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ORIGINAL ARTICLE

Evaluation of the long-term storage stability of saliva as a source of human DNA

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Abstract
Objectives The objectives of this paper are to determine the storage stability of saliva at 37 °C over an 18-month period, and its influence on the DNA yield, purity, PCR protocols and genotyping efficacy.
Materials and methods Of the 60 participants, blood samples were obtained from 10 and saliva from 50. Samples were subjected to different storage conditions: DNA extracted immediately; DNA extracted following storage at 37 °C for 1, 6, 12 and 18 months. Subsequently, DNA yield, $OD_{260/280}$ and $OD_{260/230}$ ratios were measured. The isolated DNA was used to amplify exons 0–7 of the *RUNX2* gene and subsequently sequenced. Furthermore, 25 SNPs were genotyped.
Results The mean DNA yield, $OD_{260/280}$ and $OD_{260/230}$ ratios obtained from blood were 67.4 ng/μl, 1.8±0.05 and 1.8±0.4 respectively. DNA yield obtained from saliva was significantly higher than blood ($p<0.0001$), ranging from 97.4 to 125.8 ng/μl while the $OD_{260/280}$ ratio ranged from 1.8±0.13 to 1.9±0.1. The success rates for the 25 SNPs ranged from 98 to 100 % for blood and 96–99 % for saliva samples with the genotype frequencies in Hardy–Weinberg equilibrium (>0.01).
Conclusions Saliva can be stored at 37 °C for 18 months without compromising its quality and ability to endure genetic analyses.
Clinical relevance Saliva is a viable source of human DNA to facilitate the feasibility of large-scale genetic studies.

Keywords Saliva · Blood · DNA · *RUNX2*

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Introduction

Assessment of an individual's risk to acquire a particular disease or condition is an integral part of personalized medicine. If this concept is to become a reality, the genetic risk factors that are responsible for the formation of the disease or condition need to be identified. This would then necessitate large-scale genetic epidemiological studies, with efficient and convenient methods for obtaining sufficient qualities of genomic DNA from the study participants. Although DNA is easily obtained from blood, this invasive procedure is painful, disliked by subjects, impractical for children, necessitates refrigeration and rapid processing (less than a week), moreover; it requires training in phlebotomy [1]. Therefore, to facilitate wider population samplings in large epidemiological studies and to ensure a high participation rate, non-invasive methods of DNA collection are preferable, especially for children.

Subsequently, several investigators have evaluated alternative non-invasive sources such as saliva [2–4] and buccal cells, using different procedures namely, mouthwash [5], cheek swabs [3] and cytobrush [5] to obtain DNA from subjects for polymerase chain reaction (PCR) protocols and genotyping [6, 7]. The average yield of genomic DNA obtained from 2 ml of saliva is 35 μg [1], which is much higher than the 8 μg obtained from a cheek swab [3], 0.6 to 21.7 μg from a mouthwash, and 0.4 to 9 μg from a cytobrush

[5]. Although the DNA yield from saliva (35 μg/2 ml) is less than that obtained from the same volume of blood (20 μg to 80 μg), it is still sufficient for genotyping approximately 1,750 genetic markers [1]; which is adequate for most genetic epidemiological studies evaluating specific candidate genes for disease susceptibility. Therefore, saliva is considered to be a reliable source of DNA for a wide variety of applications in population and association genetic studies [1, 3].

For complex diseases, large-scale genetic epidemiological studies involving thousands of subjects with the condition under investigation and the appropriate control groups would be necessary for the identification of genetic determinants, particularly their interaction with the environment [8]. Consequently, subject recruitment, sample collection and laboratory processing would invariably take a longer time than usually expected, especially for diseases with a low prevalence in the general population. Hence, long-term storage of samples at ambient temperatures before DNA extraction is a critical requirement for most field studies, if storage costs are to be minimized. Furthermore, epidemiologists may wish to collect a DNA sample that is both adequate for immediate use and for archiving, prior to use in the future as new assays are developed [9].

Recently, one study evaluated the stability of saliva collected using the Oragene® saliva collection kit and reported that storage of saliva over a 6-month period at 37 °C did not impact adversely on the DNA quality and genotyping [1]. Furthermore, no studies have evaluated the potential long-term storage stability of saliva at 37 °C and its effect on the quantity and quality of the resulting DNA beyond the 6 months period, which would be essential if the DNA samples are to be stored for large-scale epidemiological studies with off-site collection. Therefore, the objectives of this study were to (1) determine the storage stability of saliva at 37 °C over an 18-month period, and (2) its influence on the DNA yield, purity, PCR protocols, genotyping and sequencing efficacy of the genomic DNA.

Materials and methods

Ethical approval was attained from the Institutional Review Board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster. The parents of the 60 volunteers, aged between 5 and 10 years who participated in this study, signed the informed consent for their child's participation. Ten subjects had their dental treatment performed under general anaesthesia during which a consultant anaesthetist obtained the blood samples (2 ml per subject). The samples were then transported to the laboratory and the DNA was subsequently extracted from all the blood samples [storage condition (SC) 1] using a Qiagen® extraction kit as per the manufacturers protocol.

The remaining 50 subjects gave their saliva samples (2 ml each) using an Oragene® DNA self-collection kit [DNA Genotek Inc., Canada] over a single time period. The kit comprises of a container, into which the participants sputter up to 2 ml (level indicated by a black line on the container) of their saliva, and a lid that contains a solution [Oragene® DNA solution; the components of which are not stated by the manufacturer] which is released on screwing the lid onto the container. Upon mixing the saliva with the released Oragene® DNA solution, the DNA is immediately stabilized and this prevents bacterial growth and degradation of human DNA. The collected samples were subsequently tested for various storage conditions. These conditions involved the extraction of DNA immediately after saliva collection (SC1), or following storage at 37 °C for 1 month (SC2), 6 months (SC3), 12 months (SC4) and 18 months (SC5). DNA extraction was performed according to the manufacturer's protocol for manual purification using a purifier and alcohol precipitation.

The total amount of extracted DNA, $OD_{260/280}$ and $OD_{260/230}$ ratios were measured using a NanoDropND-1000 spectrophotometer [NanoDrop Technologies, USA] as per the manufacturer's instructions. One-way ANOVA and Tukey–Kramer test were employed to determine the influence of the different storage conditions on the DNA yield and purity, with a $p<0.05$ considered statistically significant.

Five samples from each group were randomly selected and the isolated DNA was amplified by PCR to validate its utility for genetic epidemiological studies. Eight pairs of PCR primers for exons 0 to 7 of the *RUNX2* gene (haplo-insufficiency leads to cleidocranial dysplasia) were used to amplify the DNA, which was subsequently sequenced as previously outlined [10]. Furthermore, to investigate the reliability of genotyping different genetic markers in all the samples ($n=60$), stored at different conditions, 25 single-nucleotide polymorphisms (SNPs) on eight different genes were genotyped, see Table 1. Genotyping was performed at the Genome Research Centre, University of Hong Kong using the Sequenom MassARRAY technology platform with the iPLEX GOLD chemistry (Sequenom, San Diego, California, USA). Hardy–Weinberg calculations [11] were performed to ensure that the genetic marker was within the allelic population equilibrium i.e. >0.01.

Results

The mean DNA yield and purity values for the different storage conditions are listed in Table 2. The DNA yield obtained from the saliva samples were significantly greater than that obtained from blood [$p<0.0001$, ANOVA, Tukey–Kramer test]. For the various saliva storage conditions, significant differences were only evident between condition 1 (immediate) and the remaining storage conditions. The

Table 1 Details of the 25 SNPs on 8 different genes genotyped using 10 blood DNA samples and 50 saliva DNA samples; the genotyping success rates and Hardy–Weinberg equilibrium (*P*) values for each assay

Gene	SNP identification no.[a]	Success rate (%) of genotype analyses [Hardy–Weinberg equilibrium (*P*)]					
		Blood (*n*=10)	Saliva (*n*=50)				
			SC1	SC2	SC3	SC4	SC5
Runt-related transcription factor 2 (RUNX2)	rs12214749	99 [0.28]	98 [0.96]	98 [0.55]	97 [0.78]	97 [0.35]	98 [0.48]
	rs12664261	100 [0.09]	98 [0.52]	98 [0.08]	98 [0.09]	97 [0.49]	97 [0.52]
	rs3749863	98 [0.75]	96 [0.29]	96 [0.64]	97 [0.66]	97 [0.75]	96 [0.85]
	rs16873366	100 [0.79]	99 [0.12]	99 [0.85]	98 [0.43]	99 [0.49]	99 [0,56]
	rs12664292	99 [0.42]	97 [0.65]	97 [0.53]	96 [0.67]	96 [0.83]	96 [0.78]
Adenomatous polyposis coli (APC)	rs467033	97 [0.49]	97[0.32]	96 [0.21]	98 [0.44]	98 [0.39]	97 [0.53]
	rs2439591	96 [0.52]	96[0.30]	95 [0.21]	96 [0.19]	97 [0.25]	98 [0.33]
	rs2464805	100 [0.66]	98 [0.62]	98 [0.67]	97 [0.51]	97 [0.55]	97 [0.59]
	rs41115	98 [0.56]	97 [0.33]	97 [0.31]	98 [0.39]	98 [0.40]	97 [0.29]
	rs2229995	99 [0.67]	98 [0.71]	97 [0.57]	98 [0.61]	98 [0.59]	98 [0.72]
Sonic hedgehog (SHH)	rs9333613	98 [0.45]	99 [0.29]	98 [0.19]	99 [0.30]	99 [0.37]	98 [0.29]
Wingless-type MMTV integration site family, member 1 (WNT1)	rs833843	98 [0.53]	97 [0.59]	96 [0.49]	98 [0.55]	97 [0.69]	98 [0.39]
	rs7311091	98 [0.76]	97 [0.67]	97 [0.69]	98 [0.59]	97 [0.51]	97 [0.63]
	rs10783298	100 [0.33]	98 [0.49]	98 [0.42]	99 [0.39]	99 [0.51]	97[0.35]
Paired box 6 (PAX6)	rs3026354	99 [0.29]	97 [0.31]	98 [0.34]	97 [0.49]	97 [0.44]	97 [0.46]
	rs3026398	100 [0.35]	99 [0.33]	99 [0.43]	98 [0.29]	99 [0.30]	99 [0.31]
	rs3026371	98 [0.25]	98 [0.42]	98 [0.19]	97 [0.12]	97 [0.17]	97 [0.17]
Ectodysplasin A (EDAR)	rs10865025	97 [0.31]	98 [0.52]	97 [0.55]	98 [0.61]	98 [0.35]	97 [0.49]
	rs3749097	99 [0.41]	99 [0.54]	97 [0.64]	98 [0.71]	98 [0.79]	98 [0.69]
	rs17037099	100 [0.27]	98 [0.33]	98 [0.31]	97 [0.49]	97 [0.29]	97 [0.27]
	rs899259	98 [0.37]	99 [0.39]	98 [0.44]	99 [0.41]	97 [0.36]	98 [0.50]
	rs12992554	97 [0.49]	98 [0.30]	97 [0.27]	98 [0.21]	98 [0.19]	97 [0.20]
Ectodysplasin A (EDA)	rs3764746	96 [0.51]	97 [0.59]	97 [0.60]	97 [0.47]	97 [0.45]	97 [0.57]
	rs3795170	99 [0.23]	98 [0.25]	96 [0.39]	96 [0.33]	97 [0.27]	96 [0.30]
Msh homeobox 1 (MSX1)	rs3775261	97 [0.27]	99 [0.30]	99 [0.19]	98 [0.21]	98 [0.29]	98 [0.40]

SC storage condition, *SC1* DNA extracted immediately, *SC2* DNA extracted after sample was stored at 37 °C for 1 month, *SC3* DNA extracted after sample was stored at 37 °C for 6 months, *SC4* DNA extracted after sample was stored at 37 °C for 12 months, *SC5* DNA extracted after sample was stored at 37 °C for 18 months

[a] NCBI database

average DNA yield from saliva (various storage conditions) was 116.8 ng/μl, which was approximately twice the average yield of the blood samples. The mean DNA yield, $OD_{260/280}$ and $OD_{260/230}$ ratios obtained from blood samples were 67.4 ng/μl, 1.8±0.05 and 1.8±0.4, respectively. The total amount of DNA obtained from saliva was consistent across different storage conditions ranging from 97.4 ng/μl (SC1) to 125.8 ng/μl (SC5) while the $OD_{260/280}$ ratio ranged from 1.8±0.13 (SC1) to 1.9±0.1 (SC5). There were no significant differences for the $OD_{260/280}$ and $OD_{260/230}$ ratios within and between the two sample types.

The amplification of exon 0 to 7 of the *RUNX2* gene to be sequenced resulted in a PCR fragment of 340 bp for all of the blood (*n*=10) and saliva samples (*n*=50). Electropherograms (for exons 0–7) from a representative sample for both blood and saliva, and the different storage conditions are illustrated in Figs. 1 and 2. The resulting sequencing traces were between a minimum 160 bases (exon 6) to a maximum 580 bases (exon 7) long. None of the samples provided a clear sequence at the 5′ end for the first 20 or 40 bases. No significant variations were evident in the sequencing protocols between the different storage conditions for the saliva and blood samples.

The quality assessment of both blood and saliva samples stored at various conditions was performed using genotype analyses of 25 SNPs in eight genes. The success rates for these 25 specific SNPs assays ranged from 98 to 100 % for the blood samples, and between 96 and 99 % for the saliva samples stored at various conditions. Furthermore, the genotype frequencies for all the samples were in the Hardy–

Table 2 Influence of storage conditions on yield and purity of DNA from 2 ml of saliva as assessed using NanoDropND-1000 spectrophotometer

Source (n)/storage condition	DNA yield (ng/μl) Mean (range)	DNA purity ($OD_{260/280}$) Mean±SD	DNA purity ($OD_{260/230}$) Mean±SD
Blood (n=10)			
Condition 1	67.4[a] (35.9–111.6)	1.8±0.05	1.8±0.4
Saliva (n=50)			
Condition 1	97.4[b] (39.5–154.2)	1.8±0.13	1.6±0.2
Condition 2	120.5[c] (55.4–197.8)	1.8±0.07	1.7±0.2
Condition 3	121.9[c] (48.3–198.3)	1.8±0.08	1.6±0.3
Condition 4	118.8[c] (51.2–205.8)	1.9±0.06	1.5±0.2
Condition 5	125.8[c] (57.8–215.7)	1.9±0.10	1.6±0.2

$OD_{260/280}$ ratio of optical density at 260 nm to optical density at 280 nm, a measure of protein and organic contamination, $OD_{260/230}$ ratio of optical density at 260 nm to optical density at 230 nm, a measure of salt and alcohol contamination

Different superscript letters indicate statistically significant differences between the groups (p<0.0001, ANOVA, Tukey–Kramer test)

Weinberg equilibrium (see Table 1). Of the samples that were not completely genotyped for all 25 SNPs, none of them missed more than one or two genotypes.

Discussion

The long-term stability of saliva as a potential source of human DNA was explored in this study. The impetus to investigate this issue started as a consequence of another ongoing project investigating the genetic markers responsible for the formation of supernumerary (extra) teeth with a reported prevalence of 3 % in the general population. From the practical consideration, the process of project initiation, ethical approval, sample collection, planning the workflow for laboratory analyses, etc. in large epidemiological studies, especially those in the field of dentistry, would undoubtedly take longer time than expected. Ideally, DNA from a blood sample should be either extracted immediately or stored at −20 °C for future DNA extractions. Recently, it has been reported that the quality of the DNA extracted from blood is not adversely affected by storage at 4 °C for up to 24 h [12]. Nevertheless, although both blood and extracted genetic material can be successfully stored for many years at −80 or −120 °C [13], it may not be the most cost-effective in the context of a Bio-banking project as it requires expensive sample handling systems, which can operate at low temperature. Therefore, a reliable and convenient method of sample collection that does not unduly burden the participants, has a cost-effective means of preservation for longer periods of time prior to extraction of its DNA content, or archiving subsequent to DNA extraction for further analyses are critical to the success of genetic epidemiological studies. Consequently, we evaluated the effects of different saliva storage conditions on the DNA yield, purity, PCR protocols, genotyping and sequencing efficacy.

There was no significant decrease in the purity and fragment length of DNA extracted from blood and saliva. Similarly, within the different storage conditions for the saliva samples, no differences were evident. For genotyping and sequencing, an $OD_{260/280}$ ratio of 1.7 to 2 is essential to indicate a limited protein and organic contamination, while an $OD_{260/230}$ ratio of higher than 1.5 is preferred to indicate limited salt and alcohol contamination. The $OD_{260/280}$ and $OD_{260/230}$ ratios obtained from the samples with the different storage conditions were well within these requirements. Furthermore, the storage conditions did not influence or affect the ability of all the samples to be amplified and subsequently sequenced. Based on these findings, for saliva sample stored at 37 °C, an 18-month time frame between sample collection and processing is acceptable; this could be a major factor which would allow for the shipping and storage of DNA samples for large-scale epidemiological studies with off-site collection which highlights the potential role of clinicians' in recruiting samples.

In the present study, the DNA yield obtained from the saliva was similar to that reported by Rogers and co-workers [4] who employed a similar DNA collection kit. The average DNA yield from the saliva samples was approximately twice the average yield obtained from the blood samples. However, we are aware of the fact that this is not a true comparison of the total human DNA, as saliva contains higher levels of non-human DNA from bacterial cells and viruses [14, 15]; which is its potential limitation. Nevertheless, specific quantification assays (TaqMan assays) are currently available to ascertain reliable estimates of the amount of human DNA in saliva samples. Due to cost implications, the above-mentioned assays were not employed which may be considered as a limitation of the present study. Nevertheless, two recent studies [1, 3] that employed the TaqMan quantification assays have reported that the average DNA obtained from 2 ml of saliva was approximately around 35 and 22.8 μg, respectively. Therefore, it would be logical to state that a reliable estimate of human DNA can be obtained from saliva.

Recently, Rylander-Rudqvist and co-workers [16] reported that 68 % of the total DNA obtained from saliva collected using the Oragene® DNA collection kit was of human origin. Furthermore, the manufacturers of the Oragene® DNA collection kit state that the median bacterial content of samples collected using their kit is 6.8 % and that the preserving agents in the storage solution inhibit the growth of microorganisms [17]. However, this may vary according to the cell constituents such as viruses, fungi, bacteria, food residues, etc., present in the saliva [18]. Therefore, in an attempt to minimize the effect of contaminating DNA, the participants were requested to refrain from eating and drinking half an hour prior to sample

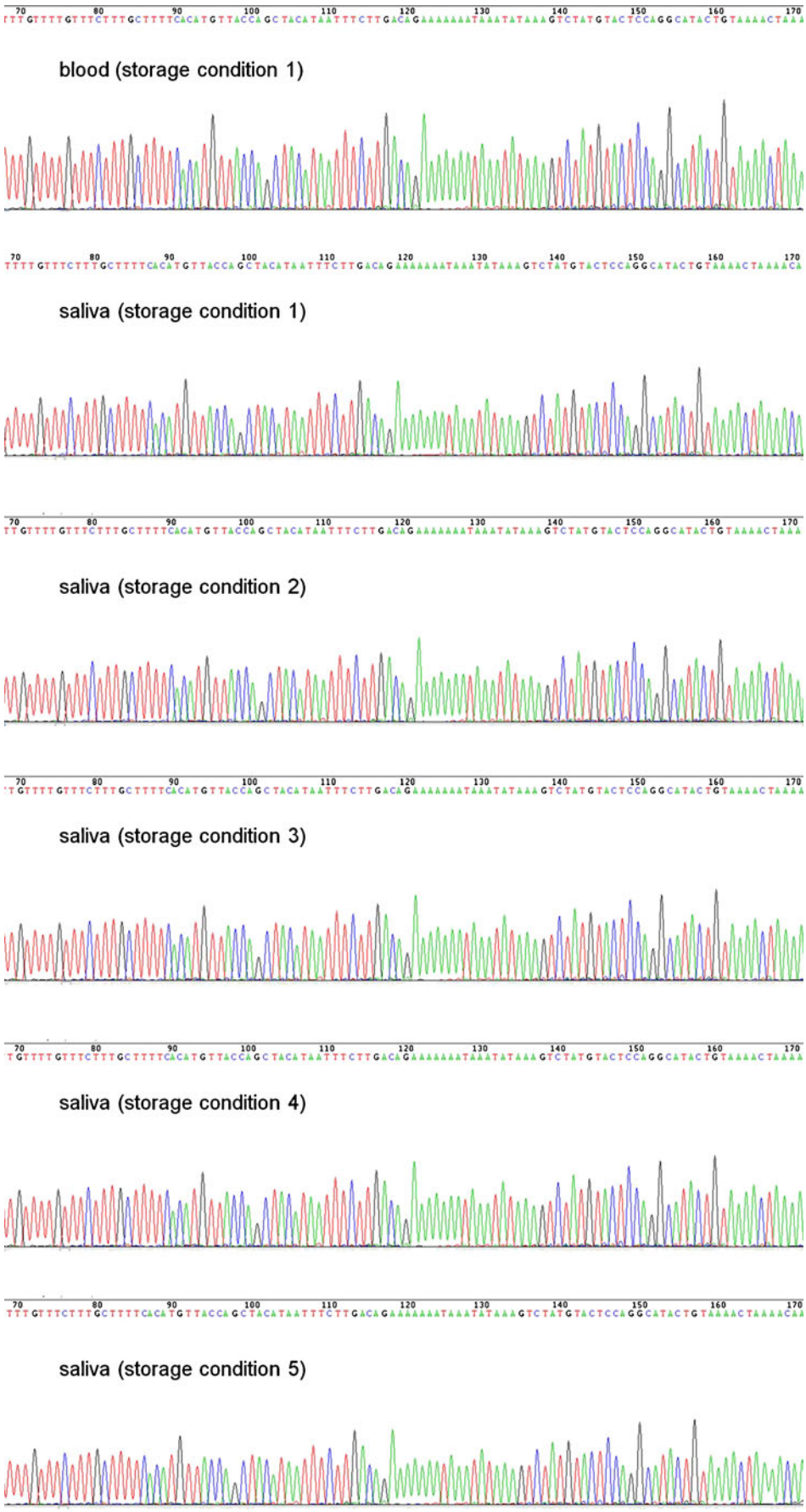

Fig. 1 Electropherograms of a representative sample, from each group, for exon 0 of the *RUNX2* gene

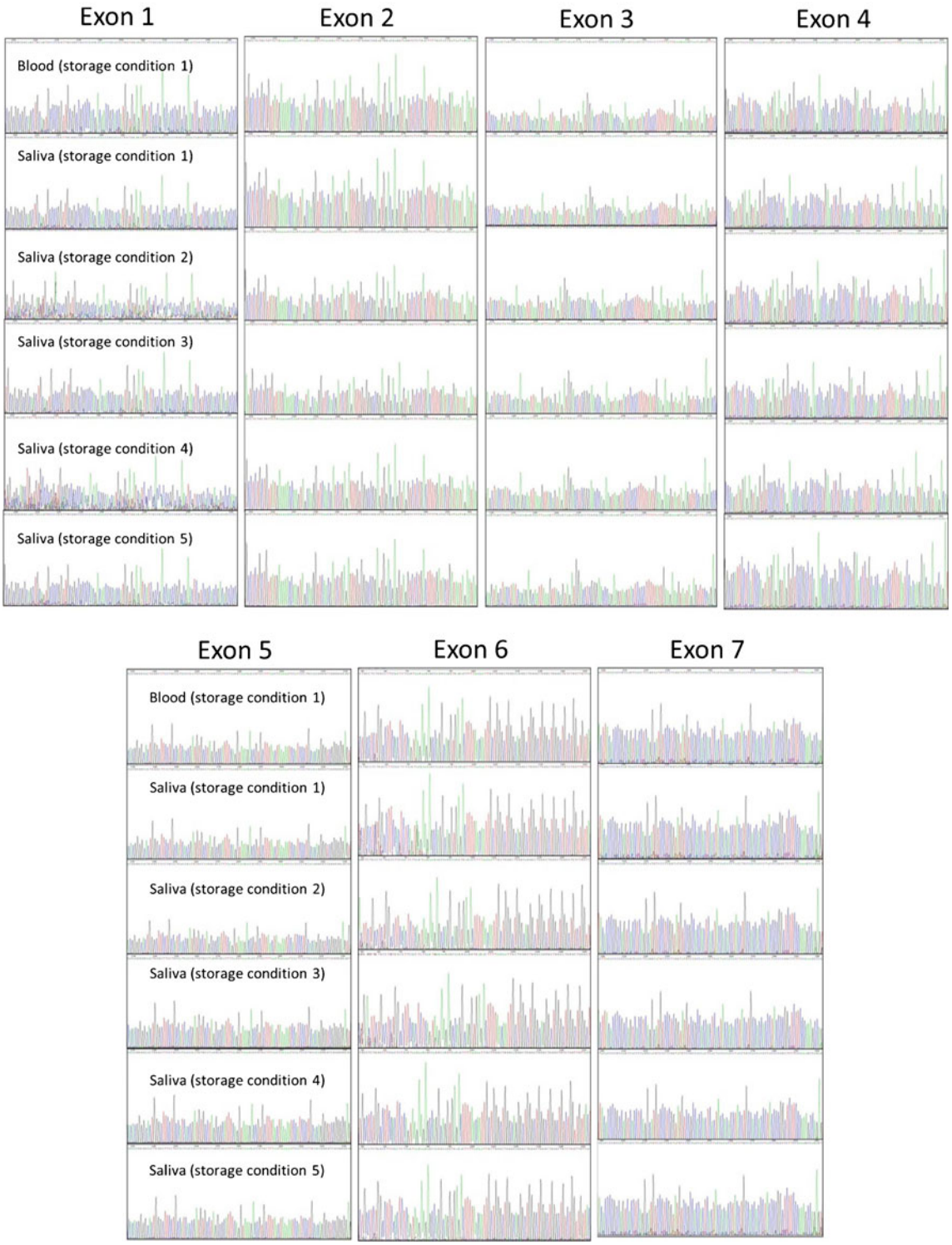

Fig. 2 Electropherograms of a representative sample, from each group, for exons 1–7 of the *RUNX2* gene

collection and to rinse their mouths out with water before donating the saliva sample. Nevertheless, even if the content of non-human DNA was higher in the saliva samples than reported, the greater overall yield would still allow more usable DNA than the other non-invasive methods such as buccal swab and a mouth rinse [4]. Moreover, there is no doubt that the total human DNA obtained from blood is greater than that, which can be obtained from saliva [1, 3]. Nevertheless, microscopy was not used to check for the microorganisms in the saliva solutions.

The reason for reduced DNA in the fresh saliva samples compared to those stored for different time periods is unknown. Nevertheless, this may reflect technical problems associated with manual DNA extraction. Although it is valuable to genotype several SNPs on different chromosomes to evaluate the quality of DNA samples, due to cost implications, we genotyped only 25 SNPs on 8 genes as this study was conducted in conjunction with another ongoing project. Therefore, PCR or sequencing results of other genes on different chromosomes should be tested in future studies. Furthermore, considering the substantial variation in the DNA yield among the saliva samples, it would be logical to use whole-genome amplification as a backup approach to generate additional DNA in future studies.

The PCR amplification protocols are usually employed to test DNA integrity [17, 19]; hence, it was used in the present study. The PCR amplification success rates (100 %) in the present study are consistent with the DNA quality estimates, as both saliva and blood samples exhibited a higher DNA quality irrespective of their storage conditions. These findings along with those of previous reports [1, 3, 4, 16] indicate that saliva collected using the Oragene® kit may be of sufficient quality to be used for various applications in population and association genetic studies. Moreover, our findings also demonstrate that the various saliva storage conditions did not negatively influence the DNA yield, purity, amplification, genotyping and sequencing results. Therefore, saliva can be a useful alternative to blood as a source of human DNA in large genetic epidemiological studies for both immediate use and archiving for future analyses.

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Conflict of interest The author(s) declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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