Collection of Buccal Cell DNA Using Treated Cards

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Abstract

We devised a simple, noninvasive, cost-efficient technique for collecting buccal cell DNA for molecular epidemiology studies. Subjects ($n = 52$) brushed their oral mucosa and expectorated the fluid in their mouths, which was applied to "Guthrie" cards pretreated to retard bacterial growth and inhibit nuclease activity (IsoCode, Schleicher and Schuell, Keene, NH). The cards are well-suited for transport and storage because they dry quickly, need no processing, and are compact and lightweight. We stored the samples at room temperature for 5 days to mimic a field situation and then divided them into portions from which DNA was extracted either immediately or after storage for 9 months at room temperature, −20°C, or −70°C. The fresh samples had a median yield of 2.3 μg of human DNA (range, 0.2–53.8 μg), which was adequate for at least 550 PCR reactions. More than 90% of the samples were amplified in all three β-globin gene fragment assays attempted. DNA extract frozen for 1 week at −20°C also performed well. Stored samples had reduced DNA yields, which achieved statistical significance for room temperature and −70°C, but not −20°C, storage. However, because all of the stored samples tested were successfully amplified, the observed reduction may represent tighter DNA fixation to the card over time rather than loss of genetic material. We conclude that treated cards are an alternative to brushes/swabs and mouth rinses for the collection of buccal cell DNA and offer some advantages over these methods, particularly for large-scale or long-term studies involving stored samples and studies in which samples are collected off-site and transported. Future studies that enable direct comparisons of the various buccal cell collection methods are needed.

Introduction

Simple methods of collecting DNA samples in large-scale community studies could extend the range of molecular epidemiological studies. Venipuncture, the standard DNA collection method, cannot be used in many situations for medical, logistic, or cultural reasons. In other situations, it is feasible but prohibitively expensive. We therefore devised a simple, noninvasive, cost-efficient technique to obtain DNA samples in large-scale community studies and assessed the quantity and quality of DNA collected.

Buccal cells provide an accessible source of germ-line DNA. Buccal cell collection techniques involving swabs, brushes, and scraping instruments (1–9) and oral rinses using water, saline, and mouthwash (4, 10–15) have been described. Although these methods are generally easy to administer, noninvasive, cost-efficient, well accepted by subjects, and safe for study personnel, the DNA collected using swabs, brushes, and scraping tools may be vulnerable to degradation if the samples are not processed or frozen soon after collection (1, 6–9).[2] The unfortunate result may be specimens that are inadequate for testing multiple genetic factors. Rinses typically provide adequate quantities of good quality DNA, but liquid samples may spill or leak during shipment. In addition, storing large numbers of rinses requires considerable space, some preservatives pose safety hazards to untrained individuals, and extracting DNA from rinses may be labor-intensive. We sought an alternative technique for collecting buccal cell samples, which would eliminate these potential drawbacks.

"Guthrie cards" (903 filter paper, Schleicher & Schuell, Inc., Keene, NH) are used to collect heelstick blood from newborns for metabolic disease screening (16); however, blood spots archived as long as 17 years, sometimes at room temperature, have also provided valuable sources of amplifiable DNA (17–20). A modified card (IsoCode, Schleicher and Schuell, Keene, NH) has been developed that is treated to retard bacterial and viral growth, inhibit nuclease activities, and release template DNA during processing (21, 22). Treated cards reliably yield amplifiable nucleic acid from blood and buccal cell samples, whereas untreated cards do not (22). In one genetic epidemiology study, PCR-based assays were performed on DNA from fingerstick blood samples collected on treated cards from >5300 subjects (23). In the present study, we used such treated cards to collect buccal cell DNA and report the quantity, quality, and stability of human DNA from samples collected in a manner similar to that which would occur in many epidemiological studies.

Materials and Methods

Subjects. Fifty-two healthy employees of the NIH (Bethesda, MD) and Westat, Inc. (Rockville, MD) provided written, informed consent to participate in this study. The study protocol was approved by the Institutional Review Boards of the NIH and Westat, Inc.

Received 9/30/98; revised 2/2/00; accepted 2/29/00.
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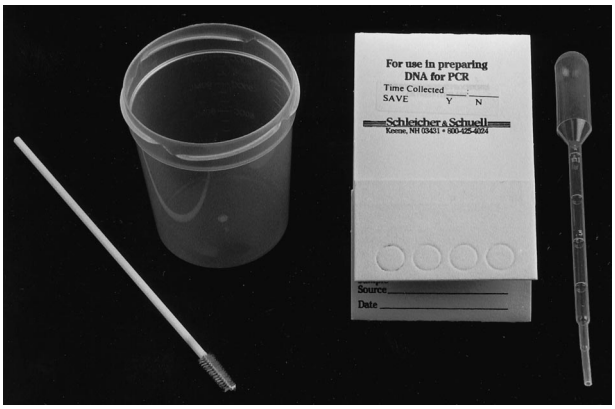

Fig. 1. Buccal cell collection kit. Subjects brushed their oral mucosa with the soft-bristled cytobrush, swished the saliva that collected in their mouths during brushing, and expectorated it into the cup. The saliva sample was transferred to the card using the disposable pipette.

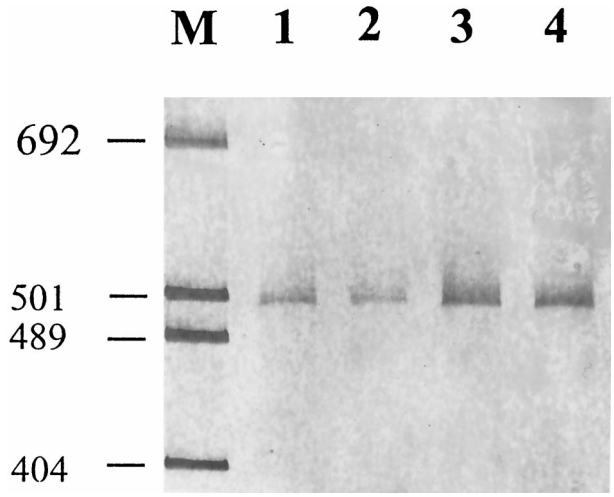

Fig. 2. A 536-bp fragment of the human β-globin gene was amplified from fresh buccal cell samples collected on treated cards. DNA extract (5 μl) from buccal cells was amplified by PCR in a 100-μl reaction mix containing 1× PCR buffer [50 mM KCl, 4.0 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5)], 200 μM of each dNTP, 100 nM of each primer (RS42/KM29), 2.5 units of Taq DNA polymerase, and 5 μl of template DNA. The DNA template was amplified by denaturing at 95°C for 1 min, annealing at 55°C for 1 min, and extending at 72°C for 2 min, with a final extension at 72°C for 5 min. Amplified product (10 μl) from four samples was separated in polyacrylamide gel and stained with silver (*Lanes 1–4*). *Lane M* contains a DNA molecular weight marker. Fragment sizes were confirmed by comparison to DNA molecular weight marker VIII (Boehringer Mannheim Corp., Indianapolis, IN; *Lane M*).

Sample Collection. We asked subjects to refrain from smoking, drinking, or eating for 1 h before sample collection to reduce the possibility that food particles or other exogenous materials would compromise the sample (15). Subjects made 20 firm strokes on their oral mucosa with a soft-bristled, sterile cytobrush (Medical Packaging Corp., Camarillo, CA; Fig. 1). We instructed the subjects to maintain contact between the brush and the inside of their cheeks and to brush as much of the inner cheek surface as possible. Subjects swished the saliva that pooled in the mouth during brushing and expectorated it into a sterile specimen collection cup (VWR, Westchester, PA). A research assistant transferred the saliva to IsoCode collection cards (Schleicher & Schuell) by use of a disposable, sterile pipette (VWR). The saliva sample was outlined using a disposable pencil. Cards were air-dried for 30–60 min and placed in a sealed plastic bag with a desiccant packet. The collection procedure, including obtaining informed consent, took ~10 min.

Sample Processing. We stored all samples at room temperature in sealed plastic bags with desiccant for 5 days. We reasoned that samples collected off-site and mailed to a laboratory by regular mail would be at room temperature that long. On the 5th day, samples were split into four portions, and DNA was extracted from one portion ("fresh samples"). The other three portions ("stored samples") were kept at different temperatures (room temperature, −20°C, and −70°C) for 9 months to assess the stability of unprocessed cards. DNA was extracted from five stored samples corresponding to the five fresh samples with DNA yields at the 20th, 40th, 60th, 80th, and 100th percentiles. Extracted DNA was stored at 4°C. After 2 months at 4°C, DNA extract from the fresh samples was stored at −20°C for 1 week ("frozen DNA") to assess the impact of freezing on extracted DNA.

DNA was extracted using the following method, which is a modification of the manufacturer's instructions. Two one-eighth-inch discs were punched from the card and rinsed twice by pulse-vortexing for 5 s in 500 μl of double-distilled H_2O. The paper puncher was sterilized between samples with alcohol and flame and by making several punches through clean filter paper. Rinsed discs were transferred into 50 μl of double-distilled H_2O, heated to 95°C for 30 min, tapped 20 times, and centrifuged for 10 s at 3000 rpm. The extraction procedure was repeated with two more discs, and the aliquots were combined to yield a total volume of 100 μl.

DNA Quantification. DNA yields (per four punches) were determined by hybridization to Alu sequences using the ACES 2.0+ DNA Quantification System (Life Technologies, Inc., Grand Island, NY). One to four serial dilutions of samples were prepared with Tris-low EDTA, and the DNA yield was quantified by comparison to human DNA standards using image analysis. The sensitivity of the Alu assay in our laboratory was 0.25 ng using the Image Analyzer. We estimated the DNA yield per card by multiplying the amount of DNA per punch (*i.e.*, one-quarter of the measured yield) by the mean number of punches per card, as determined from four randomly selected cards (mean ± SD, 110.5 ± 6.6 punches).

To assess whether the DNA was evenly distributed, we quantified the amounts of DNA in four concentric circles, which, altogether, included the entire outlined area of the sample. The innermost circle was the premarked circle to which the expectorated saliva was directly applied, and the three outermost rings comprised the area to which the saliva spread. We determined the DNA quantities in concentric circles separately for each of the four premarked circles on the card and determined the mean yields for a given ring (*e.g.*, innermost, second outermost) by averaging the results from the four replicates. We also determined the total DNA yield from each premarked circle and the concentric rings surrounding it and compared the total DNA yields for each of the four replicates.

PCR-based Assays. Portions of the β-globin gene were amplified from the extracted DNA using the PCR (24, 25) as described by Greer *et al.* (26). Briefly, in separate PCR reactions, primers GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) and PC04 (5′-CAACTTCATCCACGTTCACC-3′) were used to generate a 268-bp fragment, primers RS42 (5′-GCT-

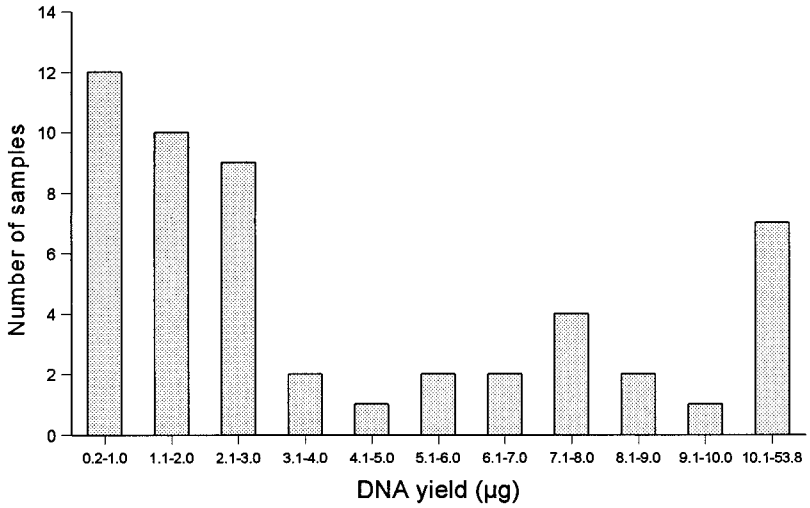

Fig. 3. Estimated DNA yields (μg) per card from samples collected from 52 subjects. The amount of DNA extracted from four 3-mm punches of the card was determined by hybridization to Alu sequences, a method which is specific for human DNA. Yields per card were estimated by multiplying the DNA yield per punch by 110.5, the average number of punches per card.

CACTCAGTGTGGCAAAG-3′) and KM29 (5′-GGTTGGCCAATCTACTCCCAGG-3′) were used to generate a 536-bp fragment, and primers RS80 (5′-TGGTAGCTGGATTGTAGCTG-3′) and RS40 (5′-ATTTTCCCACCCTTAGGCTG-3′) were used to generate a 989-bp fragment. In addition to 5 μl of extracted DNA, the 100-μl reaction volume contained 100 nM of each primer, 200 μl of each dNTP, 2.5 units of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN), and 1× PCR buffer [50 mM KCl, 4 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5)] and was overlaid with 100 μl of mineral oil. Samples underwent 40 amplification cycles (1 min at 95°C, 1 min at 55°C, and 2 min at 72°C) followed by a final extension of 5 min at 72°C. The amplicons were separated in a modified polyacrylamide matrix (GeneAmp Solution, Perkin-Elmer, Foster City, CA) and stained with silver. Up to two amplification attempts were made per sample. Samples that failed in both attempts were also attempted up to two times using 2.5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA) under the reaction conditions described above.

The quality of the DNA from the fresh samples was assessed with the 268-bp, 536-bp, and 989-bp fragment assays; representative results of 536-bp fragment assay are shown in Fig. 2. The stored samples and the frozen DNA were compared to the fresh samples using the 536-bp assay.

Statistical Analyses. The distribution of DNA yields was normalized using a natural logarithmic transformation. We evaluated differences in log (DNA yield) values among selected factors by use of unpaired *t* tests. To compare DNA yields between fresh and stored samples from the same subjects, we standardized the differences by dividing by the fresh sample DNA yield and performed paired *t* tests on the standardized differences. We assessed differences in the percentage of samples with successful PCR amplification by various factors using Fisher's exact test for unpaired samples, or the exact version of McNemar's test (equivalent to the sign test) for paired samples. All statistical tests were implemented using PC-SAS version 6.12 (SAS Institute, Inc., Cary, NC). All tests of significance were two-sided.

Results

The DNA yields from four punches of the fresh samples were variable (range, 7.6–1945.7 ng), with a median value of 83.6 ng. Assuming 110.5 punches/card, the estimated median yield per card was 2.3 μg (range, 0.2–53.8 μg; Fig. 3). One subject with a chronically dry mouth produced approximately one-quarter the amount of saliva (and therefore punches) as the other subjects. The estimated DNA yield from this subject's card was 0.4 μg of DNA. Based on the average number of punches per card and the volume of DNA extract used per PCR reaction in our study, we estimate that at least 550 such PCR reactions could be conducted per sample.

On the card from which all four premarked circles and corresponding concentric circles were all processed as fresh samples, the DNA was dispersed throughout the outlined area. The mean yields from four punches was 137.5 ng for those taken from the premarked circle (innermost circle) and 121.0 ng for those taken just within the outline (outermost circle). Comparing the yields among the premarked circles together with their concentric circles, the mean DNA yields per four punches ranged from 102.3 ng to 132.0 ng for the four replicates.

In >90% of the fresh samples, we were able to amplify human β-globin gene fragments (Table 1). Slightly more samples amplified in the 268-bp fragment assay [51 of 52 (98.1%)] than in the 536-bp fragment assay [50 of 52 (96.1%)] or the 989-bp fragment assay [47 of 52 (90.4%)]. Samples for which the 268- or 536-bp fragment assays failed also failed to support amplification in assays for all larger fragments. Samples that failed to amplify in the 989-bp fragment assay (which included all samples that failed in the 268-bp and 536-bp fragment assays) had significantly lower DNA content than those that amplified in all three assays ($P = 0.02$; Table 2). The only sample for which none of the three target sequences amplified had the lowest DNA yield (*i.e.*, 0.2 μg). One of two failures for the 536-bp fragment and one of five failures for the 989-bp fragment were successfully amplified by use of the AmpliTaq Gold enzyme, bringing the percentages of samples that supported amplification to 98.1% and 92.3%, respectively.

Eighteen (34.6%) subjects ate or drank in the hour preceding sample collection. Although the DNA yields from their cards (median, 2.0 μg) were similar to the yields from the cards of subjects who refrained from these behaviors (median, 2.4 μg; $P = 0.11$), PCR amplification failure was observed for a larger proportion of samples from subjects who drank or ate in the hour preceding collection (4 of 18; 22.2%) than from

Table 1 PCR amplification of β-globin gene fragments from DNA from fresh buccal cell samples collected on treated cards

Fresh samples[a]	*n*	Successful PCR amplification *n* (%)		
		268 bp	536 bp	989 bp
DNA extract never frozen				
Taq polymerase	52	51 (98.1)	50 (96.1)[c]	47 (90.4)
Taq polymerase or AmpliTaq Gold[b]	52	51 (98.1)	51 (98.1)	48 (92.3)
DNA extract frozen at −20°C for 1 wk				
Taq polymerase	52	N/A[d]	46 (88.5)[c]	N/A
Taq polymerase or AmpliTaq Gold[b]	52	N/A	51 (98.1)	N/A

[a] All fresh samples were stored at room temperature for 5 days, at which time DNA was extracted, and the DNA extract was stored at 4°C. Never frozen DNA extract was maintained at 4°C before PCR, whereas the DNA extract was stored at −20°C for 1 week for experiments on frozen DNA extract.
[b] AmpliTaq Gold used for samples that did not amplify by use of Taq polymerase.
[c] $P = 0.13$, McNemar's exact test; frozen DNA *versus* never frozen DNA amplified by Taq polymerase.
[d] N/A, not applicable.

Table 2 DNA yields of fresh samples by amplification status in three PCR-based β-globin gene assays

Amplification status[a]	*n*	DNA yield[b]	
		Median (μg)	Range (μg)
0 of 3	1	0.2	
1 of 3	1	0.9	
2 of 3	3	1.9	0.3–2.3
3 of 3	47	2.4	0.4–53.8

[a] Number of assays for which PCR amplification was achieved.
[b] $P = 0.02$, unpaired *t* test comparing log (DNA yields) of samples for which amplification was observed in three of three assays to DNA yields of samples in all other groups (*i.e.*, zero, one, or two successful assays) combined.

subjects who did not (1 of 34; 2.9%; $P = 0.04$). Three subjects were tobacco users, two of whom used tobacco products in the hour preceding sample collection. DNA yields from tobacco users' cards (median, 0.4 μg) were lower than those from the nontobacco users' cards (median, 2.4 μg; $P = 0.02$). Failure to amplify was observed for one of three (33.3%) tobacco users' samples, compared to 4 of 49 (8.2%) nontobacco users' samples ($P = 0.27$). One of the tobacco users was the subject with a chronically dry mouth who produced a relatively small amount of saliva; however, the sample from this subject supported PCR. The two other tobacco users produced a similar amount of saliva as the nontobacco users.

Amplification in the 536-bp fragment assay was achieved for 46 of 52 (88.5%) samples in which the extracted DNA was frozen for 1 week at −20°C, compared to 50 of 52 (96.1%) fresh samples ($P = 0.13$; Table 1). Furthermore, five of six (83.3%) failures among frozen samples amplified by use of AmpliTaq Gold polymerase.

Compared to fresh samples from the same subjects, DNA yields were generally lower for stored samples (Table 3). The reductions in yield achieved statistical significance for five samples stored for 9 months at either room temperature ($P = 0.01$) or −70°C ($P = 0.02$), but not −20°C ($P = 0.17$). The mean percent decrease in DNA yield between fresh samples and stored samples was 45.2% for room temperature storage, 29.6% for −20°C storage, and 57.2% for −70°C storage. For all storage conditions, the 536-bp fragment amplified for all five subjects.

Discussion

We demonstrated the use of a new method for the collection of buccal cell DNA using treated cards. This technique offers many advantages for DNA collection (Table 4). Like other buccal cell collection approaches (1–5, 9, 11–13), it is easy, noninvasive, limits staff exposure to blood-borne pathogens, and is less expensive than obtaining DNA from venous blood. A unique feature of the cards is their impregnation with an agent reported to retard bacterial and viral growth and inhibit nuclease activity, thereby minimizing nucleic acid degradation (21, 22). In contrast, buccal cell samples collected using brushes, swabs, or rinses are stable for weeks or months when stored in a preservative (7, 8, 10, 14), but without this measure, they may be vulnerable to bacterial growth, nucleic acid degradation, and reduced ability to PCR after as little as 4 days, particularly under warm or moist conditions (1, 6, 8, 9, 11).[2]

The DNA yields were highly variable, with an estimated median yield of 2.3 μg of human DNA/card (range, 0.2–53.8 μg). Because the DNA yields varied 100-fold, it is likely that some samples could be extended by dilution. Reported DNA yields range from <2 to 111 μg of DNA for brushes or swabs (4, 7–9) and from <2 to 240 μg of DNA for rinses (4, 12–14). Direct comparisons are difficult because the quantification methods used in these studies were not specific for human DNA, as ours was, and it is believed that some samples contain significant quantities of bacterial DNA (6, 11). Sometimes, the fraction of the sample that was used in a PCR reaction was reported, which ranged from 1 to 10% for rinses (10, 12) and from 1 to 60% for buccal brushes or swabs (1–3, 5, 9, 27). We estimate that our samples could support several hundred PCR reactions per card. It is also feasible to retain the brushes used to loosen the buccal cells as an additional source of DNA.

The cards are well-suited for transport and storage because they dry within 1 h, do not require processing, and are lightweight, compact, and flat. For example, a standard 27-ft^3 −70°C freezer holds 14,560 cards, and efficient use of storage space can substantially reduce storage costs in cohort or other large studies. Of concern is the observed reduction in DNA yields after storage for 9 months; the decline was statistically significant for storage at room temperature or at −70°C, but not at −20°C. For all storage conditions, measurable DNA was recovered and PCR amplification was successful for all samples we assessed. The reduction in extracted DNA using the standard method may reflect greater DNA fixation to the card over time, as reported by Makowski *et al.* (17) for blood samples, or may reflect loss of genetic material. Others have reported no differences in DNA yields or ability to PCR for buccal cell samples collected p.o. washes stored at −20°C for up to 6 months (14) or by brushes or swabs stored at 4°C for up to 1 month (1). Walker *et al.* (9) reported that long-term storage (12–41 months) at 4°C of buccal cell DNA samples collected using cytobrushes diminished their success in PCR assays by between 6 and 11%, although amplification in at least one PCR assay was achieved for all samples. It will be important for larger, long-term studies to investigate whether the DNA concentration of the extract from the cards truly declines over time, and if so, to determine the pattern of the reduction (*e.g.*, plateau; linear decrease to zero) and whether DNA yields can be restored through the use of more rigorous extraction techniques. Our finding that cards stored at room temperature for 9 months had comparable DNA yields and PCR results to those stored at −20°C or −70°C suggests that storage at room temperature or in regular freezers, such as in situations in which laboratory

Table 3 DNA yields and PCR amplification results from five buccal cell samples collected on treated cards and stored under various conditions

Sample type	*n*	DNA yield (μg)					*n* (%) amplified 536-bp fragment
		Card A	Card B	Card C	Card D	Card E	
Fresh	5	1.0	1.9	3.1	7.5	45.6	5 (100)
Stored 9 mo							
Room temperature[a]	5	0.4	1.8	1.5	3.9	17.8	5 (100)
−20°C[b]	5	0.7	1.7	3.9	2.0	18.3	5 (100)
−70°C[c]	5	0.9	0.5	1.8	1.4	9.6	5 (100)

[a] $P = 0.01$, paired *t* test of standardized difference in DNA yields, room temperature storage *versus* fresh.
[b] $P = 0.17$, paired *t* test of standardized difference in DNA yields, −20°C storage *versus* fresh.
[c] $P = 0.02$, paired *t* test of standardized difference in DNA yields, −70°C storage *versus* fresh.

Table 4 Advantages and disadvantages of using treated cards to collect buccal cell DNA

Feature	Advantages	Disadvantages
Collection	Easy to administer	Three-step method (brush, expectorate, transfer) *versus* one-step for swab/brush only method or rinse method
	Noninvasive	Assistance by study personnel required
	Limited exposure to blood-borne pathogens	
	Medically trained personnel not needed	
	Dries within 1 h	
Transport	No liquid sample handling	None
	Compact and lightweight	
Storage	No processing required	DNA yields using standard extraction procedure may decline after 9 mo[b]
	Efficient[a]	
	High quality DNA obtained after 9 mo[b]	
	Room temperature and cold storage comparable[b]	
DNA extraction	Fast and easy[c]	DNA extract unpurified[c]
	Relatively inexpensive	More rigorous methods may be required for stored samples (see Storage)
	Can process portion and store remainder	
DNA yield	2.3 μg of human DNA (median)	Highly variable (0.2–53.8 μg of human DNA)
	Ample for hundreds of PCR assays	
	May be increased 30–50-fold by whole genome amplification	
	Brush may contain additional DNA	
PCR amplification	High success rate (51 of 52; 98.1%)	Some samples unsuitable (1 of 52; 1.9%)
Cost of supplies	Modest: $4.72/subject[d]	More expensive than collecting up to several swabs/brushes

[a] For example, a 27-ft^3 −70°C freezer can hold 14,560 cards.
[b] DNA yields and suitability for PCR evaluated after storage at room temperature, −20°C, and −70°C for 9 months.
[c] The manufacturer's suggested extraction protocol is a fast and simple method producing a crude DNA extract; more extensive methods that include purification may be preferred.
[d] Includes card ($3.90), brush ($0.47), sterile cup ($0.26), and transfer pipette ($0.09).

facilities are not immediately available or cost considerations prohibit storage in liquid nitrogen, may be satisfactory.

Using Taq polymerase, >90% of the samples amplified in each of the three PCR-based assays, with the smallest fragment (268 bp) yielding the highest success rate (51 of 52, or 98.1%). A small number of the failures (two of eight) were recovered by use of AmpliTaq gold. One sample (1 of 52; 1.9%), which had the lowest DNA yield, failed to amplify in all assays. Other studies have validated the use of buccal cell DNA in PCR-based genotyping assays by demonstrating 100% concordance with results from paired blood samples (1, 6, 11, 12, 15).

The technique described here can be used whenever interviewers or other study personnel are on site, such as in surveys, cross-sectional studies, and case-control studies. The specimen could be collected at the beginning of the appointment and allowed to dry while the questionnaire is administered. In the present study, a staff member transferred the sample from the cup to the card using the pipette; in a self-administered situation, some subjects may not be able or willing to perform this step. Modifications to the present protocol to allow for self-collection would further enhance its use in epidemiological studies in which contact with subjects is via mail or phone, such as in some surveys, population-based case-control studies, and cohort studies. It has been shown that saliva and buccal cell samples can be collected by mail using vials, swabs, or cytobrushes (7, 9, 28). Thus, buccal cells collected on treated cards offer promise for molecular epidemiological studies involving PCR-based DNA assays, including those with large numbers of geographically dispersed subjects.

Acknowledgments

We thank Shirley Friend of Westat, Inc. for assistance with sample collection and the volunteers who participated in this study.

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Cancer Epidemiol Biomarkers Prev 2000;9:501-506.