Evaluation of DNA Extraction Methods from Saliva as a Source of PCR - Amplifiable Genomic DNA

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Saliva represents a unique body fluid, easy and non-invasive to collect and may be useful as an alternative to blood source of genomic DNA for large scale genetic studies. In this study we evaluated three different DNA isolation methods onfour different storage conditions of samples and measured the impact on the total DNA yield and purity by spectrophotometry followed by amplification of human beta-globin gene through real-time PCR.

Keywords: saliva, polymerase chain reaction, DNA extraction, genomic DNA, storage conditions

In recent times the new field of salivary diagnostics has emerged and saliva has increasingly been investigated for its potential as a diagnostic fluid. A wide range of biologically active substances such as enzymes, hormones, immunoglobulins, xenobiotics have already been quantified with great success [1]. There is no surprise that the field of salivary diagnostics is gaining recognition as long as it is non-invasive, easy to collect, needs no special treatment and acceptance by patients is very high.

As the cost of whole genome sequencing and genotyping methods is continuously decreasing and the interest in understanding the genetic basis of diseasesand human microbiota has increased, the number of large genetic population studies also increased. This studies also addresses questions concerning population history, migrations, environmental adaptation, genetically modified foods, pollution contaminants detection and each of this study require good quality DNA [2]. Traditionally the main source of human genomic DNA is the blood as it provides high amount of DNA. The act of phlebotomy requires trained personnel, its invasiveness and risks makes blood unsuitable for large scale genetic studies as it increases the cost of the study, the acceptance by the study subjects may be problematic especially if it involves newborns, extreme ill persons, handicapped individuals and hemophilic subjects [3,4]. Saliva is a viable source of high quality genomic DNA and its non-invasiveness makes it more suitable for such studies.

The potential of saliva as a source of high quality human genomic DNA is obvious as the mean number of epithelial cells per 1 mL of saliva is 4.3×10^5, whereas the number of nucleated cells in 1 mL of whole blood is approximately $4.5\text{-}11 \times 10^5$. The turnover of oral epithelial cells is quite dynamic, on average, the surface layer of epithelial cells is replaced every 2.7 hours suggesting that there is a very high probability to isolate intact genomic DNA from saliva at any point of the day [5, 6]. A potential drawback of saliva is that a large proportion from total isolated DNA is of microbial origin but this is ideally for oral microbiology studies and for molecular diagnostic of periodontitis as the bacterial content of saliva is approximately 1.7×10^7/mL of saliva [5, 7-10].

Collection of saliva may be done in several ways, including mouthwash, swabs, whole saliva collection and cytological brushes [11,12]. Many commercially available collection kits contain also a preservation solution and the sample may be stored for up to a year at room temperature before DNA extraction which is ideal for field studies but their cost is high [13].

The commercially available DNA extraction kits are non-toxic, simple to use, are mainly produced in industrialized countries and may not be available at affordable prices in developing and underdeveloped countries, where diagnosis and epidemiological surveys based on DNA analysis may be a key factor in planning and establishing disease treatment and community disease-prevention programs. Our aim was to evaluate the DNA yield and the suitability of isolated DNA for real-time PCR amplification (which is the most used molecular technique in genetic studies) using an automatic nucleic acids extractor, a commercial DNA extraction kit and a simple cost effective protocol with ammonium acetate under different storage conditions of saliva samples.

Experimental part

Saliva collection and storage

A group of 20 volunteers of both genders (age range: 19-57) were enrolled in the present study. The study subjects were advised not to brush their teeth, to smoke to eat or drink at least 2 h prior sample collection. Each individual was asked to provide 10 mL of whole saliva and was oriented to rub the tongue vigorously on the teeth and whole oral mucosa surface.

In order the sample to be more representative and to avoid sub-sampling errors the participants were also asked to rinse vigorously their mouth for 45-60 swith 15 mL of a 2% saline solution. Collection of the oral rinse was done in the same 50 mL Falcon tube with the priory collected saliva with an interval of 5 min between the two different sample collections.

In order to assess the DNA integrity over time the samples were divided into 4 tubes containing equal amount of sample and were submitted to different storage

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conditions. The first tube was used for immediate DNA extraction (C1), the second tube was stored at -20°C for 1 week (C2), the third tube was immediately centrifuged at 10000 rpm for 5 minutes and the cell pellet stored at -20°C for 1 week (C3) and the final aliquot was mixed with ethanol to a final 70% concentration and stored at room temperature for 1 week (C4). A total number of 20 samples was included for the study, 5 samples for each storage conditions category [5 x (C1+C2+C3+C4)].

DNA extraction

Genomic DNA was extracted in three different ways:

a) using an automated nucleic acids extractor, MagCore® Super (RBC Bioscience Corp., Taiwan) with the MagCore®Genomic DNA Tissue Kit, cartridge code 401 following manufacturer's instructions (E1);

b) QIAamp® DNA Mini Kit (QIAGEN Group), spin protocol following manufacturer's instructions (E2),

c) Following the modified Aidar and Line protocol reported by Kuchler *et all*with minor modifications (E3) [14,15].

Briefly we pelleted the buccal cells by centrifugation at 10000 rpm for 5 min and washed the pellet 2 times with PBS and 1 time with molecular biologypure water by centrifugation at 3500 rpm for 3 min and the supernatant was discarded.The pellet was transferred to 2 mL micro-centrifuge tube and a 1 mL of cell lysis solution [10mM Tris, *p*H 8.0, 5mM EDTA, 5mM sucrose, 0.5% SDS] containing proteinase K (150 ng/mL) was added and incubated for 5 h at 57°C. After the incubation the proteins and other contaminants were removed by adding 400 µL of 10mM ammonium acetate followed by vigorous vortexing for 15 s and centrifuging at 17000 x g for 10 min.

The supernatant was transferred to a new tube to which 800µL of isopropanol was added and DNA was precipitated at -20°C for 30 min. After a centrifugation step at the supernatant was poured of followed by a washing step with 1mL of 70% ethanol and centrifugation at17000 g for 10 min at 4°C, the supernatant was discarded, the tube was inverted and air-dried for 30 min on absorbent paper. And finally the DNA was resuspended in 150 µL of TE buffer [10mM Tris (*p*H 7.8)and 1mM EDTA]. A total number of 60 DNA extractions were performed (each sample was extracted by the 3 different methodologies, E1, E2 and E3). All the chemicals used in this protocol were molecular biology grade and were acquired from Sigma Inc.

DNA quantification

The total DNA yield and purity was determined by spectrophotometry. We used the incorporated spectrophotometer in the MagCore® Super automated nucleic acids extractor (RBC Bioscience Corp., Taiwan). The DNA concentration is obtained by readings of optical density (OD) at 260 nm and the ratio of ODs at 260nm/280nm is used to estimate the DNA purity. In general a 1.7-2.0 value of ODs 260nm/280nm ratio is indicative for acceptable DNA purity.

Real-time PCR

In order to assess the DNA quality of samples after different storage conditions and different DNA isolation methodologies in terms of their real-time PCR amplification efficiency the samples were submittedto a PCR reaction targeting a 268 bp fragment of human beta-globin gene. Real-time PCR reactions were performed on a DT prime, plate type real-time cycler(DNA-technology, Russia) using a commercial human beta-globin control assay (PC04 and GH20 primer set, Life Technologies), following the manufacturer's instructions. This internal control assay contains all the reagents necessary for amplification and quantification of a 268 bp fragment of human beta-globin gene.

Results and discussions

The total DNA yield, purity and quality in extracts from saliva samples are listed in table 1. The total DNA yield was generally consistent across different storage conditions, except the C4 conditions where an important decrease in the amount of DNA regardless the extraction methodology was observed.

The reason for the decrease of DNA yield in C4 samples may reflect technical problems with these extractions and also may be due to the different sampling methods and sample amount submitted to DNA isolation in comparison with Kuchler *et all* and Aidar and Line studies. Also the real-time PCR C_T value was slightly modified for the C4 samples but the difference didn't have a negative impact on final amplification of the target proving that even in such conditions we can obtain real-time PCR amplifiable human genomic DNA from saliva.Obtaining PCR-amplifiable DNA from saliva can be done at reduced costs, according to the ammonium acetate DNA extraction protocol (E3), and in this way we can scale-up the studies

Storage conditions	DNA extraction method		Total DNA yield (µg)	DNA purity $OD_{260/280}$	DNA quality real-time PCR C_T value
C1 Immediate extraction	E1	automated extractor	32.83 (23.11-53.42)	1.82 (1.7-1.9)	23.57 (23.27-23.84)
	E2	QIAamp DNA extraction mini	28.31 (22.88-55.45)	1.87 (1.73-1.94)	
	E3	ammonium acetate protocol	27.88 (15.00-52.33)	1.97 (1.7-2.01)	
C2 immediately stored at -20°C for 1 week	E1	automated extractor	32.35(22.47-50.92)	1.87 (1.74-1.92)	23.68 (23.34-23.82)
	E2	QIAamp DNA extraction mini	29.42 (21.24-54.83)	1.85 (1.7-1.95)	
	E3	ammonium acetate protocol	25.62 (18.39-46.28)	1.95 (1.65-2.00)	
C3 The pellet stored at -20°C for 1 week	E1	automated extractor	35.45 (22.12-48.32)	1.85 (1.72-1.94)	23.65 (23.29-23.81)
	E2	QIAamp DNA extraction mini	30.84 (19.32-49.34)	1.89 (1.75-1.9)	
	E3	ammonium acetate protocol	27.49 (17.56-48.57	1.97 (1.65-2.02)	
C4 70% ethanol at room temperature for 1 week	E1	automated extractor	7.28 (1.85-15.42)	1.92 (1.76-1.98)	26.44 26.02-26.94
	E2	QIAamp DNA extraction mini	8.7 (2.37-10.78)	1.87 (1.72-1.95)	
	E3	ammonium acetate protocol	6.56 (1.53-8.34)	2.0 (1.62-2.02)	

Table 1
EFFECT OF STORAGE CONDITIONS AND DNA EXTRACTION PROTOCOLS ON YIELD, PURITY AND QUALITY OF GENOMIC DNA FROM SALIVA. DATA PROVIDED AS MEAN VALUES AND (RANGE)

in order to obtain more reliable data. The results obtained by E3 protocolare comparable with the others protocols used in the study (E1 and E2), doesn't use toxic reagents (phenol or chloroform), several samples can be processed in parallel and obviously implies much lower costs.

The use of an automatic nucleic acids extractor (E1) is generally more advantageous over the commercial DNA extraction methods (E2). Using such technologies the hands-on time is drastically reduced,and the risks of cross-contamination of samples, nucleic acids contamination of the laboratory facility are also reduced. The costs between E1 and E2 protocol are comparable as long as E1 protocol needs less additional materials and is suitable for high-throughput pipelines.

Conclusions

Saliva is a viable alternative source for real-time PCR amplifiable DNA and represents a flexible body fluid to work with. Even storage in 70% ethanol solution for one week at room temperature, still provided DNA sufficient for several real-time PCR reactions without affecting the PCR amplification results. We conclude that collection, preservation and isolation of DNA from saliva can be done at low costs and provides flexibility for the clinical and laboratory workflow.

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