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Comparison of methods for DNA extraction from paraffin-embedded tissues and buccal cells

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Abstract

Both paraffin-embedded tissue specimens and buccal cells are excellent resources for large-scale molecular epidemiological studies. In order to identify the optimal method for DNA extraction, we compared three methods: (1) modified phenol–chloroform protocol; (2) simple boiling method; and (3) DNA Extraction Mini Kit. For paraffin-embedded tissue specimens, amplification of the β-globin gene sequence was successful in 30 of 34 (88.2%) by the simple boiling method, 29 of 34 (85.3%) samples using DNA extracted by the phenol–chloroform method, and 18 of 34 (52.9%) by the DNA Mini Kit. For buccal cells, amplification of the β-globin gene sequence was successful in 16 of 17 (94.1%) DNA samples extracted by the phenol–chloroform method, 2 of 16 (12.5%) by the simple boiling method, and 12 of 16 (75%) by the DNA Mini Kit. Both the simple boiling method and the phenol–chloroform method are better methods for DNA isolation from paraffin-embedded tissue specimens, and the phenol–chloroform method is the best method for DNA extraction from buccal cells.

Keywords: DNA extraction; Paraffin-embedded tissue; Buccal cell; Polymerase chain reaction; Epidemiology; β-Globin gene

1. Introduction

There is a rapidly increasing need to evaluate a range of susceptibility and tumor markers in formalin-fixed, paraffin-embedded tissue specimens in molecular epidemiological studies. However, there are several reasons for the failure of polymerase chain reaction (PCR) using DNA isolated from paraffin-embedded tissues [1]: (1) the absence of a detectable amount of target DNA in the small tissue samples or biopsy specimens [2]; (2) the presence of inhibitory substances such as hemoglobin [3]; (3) the degradation of target DNA, which may occur due to long time lapses between surgical tissue removal and fixation, the type of fixative used, and the duration of the fixation; and (4) the fragmentation of nucleic acids due to formalin fixation [4]. These factors would limit the use of PCR analysis of DNA from formalin-fixed, paraffin-embedded tissues in molecular epidemiologic studies. Identifying a better method for DNA isolation from paraffin-embedded tissue specimens is of importance for molecular epidemiological studies using tissue specimens.

Besides getting DNA from tissue samples, peripheral blood samples have traditionally been used for genomic DNA extraction for molecular epidemiological studies [5–9]. However, collection of blood samples is considered invasive, which may lead to a lower response rate for epidemiological investigations and may not be feasible for large-scale population-based studies. For other types of specimens such as urine, DNA extraction may be difficult and the amount and quality of DNA may not be adequate for proposed molecular markers [5]. Obtaining buccal cells by a mouthwash rinse method provides a non-invasive method for specimen collection for genomic DNA [5,6]. In addition, for studies of smoking-related cancers, DNA isolated from oral buccal cells may also be informative on potential somatic alterations because the oral cavity may have a high exposure of tobacco smoke and buccal cells may demonstrate the early genetic alterations such as methylations of certain genes. For studies that involve large sample sizes, collecting buccal cells by a mouthwash method may provide a non-invasive, inexpensive and time-saving method for genomic DNA. However, few studies have evaluated

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the existing DNA extraction protocols to identify a better method for DNA isolation from buccal cells.

Although several DNA isolation methods for paraffin-embedded tissues and buccal cells have been proposed [5,6,9–11,16–18], few studies have been conducted to compare these existing methods in order to identify better methods for DNA isolation. The objective of this study was to compare three existing DNA isolation methods for both buccal cells collected by a mouthwash and paraffin-embedded tumor specimens on the yield, purity and suitability of DNA for the amplification of selected genes sequences.

2. Materials and methods

2.1. Tissue preparation

Sixteen human bladder and 18 human lung paraffin-embedded specimens were obtained from patients with bladder or lung cancer. Using a standard microtome with disposable blades, serial sections of 10 μm thickness obtained from the top of the block were placed on plain glass slides and compared with each other visually to confirm that the surface area of each was equivalent. Two 5 μm sections of representative areas of the specimen (one from the top and one from the end) were cut from paraffin-embedded tissue, stained with hematoxylin and eosin and then examined under a microscope to verify the tumor lesions. The lesions of interest were identified and encircled using a fine-tip indelible marker on the hematoxylin and eosin stained slides. The stained and encircled slides were then overlapped with each unstained slide to identify the lesion of interest. Microscope-guided dissection techniques were used to selectively analyze specific areas such as tumor and adjacent normal tissue specimens. Paired normal and tumor tissues were then dissected under microscope for the DNA extraction. We used the same amount of tissues for all three DNA isolation methods for tissue specimen from each patient.

2.2. Collection of buccal cell samples

A modified protocol for buccal cell collection was used [5]. Briefly, the subject rinsed his or her mouth with approximately 20 ml of Scope mouthwash to reduce bacteria or food residual interference. The subject was then asked to brush the inside of each cheek 20 times and take 20 ml Scope mouthwash again. The mouthwash was swirled back and forth, then left to right for 30 s and released into a 50 ml conical tube (Fig. 1). Buccal cells from 49 anonymous normal individuals from UCLA School of Public Health and 125 lung cancer patients were collected. For the 49 anonymous individuals, DNA was extracted immediately after collection. Buccal cell samples from the 125 lung cancer patients were stored for different durations in certain temperatures before extracting DNA. The buccal cells were kept

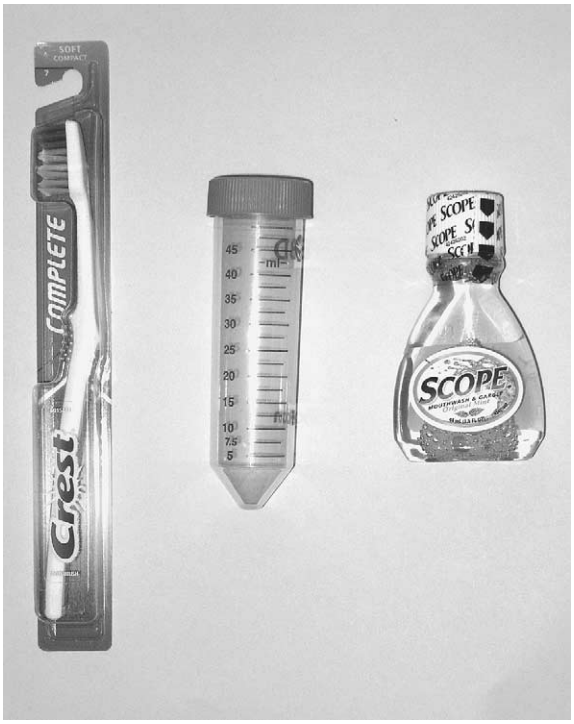

Fig. 1. Buccal cell collection kit for mouthwash DNA extraction method.

at room temperature before the samples were received in the laboratory. Most of the buccal cell samples were kept at room temperature for less than 1 week ($n = 80$), while the rest of samples were at room temperature for over a week ($n = 45$). After the buccal cells samples were received in the laboratory, they were kept at 4 °C for less than 1 week ($n = 109$) or over a week ($n = 16$). Most buccal cell samples were then stored at −80 °C before DNA extraction; 28 samples were never frozen, 43 samples were in the freezer for less than or equal to 10 weeks, 54 samples were in the freezer for more than 10 weeks before DNA isolation.

To prepare the samples for DNA extraction, the 50 ml tube with mouthwash and buccal cells was centrifuged at 7500 rpm for 20 min. The supernatant was decanted and the pellet was washed twice with 1.5 ml of TE buffer (10 mM Tris–Cl (pH 8.0), 1 mM EDTA (pH 8.0)). After every wash, the suspension was centrifuged at 14,000 rpm for 20 min. The supernatant was discarded and the pellet was used for DNA extraction by the following methods.

2.3. The phenol–chloroform method

The phenol–chloroform method was modified according to Diaz-Cano et al. [10]. Briefly, to digest the tissue sample, the pellet was resuspended in 200 μl of digestion buffer (100 mM Tris–Cl (pH 8.0), 5 mM EDTA (pH 8.0), 1% SDS) containing freshly thawed proteinase K (500 μg/ml). The sample was then incubated at 55 °C in a water bath overnight. After the incubation, DNA was isolated with an equal volume of Tris–saturated phenol–chloroform–isoamylalcohol

solution (25:24:1) and precipitated with two volumes of ice-cold absolute ethanol. The sample was placed in the freezer (−20 °C) for at least 1 h and then centrifuged at 14,000 rpm for 20 min. The supernatant was carefully removed without touching the pellet or the area where the pellet was expected to be. The pellet was washed by adding 1 ml of 70% ethanol and then centrifuged for 15 min at 14,000 rpm. The supernatant was then carefully removed, and washed again with absolute ethanol. The sample was centrifuged again for 15 min at 14,000 rpm. Finally, the DNA was resuspended in 100 μl of distilled water and stored at 4 or −20 °C. (For the detailed protocol, refer to Appendix A for buccal cells and Appendix B for the paraffin-embedded tissue.)

2.4. The simple boiling method

The simple boiling method was modified according to Merkelbach et al. [11]. The pretreated pellet was washed with 1 ml 10 mM TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA) and then incubated with 100 μl of digestion buffer (10 mM TE/1% Tween 20, proteinase K 200 μg) at 55 °C for 24 h. The proteinase K was inactivated by heating to 97 °C for 10 min. The samples were centrifuged for 5 min at 14,000 rpm and the supernatant was transferred to a new tube and stored at 4 or −20 °C. (Please refer to Appendix C for the detailed protocol.)

2.5. The DNA Mini Kit

QIAGEN Inc.'s DNA Mini Kit was used to isolate tissues and buccal cells DNA (Valencia, CA, USA). The extraction procedure was performed according to the manufacturer's instruction (QIAamp). The pellet was lysed with proteinase K at a concentration of 2 μg/μl at 55 °C overnight and loaded onto a spin column. DNA was then absorbed by short centrifugation onto the QIAamp silica membrane, washed and eluted with 100 μl water.

3. Concentration measurements

Spectrophotometric determination of the amount and purity of DNA was conducted. Readings were taken at wavelengths of 260 and 280 nm. The reading at 260 nm was used to calculate concentration (yield). The ratio of the readings at 260 and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the DNA.

4. Polymerase chain reaction

The 256 bp fragment of the β-globin gene, the 219 bp fragment of the GSTM1 gene, the 459 bp fragment of the GSTT1 gene, and the 179 bp fragment of the GSTP1 gene were amplified from isolated DNA using PCR. The total volume of the reaction mixture was 20 μl with 4 μl of extracted DNA, 0.2 mM of each dNTP, 0.5 μM each of primers, 1 U Taq polymerase, 1× Taq buffer (with 1.5 mM $MgCl_2$; supplied with the enzyme). PCR products for β-globin, GSTM1, and GSTT1 were separated on 4% NuSieve 3:1 plus agarose (BMA). The PCR product of GSTP1 was further digested with 5U Alw261 (Promega, Madison, WI, USA) for a total volume of 20 μl and the products were separated on a 3.5% agarose gel containing a concentration of 0.5 μg/ml ethidium bromide to visualize the bands. Information regarding the primers is given in Table 1 [12–15].

5. Statistical methods

We used the analysis of variance (ANOVA) to compare the means of OD ratios and DNA yields for the three DNA extraction methods and Student's *t*-test for two DNA extraction methods. To examine the differences of successful DNA amplifications of the β-globin gene sequence and the GSTs using these DNA extraction methods, we used the two-tailed Fisher's exact test.

6. Results

6.1. Paraffin-embedded tissues

Spectrophotometric determination of the yield and purity of DNA was conducted, as shown in Table 2. Based on DNA extracted from 34 tissue specimens (16 bladder specimens and 18 lung specimens), the ranges of OD ratios (OD_{260}/OD_{280}) were between 1.5 and 2.0 (mean = 1.79) for the phenol–chloroform method, between 0.9 and 1.4 (mean = 1.13) for the simple boiling method, and between 1.6 and 2.0 (mean = 1.71) for the DNA Mini Kit. Both the phenol–chloroform method and the DNA Mini Kit

Table 1
Primers used for PCR

Gene target	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)	Annealing temperature (°C)
GSTM1	5′-GAA CTC CCT GAA AAG CTA AAG C-3′	5′-GTT GGG CTC AAA TAT ACG GTG G-3′	219	63
GSTT1	5′-TTC CTT ACT GGT CCT CAC ATC TC-3′	5′-TCA CCG GAT CAT GGC CAG CA-3′	459	63
GSTP1	5′-ACC CCA GGG CTC TAT GGG AA-3′	5′-TGA GGG CAC AAG AAG CCC CT-3′	176	55
β-Globin	5′-CCA CTT CAT CCA CGT TCA CC-3′	5′-GAA GAA CCA AGG ACA GGT AC-3′	256	63

Table 2
Comparisons of the OD ratio and DNA yield with different DNA extraction methods from paraffin-embedded tissue

Method	Average (OD_{260}/OD_{280})	Yield (μg ± S.E.)	β-Globin	
			+	−
Phenol–chloroform method	1.79	6.0 ± 1.2	29	5
Simple boiling method	1.13	48.6 ± 5.7	30	4
DNA Mini Kit method	1.71	9.0 ± 1.6	18	16
P-value	0.0001*	0.0001*	0.0020**	

(+): Results obtained; (−): no results.
* ANOVA.
** Two-tailed Fisher's exact test.

achieved higher OD ratios than the simple boiling method ($P < 0.0001$) (Table 2). The mean yields and standard errors (S.E.) were 6.0 μg (1.2) for the phenol–chloroform method, 48.6 μg (5.7) for the simple boiling method, and 9.0 μg (1.6) for DNA Mini Kit ($P = 0.0001$). The simple boiling method had the lowest OD ratio, but the highest yield, which suggests that the products may represent a mixture of DNA and protein in the sample.

To evaluate the suitability of DNA extracted from paraffin-embedded tissue specimens, DNA was used to amplify a 256 bp product from the β-globin gene. In all samples, the yields of DNA were sufficient for PCR amplification, which was performed with equal volumes of DNA (4 μl). Amplification of the 256 bp fragment of the β-globin gene (Fig. 2) was successful in 29 of 34 (85.3%) using DNA extracted by the phenol–chloroform method, 30 of 34 (88.2%) by the simple boiling method, and 18 of 34 (52.9%) by the DNA Mini Kit ($P = 0.0020$). Both the simple boiling and phenol–chloroform methods had a significant higher proportion of successful amplification of the β-globin gene sequence than DNA Mini Kit when separate comparisons were made by Student's *t*-test ($P < 0.0001$), and there was no statistical difference between simple boiling and phenol–chloroform methods in terms of the successful amplification rates.

6.2. Buccal cell samples

DNA samples were extracted from 49 buccal cell specimens by the three methods. Amplifying the 256 bp fragment

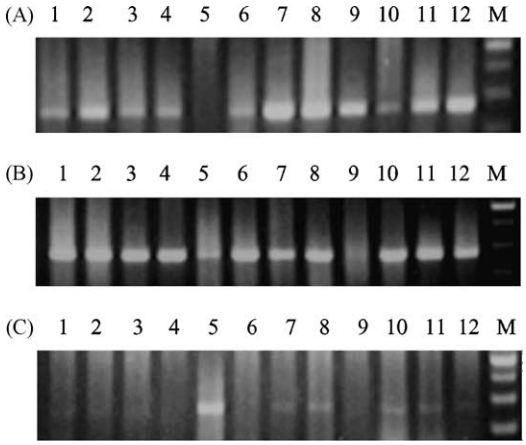

Fig. 2. A 256 bp fragment of the β-globin gene sequence was amplified from DNA extracted from paraffin-embedded tissue, by three different extraction methods: (A) phenol–chloroform method; (B) simple boiling method and (C) DNA Mini Kit method.

of the β-globin gene was successful in 16 of 17 (94.1%) samples extracted by the phenol–chloroform method, 2 of 16 (12.5%) by the simple boiling method, and 12 of 16 (75%) by the DNA Mini Kit method (Table 3) ($P < 0.0001$). The proportion of successful amplification of the β-globin gene sequence by the simple boiling method was significantly lower than other two methods when it was compared with the proportions of other two methods separately ($P < 0.0001$ for both comparisons). There was no significant difference of the proportions of successful amplification between the phenol–chloroform and the DNA Mini Kit method ($P = 0.175$).

To evaluate the suitability of DNA extracted from buccal cells, different sizes of gene fragments were amplified. The 459 bp fragment of the GSTT1 gene, 219 bp fragment of the GSTM1 gene, and 176 bp fragment of the GSTP1 gene were successful in 16 of 17 (94.1%) samples extracted by phenol–chloroform method, 12 of 16 (75%) by DNA Mini Kit, and none of the 16 samples by the simple boiling method ($P < 0.0001$) (Table 3). Similar to the β-globin results, simple boiling method had yielded the lowest proportion of successful amplification when compared with other two methods individually ($P < 0.0001$), while there was no statistical difference between the phenol–chloroform and the DNA Mini Kit method ($P = 0.175$).

Table 3
Amplification of β-globin, GSTT1, GSTM1 and GSTP1 polymorphism from buccal cells

	β-Globin		GSTT1		GSTM1		GSTP1	
	+	−	+	−	+	−	+	−
Phenol–chloroform method	16	1	16	1	16	1	16	1
Simple boiling method	2	14	0	16	0	16	0	16
DNA Mini Kit method	12	4	12	4	12	4	12	4
P-value*	<0.0001		<0.0001		<0.0001		<0.0001	

(+): Results obtained; (−): no results.
* Two-tailed Fisher's exact test.

Using DNA extracted from buccal cells by the phenol–chloroform method, 125 lung cancer subjects were genotyped by PCR-based assays for polymorphisms of the GSTT1, GSTM1 and GSTP1 genes. The proportions of the successful amplification were 94.4% for β-globin, 94.4% for GSTM1 and GSTT1, and 100% for GSTP1. Among the 125 lung cancer patients' DNA samples, PCR amplification of GSTP1 was repeated for 21 samples with an adjusted quantity of DNA and PCR conditions because bands did not appear. Similarly, the PCR amplification for GSTM1/T1 and β-globin was repeated for 17 samples for which we did not obtain results from the first PCR reaction. Bands appeared for most of the samples that were repeated (21/21 for GSTP1, 10/17 for GSTM1/T1 and 10/17 for β-globin). The durations of storage at room or refrigerator or freezer temperatures had little impact on the DNA amplification in buccal cell samples from lung cancer patients.

7. Discussion

For paraffin-embedded tissues, DNA extracted by the simple boiling methods and the phenol–chloroform protocol yielded higher proportions of successful gene amplifications (88.2 and 85.3%, respectively) than did the DNA Mini Kit (52.9%). The DNA Mini Kit yielded a good OD ratio, but DNA yields were lower than that from the simple boiling method, the fraction of DNA suitable for amplification of the β-globin gene sequence was also relatively low. Chan et al. [20] reported a study on microwave and DNA Mini Kit methods to isolate DNA (which is equivalent to the simple boiling method) from paraffin wax-embedded cervical squamous cell carcinoma. The results showed that the highest positive rate of β-globin PCR is from the phenol–chloroform. On the other hand, DNA Mini Kit was the most efficient for HPV DNA PCR. Our study suggested that the simple boiling and phenol–chloroform methods had significantly high proportion of successful amplification of the β-globin gene sequence. For research purposes, it is of importance that PCR can amplify specific gene fragments from target DNA samples. Sepp et al. [2] found that DNA extracted by the simple boiling method allowed amplifications only up to 400 bp fragments. We were able to amplify the β-globin gene (265 bp) sequence by the simple boiling method in most tissue specimens. Whether DNA extracted by the DNA Mini Kit is suitable for amplification of genes of other sizes needs to be further studied. Based on our observation, both the simple boiling and the phenol–chloroform methods are better methods for PCR amplification less than 256 bp fragments for paraffin-embedded tissue specimens than DNA Mini Kit.

Blood samples have been the specimens of choice for genomic DNA in molecular epidemiological studies. Various methods are currently available to extract DNA from blood lymphocytes with phenol–chloroform [7]. However, collecting blood samples is both invasive and expensive. In comparison with blood collection, buccal cell sample collection is relatively simple, feasible, non-invasive, and more acceptable by study participants. For epidemiological studies, buccal cell collection may be an efficient method of obtaining DNA for a large study population. Because of the non-invasive nature, the response rate of donating buccal cells may be higher than that of donating blood sample. However, these advantages may also compensate with a relatively high PCR failure rates of the DNA extracted from buccal cells: 5.9% for β-globin by the phenol–chloroform method, 25% by the DNA Mini Kit method, and 87.5% by the simple boiling method. For amplifying other genes such as GSTP1, GSTT1, and GSTM1, PCR failure rates were the same for each of the three methods. The PCR failure rate of the phenol–chloroform method is much lower than these of the other two methods. In a previous study on DNA isolation from buccal cells by the phenol–chloroform method, Lum and Le Marchand [5] studied 60 subjects for six polymorphisms. The PCR failure rates were 2% for CYP1A1 MSP1, 8% for CYP1A1 ile-val, 5% for CYP2E2 RSAI, 12% for NQO1, and 2% for GSTM1/GSTT1; these results are consistent with ours. Garcia-Closa [21] compared the total and human DNA yields from phenol–chloroform and QIAamp kit. For the phenol–chloroform method, total and human DNA median yields were 57.3 and 27.5 μg per mouthwash, respectively. The proportion of median human DNA yield was 48% (27.5/57.3). For the QIAamp Kit method, total and human DNA median yields were 35.2 and 10.6 μg per mouthwash, respectively. The proportion of median human DNA yield was 30.1% (10.6/35.2). Therefore, the proportion of median human DNA yields from the phenol–chloroform method was higher than from the DNA Mini Kit method. Successful PCR amplification was an important DNA suitability criterion for the selection of a DNA extraction method. From previous published studies and our results, the phenol–chloroform method is the best available DNA extraction method for buccal cells.

Several factors regarding the quality of DNA extracted from buccal cells may be related to the failure of subsequent PCR, including lack of buccal cells due to insufficient rinsing of the mouth, the co-purification of inhibitory substances, age, gender, race, long storage time and the degeneration of target DNA. For the boiling method, further purification of the DNA may be required if restriction enzyme digestion or labeling is planned [19,20]. In our study, amplification of the 256 bp fragment of β-globin gene sequence was only successful in 2 out of 16 samples extracted by the simple boiling method. For the two DNA samples that could be amplified, we further assayed for the GSTT1, GSTM1 and GSTP1 gene. PCR products of GSTP1 were digested with 5U Alw261 (Promega, Madison, WI, USA) but bands did not appear, suggesting that the DNA structure might have been affected during the extraction process with the boiling method. Our results indicate that the boiling method is not a good method for DNA isolation from buccal cells.

Long-term storage may lead to the degradation of human DNA. Our study showed that whether we extracted DNA

from the buccal cells immediately after collection or stored the buccal cells at −80 °C freezer for 10 weeks, no obvious difference was observed for PCR amplification. Feigelson et al. [23] reported that buccal cell samples collected with mouthwash held for 10 and 30 days at room temperature had significantly less human DNA than those processed after 1 day ($P = 0.01$). However, 1 week of storage at room temperature did not affect DNA yields of extraction or amplification of PCR in their study. Lum and Le Marchand [5] and Le Marchand et al. [22] came to the same conclusion. Garcia-Closas et al. [21] noted that storage of unprocessed samples at −80 °C for up to 1 year would not significantly reduce the human DNA yields. Harty et al. [6] reported that PCR amplification was successful in all samples regardless of storage times, though long storage times may reduce the DNA yield. It may be another advantage for large-scale studies that the collected buccal cells can be stored in a room temperature or in a freezer for a short period of time before DNA extraction.

Currently, GSTM1, GSTT1 and GSTP1 are commonly used molecular markers in epidemiological studies. In order to evaluate the suitability of DNA size for PCR amplification from the different extraction methods, we used markers of different sizes: GSTP1 (176 bp), β-globin (256 bp), GSTM1 (219 bp), and GSTT1 (459 bp). If the length of DNA extracted was sufficient, we would be able to amplify these markers. GSTP1 has a polymorphism that can be examined by the RFLP method. Because the RFLP method requires DNA samples with relatively higher quality, the RFLP assay of GSTP1 may help us to evaluate further the quality of the DNA extracted by the different methods. For 125 lung cancer patients' buccal cell samples, we compared the PCR results for different storage conditions. All of the samples (100%) were successfully amplified for the GSTP1 polymorphism, and the majority of the samples (94.4%) were successfully amplified for β-globin and the GSTM1/T1 genes, which is very close to our results on anonymous normal individuals (94.1%). The difference in successful amplification may be related to the size of the gene fragment amplified. The GSTP1 gene is the smallest gene fragment that we amplified (176 bp), while GSTM1 (219 bp), β-globin (256 bp), and GSTT1 (459 bp) are longer. The DNA extracted may not be long enough; thus, the amplification of smaller gene fragments may be successful, but the amplification of longer gene fragments may be slightly limited.

In conclusion, both the simple boiling and phenol–chloroform methods are considered better methods for DNA extraction from paraffin-embedded tissue specimens and the phenol–chloroform method is the best available method for DNA extraction from buccal cell specimens.

Acknowledgements

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Appendix A. Isolation of DNA from buccal cells: modified phenol–chloroform method

1. Centrifuge the 50 ml conical tube at 7500 rpm for 40 min at 4 °C.
2. Remove the supernatant carefully.
3. Add 1.5 ml TE buffer, mix.
4. Centrifuge the suspension at 14,000 rpm for 20 min. Decant the supernatant.
5. Resuspend the pellet in 200 μl of digestion buffer containing freshly thawed proteinase K.
6. Close the tubes tightly and put paraffin around the caps of the tubes. Mix the samples.
7. Incubate at 55 °C overnight.
8. Spin the tube for 5 s and add 200 μl of phenol–chloroform–isoamylalcohol. Vortex vigorously for 15 s, and spin for 5 min at high speed.
9. Remove the upper phase and put it into a new tube.
10. Repeat steps 8 and 9.
11. Add 200 μl of chloroform–isoamylalcohol. Vortex for 15 s and spin for 5 min at high speed. Remove the upper phase and place into a new tube.
12. Precipitate with ethanol by adding 5 μl of 3 M sodium acetate and 400 μl of ice-cold, 100% ethanol. Invert several times.
13. Place the sample in a freezer at the lowest temperature (−20 °C) for about 1 h and then spin the sample at high speed in a microcentrifuge for 20 min.
14. Carefully remove the supernatant without touching the pellet or the area where the pellet is expected to be.
15. Add 1 ml of 70% ethanol, invert several times, spin for 20 min at high speed and carefully remove the supernatant.
16. Add 1 ml of 100% ethanol, invert several times, spin for 15 min and carefully remove the supernatant as completely as possible.
17. Resuspend the pellet in 100 μl of storage buffer and store at 4 °C.

Appendix B. Isolation of DNA from paraffin-embedded tissue: modified phenol–chloroform method

1. Cut several section and place into microcentrifuge tube using clean forceps.
2. Add 1 ml of xylene, invert several times and incubate at RT.
3. Centrifuge for 5 min at high speed in a microcentrifuge to pellet the tissue.
4. Repeat steps 2 and 3.

5. Wash the pellet by adding 1 ml 95% ethanol, invert the tube several times and centrifuge at high speed for 3 min. Remove the supernatant.
6. Repeat steps 5.
7. Wash the pellet again with 1 ml of 70% ethanol, invert several times, and pellet tissue by centrifugation at high speed for 3 min.
8. Remove the supernatant as completely as possible.
9. To digest the tissue, resuspend the pellet in 150 μl of digestion buffer containing freshly thawed proteinase K.
10. Incubate at 55 °C overnight.
11. To extract the DNA, add 150 μl of buffered phenol and 150 μl of chloroform–isoamylalcohol, vortex vigorously for 15 s, and spin for 5 min at high speed.
12. Remove the upper phase and put into new tube.
13. Repeat steps 11 and 12.
14. Add 150 μl of chloroform–isoamylalcohol, vortex for 15 s and spin for 5 min at high speed. Remove the upper phase and place into a new tube.
15. Measure the final volume, precipitate with ethanol by adding 1/10 volumes of 3 M sodium acetate and two volumes of ice-cold, 100% ethanol. Invert several times and vortex briefly.
16. Place the sample in a freezer at the lowest temperature available (−20 °C) for 1 h and then spin the sample at high speed in a microcentrifuge for 20 min.
17. Carefully remove the supernatant without touching the pellet.
18. Wash the pellet by adding 1 ml of 70% ethanol, invert several times. Spin for 15 min at high speed and carefully remove the supernatant.
19. Wash the pellet by adding 1 ml of 100% ethanol, invert several times. Spin for 15 min at high speed and carefully remove the supernatant.
20. Resuspend the pellet in 100 μl of storage buffer and store at 4 °C.

Appendix C. Isolation of DNA from paraffin-embedded tissue: boiling method

1. Cut several sections and place into microcentrifuge tubes using clean forceps.
2. Add 1 ml of xylene, invert several times and incubate at room temperature.
3. Centrifuge for 5 min at high speed in a microcentrifuge to pellet the tissue.
4. Repeat steps 2 and 3.
5. Wash the pellet by adding 1 ml 100% ethanol, invert the tube several times and centrifuge at high speed for 5 min. Remove the supernatant.
6. Repeat step 5.
7. Add 1 ml of 10 mM TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA). Invert the tube several times. Centrifuge at high speed for 5 min. Remove the supernatant.
8. Add 100 μl of 10 mM TE/1% Tween 20 containing 200 μg of proteinase K.
9. Incubate at 55 °C overnight.
10. Heat at 97 °C for 10 min to inactivate proteinase K.
11. Centrifuge at high speed for 5 min.
12. Remove the supernatant into a new tube and store at −20 or 4 °C.

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