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METHODOLOGICAL NOTE

Evaluation of buccal cell collection protocols for genetic susceptibility studies

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Buccal cells are increasingly used as a source of quality DNA to improve participation rates in molecular studies. Here, three buccal cell collection protocols were compared to determine factors affecting the yield of cells, total DNA per sample, and DNA yield per cell. In addition, kinetic quantitative polymerase chain reaction (PCR) (TaqMan™) was used to quantify human DNA available for PCR. The method of collection used influenced the overall DNA yield per sample. The collection buffer used influenced the number of cells but not the overall DNA yield per sample. Repeated freezing and thawing did not affect overall DNA yield per sample, DNA yield per cell, or the total number of cells collected. Mouthwashes had the highest DNA yield per sample (20.8 μg) compared with cytobrush samples (1.9 μg from three cytobrushes) and tongue depressors (0.8 μg from three tongue depressors). However, mouthwash samples may contain significant non-human DNA and other contaminants that could interfere with some molecular studies. Spectrometry grossly overestimated the total DNA recovered from mouthwash samples compared with fluorometry or quantitative PCR.

Keywords: buccal cells, susceptibility, polymorphisms, molecular and genetic epidemiology

Introduction

Traditionally, DNA has been extracted from white cells or whole blood. However, buccal cells are now increasingly used to improve participation rates in molecular studies. Generally cells are collected using tongue depressors, cytobrushes or mouthwashes, and then placed into a storage or DNA extraction buffer. Once the DNA is extracted it is primarily used for polymerase chain reaction (PCR) based assays to examine polymorphic regions of different genes. Here we evaluate different protocols (Moore *et al*. 1993, Richards *et al*. 1993, Lum and Marchand 1998) to determine which method yields the best quality and quantity of DNA. We also tested the effect of different storage buffers and freeze-thawing on cell number, DNA yield and DNA yield per cell for cells collected using brushes and tongue depressors. In addition, a novel technique called kinetic quantitative PCR was used to quantify human DNA available for PCR. This

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method is based on fluorescent TaqMan™ methodology and an ABI Prism 7700 Sequence Detection System.

Materials and methods

In table 1, a total of 24 buccal cell samples were collected from three subjects (one male aged 23 years and two females aged 35 and 37 years) using either a cytobrush or a tongue depressor. Cell collection with the cytobrush was performed as previously described by Richards *et al.* (1993). The second method employed tongue depressors (Moore *et al.* 1993). Two samples were collected from each participant per day (one on each side of the mouth) using a brush and a tongue depressor over two consecutive days. Each sample was placed in one of four buffers (10 × TE, 1 × TE, 0.9% NaCl or H_2O) and immediately frozen for approximately 4 weeks before the DNA was extracted using the QIAamp DNA Mini kit (catalogue no. 51304, QIAGEN, Valencia, California, USA).

To determine the quantity of DNA from the mouthwash samples, a published protocol by Lum and Marchand (1998) was used. Cells were collected from four subjects (two males aged 23 and 26 years and two females aged 30 and 37 years). Briefly, 1 h after teeth brushing and mouth rinsing, each subject swished 10 ml of undiluted mouthwash (FreshBurst Listerine™) throughout the mouth for 60 s. The DNA was extracted using a standard phenol chloroform extraction as described in the same protocol. A Wizard™ clean-up column was used for DNA purification according to the manufacturer's instructions (catalogue no. A7280, Promega, Madison, Wisconsin, USA).

To perform the freeze-thaw experiment, buccal cells were collected from one individual using three tongue depressors or three cytobrushes on opposite sides of the mouth over two consecutive days. One sample was repeatedly frozen and thawed while the comparison sample remained frozen to control for storage time. Three additional samples, one using brushes and one using tongue depressors, were taken in the same manner at 1 week intervals. Each time a new sample was taken, the samples taken on the previous weeks (except for the comparison sample) were removed from the freezer and completely thawed and refrozen. All cell samples were counted using a haemocytometer. After pipetting to disperse the cells, an aliquot of the cell suspension was placed into the chamber by capillary action. Cells in the centre square were counted and the number of cells per millilitre was calculated by multiplying the number of cells counted by 10^4. All DNA samples were quantified using fluorometry (TKO100, Hoefer Scientific Instruments), spectrophotometry (Gene Quant II, Pharmacia Biotech) or quantitative PCR/TaqMan™ (ABI Prism 7700).

Blood was collected in a heparin tube and DNA was extracted using a QIAamp Blood Mini Kit (catalogue no. 51106, QIAGEN) according to the kit instructions. The purified DNA was quantified using spectrophotometry and had a A_{260}/A_{280} ratio of 2.0, indicating the absence of contaminating proteins. It was also quantified using fluorometry. The ability to amplify the DNA was inferred using quantitative PCR by comparing test DNA amplification to that of a known quantity of lymphocyte DNA as previously described (Bieche *et al.* 1998). Probes for the reference housekeeping gene fatty acid binding protein (FABP), which map to chromosome 4q, were used with the following primers: forward 5′-TGGAGCCTTGAGGGAAATAAAC-3′, reverse 5′-TTTCTCGGACAGTATTCAGTTCGT-3′ and probe 5′-AATGGAAAATTCAAACGGACAGACAATGGA-3′. This gene was selected since previous testing only showed negligible inter-experimental variation in Cycle threshold (C_t) values in whole human blood DNA.

PCR was performed on exon 6 of the p53 gene using the primers 5′-TGTAAAACGACGGC-CAGT-3′ and 5′-CAGGAAACAGCTATGACC-3′. PCR was performed in a Gene Amp PCR 9700 Thermal Cycler (Perkin Elmer) in 25 µl reaction volumes. Each PCR reaction contained 3.0 mM $MgCl_2$, 0.2 mM deoxynucleotide (dNTP), 0.625 U Taq Gold polymerase (Perkin Elmer), 1 × PCR BufferII, 0.4 µM each of the forward and reverse primers, and 20–40 ng of genomic DNA. PCR cycling conditions were as follows: 95°C for 15 min, followed by 32 cycles of 95°C for 15 s, 58°C for 15 s and 72°C for 30 s, with a final hold at 72°C for 10 min.

Statistical analysis

Analysis of variance was used to determine whether collection variables significantly affected the total number of cells collected, the DNA yield per sample, and the DNA yield per cell.

Results and discussion

Table 1 compares the effect of collection method (tongue depressor or cytobrush) and storage buffer on DNA isolation. The number of cells recovered was highest in the 10 × TE buffer and lowest in 0.9% NaCl. Brushes offered both a

Table 1. DNA isolation results with various cell collection variables.

Variable tested	No. of samples	Total cells collected ($\times 10^5$)				DNA yield/sample[a] (ng)				DNA yield/cell (pg)				Group average DNA yield/cell (pg)
		Mean	Range	Median	p value[b]	Mean	Range	Median	p value[b]	Mean	Range	Median	p value[b]	
Cell collection method														
Tongue depressor	12	2.2	1.2–6.0	1.9	0.27[b]	257	90–510	230	0.003	2.0	0.7–8.5	1.6	0.05	1.2
Cytology brush	12	1.5	0.2–3.4	1.2		686	40–1290	675		12.1	0.4–55.0	3.8		4.5
Person														
1	8	2.3	0.8–4.4	1.9	0.20	369	40–900	270	0.47	1.5	0.5–3.3	1.4	0.22	1.6
2	8	1.9	0.2–2.8	1.1		570	120–375	375		9.1	0.7–30.0	4.0		3.2
3	8	1.5	0.2–6.0	1.6		478	90–1290	440		10.5	0.4–55.0	4.4		3.3
Storage buffer														
10 × TE	6	2.6	1.8–6.0	2.6	0.002	320	100–540	310	0.51	0.9	0.4–8.5	1.2	0.10	1.3
TE	6	1.6	0.8–2.8	1.6		447	40–1260	290		3.3	0.5–11	1.4		4.2
0.9% NaCl	6	1.1	0.2–2.0	1.1		547	180–1100	609		16.3	1.8–55.0	2.9		4.1
H_2O	6	1.8	0.2–4.4	1.8		575	90–1290	485		7.7	0.5–25.0	5.2		1.7
Total	24	1.9	0.2–6.0	1.8		472	40–1290	330		7.1	0.4–55.0	2.2		2.5

[a] Measured using fluorometry.
[b] ANOVA (analysis of variance).

Table 2. Comparison of three buccal cell collection protocols.

Method	Sample no.	Total yield[a] (μg)	Fluorometry concentration (ng/μl)	TaqMan™ concentration (ng/μl)	PCR result
1-Cytobrush/NaOH DNA extraction	1	3.6	36	10	+
	2	1.5	15	7	+
	3	2.3	23	17	+
	4	1.2	12	4	+
	5	1.0	10	1	−
Mean ± SD		1.9 ± 1.1	19 ± 11	8 ± 6	80%
2–Tongue depressor/QIAamp kit DNA extraction	1	0.5	9	10	+
	2	0.6	10	7	+
	3	0.7	12	16	+
	4	1.2	20	21	+
	5	1.0	16	4	+
Mean ± SD		0.8 ± 0.3	13 ± 5	12 ± 7	100%
3–Freshburst™ mouthwash/ proteinase K PCI[a]	E1	15	57	0	−
DNA extraction	M1	8	23	0	−
	B1	14	93	0	−
	C1	13	44	0	−
Mean ± SD		13 ± 3	54 ± 4	0	0%
Repeat–Freshburst™ mouthwash/proteinase K PCI[b]	E2	22	182	0	−
DNA extraction	M2	10	123	0	−
Mean ± SD		16 ± 8	153 ± 42	0	0%

[a] Measured using fluorometry.
[b] Phenol chloroform isoamyl alcohol.

higher quantity of DNA per cell and a higher quantity of DNA per sample than tongue depressors, although the average number of cells collected was similar.

We then measured the same parameters (DNA yield, DNA per cell and number of cells) for the mouthwash or 'swish and spit' method of cell collection (Lum and Marchand 1998) (data not shown). When the results for cytobrushes and tongue depressors (table 1) are compared with those using the mouthwash method, each mouthwash sample yielded almost 2.7 times more cells (5.1×10^5 versus 1.9×10^5), 27.7 times more DNA (13 μg versus 0.47 μg), and 4.9 times more DNA per cell (35 pg versus 7 pg) than samples collected with a tongue depressor or a cytobrush.

Next the ability to amplify the extracted DNA using the TaqMan™ was compared (table 2). Ten samples (five each) were randomly selected from two genetic susceptibility studies currently underway in our laboratory; the samples had been collected and extracted using published protocols (Moore *et al.* 1993, Richards *et al.* 1993, Lum and Marchand 1998). In each sample the amount of amplifiable DNA using the TaqMan™ was less than that measured by fluorometry. In addition, the DNA collected and extracted using methods 1 (cytobrush/NaOH) and 2 (tongue depressor/QIAamp) was amplifiable using quantitative PCR (TaqMan™) and regular PCR of exon 6 of the p53 gene. In contrast, the DNA collected using method 3 (mouthwash/phenol chloroform extraction) could not be quantified with quantitative PCR, even though both spectrophotometry and fluorometry measured ample amounts of DNA and the A_{260}/A_{280} ratios were high (≥1.8 for all but sample B1). In a separate experiment, buccal cells were

collected from two more individuals and DNA was extracted. In each case, ample amounts of DNA were obtained that could be quantified using spectrophotometry and fluorometry but not with quantitative PCR. Similarly, no product was detected after amplification of exon 6 of the p53 gene. The A_{260}/A_{280} ratios were 1.9 and 1.8, respectively.

Four additional mouthwash samples were collected, and the same DNA extraction procedure was repeated using entirely new solutions (Lum and Marchand 1998). From each sample, 20 µl was removed for DNA quantification and PCR. The remaining 100 µl was cleaned and filtered using a Wizard™ clean-up column. PCR and Taqman™ were only successful after clean-up; however, the average DNA yield from the samples was reduced to 33% of its original value (27 µg in 100 µl versus 9 µg).

Freezing and thawing did not affect any of the outcome variables measured.

This paper demonstrates that more DNA was collected per brush than per tongue depressor when the same DNA extraction procedure was used (686 ng versus 257 ng DNA per tongue depressor or brush; table 1). Although the number of cells collected was similar with a brush or tongue depressor, eight out of 12 brush samples contained over 500 ng of DNA. In contrast, the maximum yield for a tongue depressor was 510 ng/sample. When three brushes and three tongue depressors were used (table 2), brushes and tongue depressors still yielded about the same amount of DNA per sample as in table 1 (about 600 ng per brush and 250 ng per tongue depressor). Cytobrushes may yield more DNA because they remove healthier cells from deeper mucosal layers than tongue depressors. Only one out of 24 samples failed the PCR reaction. This sample was collected with a cytobrush and contained the least number of cells (80,000 cells/sample) and DNA (40 ng).

We also demonstrated that the method published by Lum and Marchand (1998) produced the highest quantity of DNA, but neither PCR nor quantitative PCR could be performed on these samples until an additional purification step was included in the protocol. These failures could be caused by polymerase inhibition or blocking of its active site by contaminants. Taq Gold™ was used in these experiments because it has been previously shown to enhance PCR amplification and specificity (Kebel Mann Betzing *et al.* 1998, Moretti *et al.* 1998). It has also been shown to be more sensitive to contamination, undigested proteins and salts compared with other polymerases (Al Sound *et al.* 1998). It was difficult to evaluate which polymerase was used in the previous study because the type used was not specified (Lum and Marchand 1998). Failures could also be caused by bacteria in the sample.

Unlike blood, the tongue and mouth crevices are covered in bacteria. Bacterial DNA could compete as a substrate for the PCR probes, thus inhibiting a PCR reaction, or cause non-specific banding, a problem that can sometimes be alleviated by diluting DNA in a sample. Unfortunately both fluorometry and spectrophotometry do not exclude non-human DNA when a sample is quantified. Alternatively, quantitative PCR can only measure the amount of amplifiable DNA in a sample. For method 2 in table 2 it is noteworthy that the fluorometry concentration closely approximates the TaqMan™ concentration (13 µg/µl versus 12 µg/µl). Similarly, after clean-up, the mouthwash sample DNA yield also closely approximated the TaqMan™ yield after clean-up (9 µg/sample versus 8 µg/sample), suggesting that after clean-up the majority of the DNA is amplifiable.

This is the only study in which cells were counted per sample, enabling calculation of the average DNA yield per cell. Generally the average mammalian cell contains about 6.3 pg of DNA per cell (Sambrook *et al.* 1989). However, in this study the mouthwash samples contained 35 pg per cell (versus 7 pg per cell in tongue depressor and brush samples combined; table 1), suggesting an overestimation of DNA per cell. Furthermore, the mouthwash samples yielded 2.7 times more cells (5.1×10^5 versus 1.9×10^5) yet 27.7 times more DNA (13 µg versus 0.47 µg), again corroborating that something other than human DNA is being measured.

The DNA yield per brush reported in this study was similar to that in a recent abstract presented by Garcia-Closas *et al.* (2000), in which phenol-chloroform ($n = 45$) and NaOH ($n = 45$) extraction from cytobrush samples produced a median human DNA yield of 450 and 500 ng/brush, respectively. The median human DNA yield was 26.1 µg per mouthwash sample ($n = 40$) when measured using human Alu sequence hybridisation, which measures human DNA but not the amount of amplifiable DNA as in quantitative PCR. Lum and Marchand (1998) reported an average of 49.7 µg of DNA ($n = 60$) for mouthwash samples using a spectrophotometer.

In conclusion, this evaluation has demonstrated that samples collected using the method of Lum and Marchand (1998) contain more DNA per sample than those collected with brushes or tongue depressors, but they contain significant non-human or unamplifiable DNA and contamination that inhibited PCR unless further purification steps were performed. In large studies, an extra purification step could be both costly and time consuming. Brushes and tongue depressors may be more appropriate for use with specific types of participants and for certain but not all types of molecular assays. Although these methods yield less DNA per sample, the quantity was sufficient to perform many PCR reactions. Moreover, it is always possible to increase DNA quantity with whole genome amplification if the original DNA quality is good. Lastly, spectrometry grossly overestimated the total DNA recovered from buccal cell samples. Instead, fluorometry or quantitative PCR should probably be used as a final estimate of the amplifiable DNA concentration.

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