeks later. Samples (27) were randomly selected for extraction. Extracted DNA was stored at 4°C.

Toothbrush-rinse Method. Samples were collected in March and April of 1999 at 22 middle schools in Wuhan, China. Students were enrolled individually with a technician providing instruction. We asked each student to gently swish and swallow 30 ml of bottled drinking water to reduce food particles. The student then dipped a new soft toothbrush into a cup containing 10 ml of bottled drinking water to soften the bristles. The subject brushed the right buccal surface 10 times (one time consists of one up and one down stroke), while the technician counted, and then expectorated the saliva into a sterile 50-ml polypropylene centrifuge tube. The student repeated the process on the opposite buccal surface. After swishing vigorously with 10 ml of water for 60 s, with the technician timing, the subject expectorated into the 50-ml tube. The technician slowly poured 5 ml of 99% ethanol over the bristles of the toothbrush into the same 50-ml tube. The samples were stored at room temperature until return to the laboratory, where they were stored at 4°C before processing. The enrollment procedure took < 3 min/student, including instruction time.

Before finalizing the above protocol for use among 4888 students at 22 schools in urban and suburban Wuhan, China, we collected pilot samples on 200 students from four schools. Sixteen of these samples, 4 from each school, were randomly selected from these 200 samples for DNA extraction.

Sample Processing and DNA Extraction

The water-rinse samples were centrifuged for 20 min at $1000 \times g$. The pellet was transferred to a 1.5-ml centrifuge tube. DNA was extracted using a standard phenol chloroform procedure (9) and then stored at 4°C.

The toothbrush-rinse samples were centrifuged for 10 min at $1000 \times g$. The pellet was transferred to a 1.5-ml centrifuge tube, along with a small amount of the supernatant. The samples were stored at 4°C for ≤ 2 months before transport to NIEHS. The transport at room temperature took 1 week for the 4888 full-study samples. The 16 pilot samples were kept at room temperature for 3 weeks to simulate maximum shipping delay and then extracted without freezing using phenol-chloroform extraction (9). Buccal samples on the 4888 students were stored at -70°C for 13–14 months before extraction. From these 4888 samples, we selected a case-cohort set of 1563 samples for DNA extraction.

The DNA from these 1,563 samples was extracted using a modification of a Genra Puregene protocol (#00922 Genra Systems, Minneapolis MN).⁴ Briefly, the buccal samples were centrifuged to remove the alcohol. The pellets were resuspended in Cell Lysis Solution and incubated with Proteinase K

solution at 55°C for 2 h, with vigorous pipetting every 20–30 min. To precipitate protein, samples were chilled for 10 min at -20°C , and Protein Precipitation Solution was added to the cell lysate. Samples were vortexed vigorously at high speed for 20 s, chilled for 10 min at -20°C , then centrifuged at $14,000 \times g$ for 10 min to precipitate the proteins. To precipitate DNA, the supernatant containing the DNA was decanted into a tube containing 2-propanol, and Glycogen Solution was added. The tubes were inverted gently 50 times at room temperature and centrifuged at $14,000 \times g$ for 10 min to pellet the DNA. Tubes were inverted and drained and air dried for 10–15 min, and the DNA pellets were resuspended in DNA Hydration Solution.

For all collections, we determined the total DNA yield by measuring the A260 absorbance and also calculated the A260:A280 ratio. Genomic DNA check gels of 1 μg of DNA verified that the genomic DNA was in excess of 23 kb.

Methods for PCR Assays Used to Demonstrate DNA Quality

β -hCG. Samples from the water-rinse were assayed by PCR for the β -hCG gene. The forward and reverse primers were 5'-TACTGCCCCACCATGACCCG-3' and 5'-AGGGGCCTTTGAGGAAGAGGA-3' respectively, resulting in a 263-bp PCR product. One μl of the amplification products was reamplified using the forward and reverse nested primers: 5'-CCTCAGGTGGTGTGCAACTA-3' and 5'-GCACATTGACAGCTGAGAGC-3'. The size of the PCR product using the nested primers was 115 bp. We used 50 ng of DNA/PCR.

NAT2. Samples from the toothbrush rinse were subjected to PCR amplification of NAT2 as described by Bell *et al.* (20) with slight modification. We used 50 ng of total DNA/reaction. The PCR product size was 1093 bp.

IL-13 +2044 Polymorphism. From the toothbrush-rinse samples, we determined IL-13 +2044 genotypes by a PCR-RFLP technique as previously described (21) with the modification that we used $1 \times$ PCR buffer (Promega, Madison WI). We used 50 ng of total DNA. The PCR product size was 236 bp. The three possible genotypes are defined by three distinct banding patterns: G/G (178-bp fragment), G/A (178- and 210-bp fragments), and A/A (210-bp fragment).

Albumin and CYP1A1. Samples from the water rinse were assayed using real-time PCR technology with a TaqMan 7700 apparatus (Applied Biosystems, Foster City, CA). In two sets of independent experiments, we amplified albumin and a polymorphism in exon 7 of the CYP1A1 gene (22). For albumin amplification, we used forward and reverse primers 5'-CGATTTTCTTTTATAGGGCAGTAGC-3' and 5'-TGAAACTTCTGCAAACTCAGC-3', respectively, and the probe 6FAM-CGCCTGAGCCAGAGATTTCCCA-TAMRA. The reverse primer used for amplification of CYP1A1 was 5'-ACGCTGAATTCCACCCG-3', and the probe was 6FAM-CTGGGAGGTCTTTCTTCTTCTGGCT-TAMRA. Two different forward primers were used: the sequence 5'-GGAAGTG-TATCGGTGAGACCA-3' allowed specific amplification of the wild-type allele, whereas the sequence 5'-GAAGTG-TATCGGTGAGACCG-3' allowed specific amplification of the variant allele. The PCR product size was 70 bp for albumin and 92 bp for CYP1A1. We used 50 ng of total DNA for each of these two assays.

MPO. In the toothbrush-rinse samples, we assayed an A/G polymorphism at position -463 of the MPO promoter (23) with real-time PCR by a TaqMan protocol. The forward and reverse primers were 5'-GCTGAGAAATCTTGGGCTGGT-3' and 5'-

⁴ Internet address: <http://www.genra.com>.

Table 1 Total DNA yields, in μg , for three buccal cell collections

	Collection method		
	Water rinse	Toothbrush-rinse pilot	Full study
No. of subjects	27	16	1563
Extraction method	Phenol-chloroform	Phenol-chloroform	Genra
Total DNA yield (μg)			
Minimum	6	9	14
10th %	9	15	46
25th %	10	21	72
Median	17	29	108
75th %	26	60	152
90th %	47	145	199
Maximum	64	220	416

GGCCAGGCTGGTCTTGAAC-3', respectively. The probe for the wild-type "G" allele was TET-TGATCCACCCGCCT-CAGCCTCC-TAMRA. The probe for the variant "A" allele was FAM-TGATCCACCTGCCTCAGCCTCC-TAMRA. The PCR product size was 142 bp. Each reaction contained 10 ng of DNA.

Statistical Analysis. *P*s for differences in total DNA yields were calculated using the Wilcoxon's rank-sum test (SAS, Version 8, Cary, NC).

Results

Total DNA yield results from the three collections are shown in Table 1. For the 27 water-rinse samples, the median total DNA yield was 17 μg compared with values for the toothbrush-rinse method of 29 μg for the 16 pilot samples ($P = 0.016$) or 108 μg for the 1563 samples in the full study ($P < 0.001$ compared with either the water-rinse or pilot samples).

Table 2 shows the success rates for various PCR assays for the three sample sets. For a standard PCR method, as opposed to real-time PCR, the success rate was slightly lower for the water-rinse samples. All toothbrush-rinse samples, whether from the pilot or full study, gave the 1093-bp PCR product for NAT2 on the first attempt. In the full study, we obtained a result for the *IL-13* +2044 genotype by a PCR-RFLP method on 98.8% of 1563 samples that had been stored for 6 months at -70°C after extraction. The successful result represented a repeat for 62 samples that initially produced only a faint signal and for 19 samples that did not amplify initially. The frequencies were 0.334 for the A allele and 0.666 for the G allele, and the genotype frequencies were in Hardy-Weinberg Equilibrium.

For real-time PCR, the success rates were very high for both the water-rinse (96.3%) and toothbrush-rinse samples (99.7%; Table 2). For the *MPO* genotype on the 1563 full study samples, the frequency of the A allele was 0.129 and the G allele was 0.871, and the genotype frequencies were in Hardy-Weinberg Equilibrium.

To determine whether the extracted DNA was stable over time, we reassayed the water-rinse samples, because these have been stored for the longest time: 31 months at 4°C . All 24 samples that gave a PCR product for β -*hCG* in 1998 could be amplified for this product 31 months later.

Discussion

We have developed a new method for collection of DNA samples in children as young as 12 years. The method uses a toothbrush to gently abrade the buccal mucosa followed by a

60-s water rinse. The method yields a large amount of high-quality DNA after storage for 13–14 months at -70°C , after transport at room temperature for 1 week.

We chose the toothbrush-rinse method for the full study in China initially because the water-rinse method we used in the United States required students to come back 20 min after the cleaning rinse. However, we also prefer the toothbrush method because of the higher total DNA yields and the greater success in generating PCR products. Both methods gave results in nearly all samples using real-time PCR.

The toothbrush-rinse protocol has useful features for a large school-based study. The required supplies are inexpensive and widely available: toothbrush, 50-ml centrifuge tube, cup, ethanol, and drinking water. The supplies cost \sim \$0.40/subject at United States prices.

Yields of total DNA from the toothbrush rinse compare favorably to published methods. From in-person collections in school children, Hagerman *et al.* (7) obtained median total DNA yields in the range of 5–7.5 μg for 1 min cytobrush abrasion, 0–2.5 μg for Gatorade rinse, and 2.5–5 μg for wooden swab plus Gatorade rinse. Typical yields of total DNA are \sim 2 μg from cotton swabs, in either children (5) or adults (5, 13), and from cytobrushes (Genra Protocol #00920).⁴ Mouthwash-rinse protocols in adults have produced mean yields of \leq 54.5 μg of total DNA (6, 10, 12, 24).

The total DNA yield we measured includes both human and bacterial DNA. Mouthwash samples collected by mail have been shown to contain a median of 50% human DNA (6). Although we did not determine the percentage of human DNA in our samples, 50 ng of total DNA for a standard PCR assay and 10 ng of total DNA contained sufficient human DNA to generate high-quality results. Therefore, the median sample, with a yield of 108 μg of total DNA, would suffice for 2,160 standard PCR or 10,800 real-time PCR assays, regardless of the percentage of human DNA in the amount used.

The yield of human DNA in buccal cell samples has been estimated in two studies (6, 8). The median yield of human DNA collected onto modified Guthrie cards was 2.3 μg (8). Compared with rinse methods, this method avoids the need to centrifuge the rinse fluid and extract the DNA. In mouthwash rinse samples from two mailed collections in adults, median human DNA yields were estimated at 16.6 and 27.5 μg (6). If our samples also contain a median of 50% human DNA (6), our toothbrush-rinse method generates higher yields, but the supervised in-person collection may contribute.

The lower total DNA yields for the toothbrush rinse in the pilot study, compared with the full study, might reflect degradation because of longer time at room temperature (3 weeks *versus* 1 week) or the use of phenol-chloroform extraction instead of the Genra Puregene protocol. After the pilot study, we evaluated the Genra Puregene protocol for reasons of convenience and obtained higher total yields. Alternatively, there may have been a training effect as the field collection team gained experience. However, the total yield is more reliably estimated among 1563 samples than among 16 samples, regardless of the statistical significance of the difference. Nonetheless, because the 16 pilot samples also gave acceptable total yields and PCR products, we conclude that the method is robust to variations in technique.

We recommend the toothbrush-rinse method for large-scale epidemiological studies with in-person collection in children or adults in combination with the modified Genra Puregene protocol for DNA extraction. The method requires only inexpensive and commonly available materials and produces samples that are stable for transport at room temperature and

Table 2 PCR success rates for three buccal cell collections

Gene	PCR product size (bp)	Assay	Water rinse	Toothbrush-rinse pilot	Full study
<i>β-HCG</i>	236	PCR	24/27 (88.9%)		
<i>NAT2</i>	1093	PCR		16/16 (100%)	77/77 (100%)
<i>IL-13</i>	236	PCR-RFLP			1545/1563 (98.8%)
<i>Albumin</i>	70	Real-time PCR	26/27 (96.3%)		
<i>CYP1A1</i>	92	Real-time PCR	26/27 (96.3%)		
<i>MPO</i>	142	Real-time PCR			1558/1563 (99.7%)

yield a large amount of high-quality DNA for future PCR assays.

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