DNA Analysis of Saliva Traces on Cigarette Butts Exposed to Indoor and Outdoor Environmental Conditions

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ABSTRACT: The examination of saliva traces left on cigarette butts as evidences are complicated due to the availability of biological material in trace amounts and its rapid degradation due to extreme effects of environmental factors. This study is aimed to assess the DNA purity and quantify the amount of DNA preserved in saliva found on cigarette butts subjected to various temperatures and humidity. Cigarette butt samples were smoked collected and were exposed to outdoors and indoors for 1 day, 3 days and 7 days, respectively. The samples were subjected to DNA extraction, quantification and DNA profiling. The DNA purity on cigarette butts in indoor experiment was found to be higher ($A_{260/280}$ 1.76 – 1.91) than the purities of outdoor experiment ($A_{260/280}$ 1.26 – 1.65). The concentration of DNA found on the saliva traces on cigarette butts can be very variable in outdoor experimental set-up (377.99 – 585.83 ng/µL) compared to indoor (266.38 – 290.18 ng/µL); attributable to the differences in humidity as well as temperature. In conclusion, the purity obtained in this study ranged from low to high, and samples with intermediate to high purity was proven, allowing DNA profiling. Since the concentration of DNA reported in this study may constitute human as well as non-human DNA, the interpretation of the DNA purity is a better mean for elucidating its potential value in forensic aspect compared to DNA concentration.

Keywords: Saliva, Cigarette Butt, DNA, Environment

Introduction

Cigarette butts are one of the common carriers of saliva traces in forensic practice. Today, approximately 1.2 billion people worldwide smoke tobacco [1] and they smoke when they are nervous (*i.e.* when involved in a crime) [2,3]. Cigarette butts found at crime scene may contain traces of saliva and attached mucosal epithelium cells from the lips of the smoker, which allows for DNA identification by profiling [4,5].

Saliva is an evidence which can be encountered in forensic casework. It has a high evidentiary value in identifying victims and suspects as well as exonerates an innocent individual [6]. A healthy adult produces 500 – 1500 mL of saliva in a day, at a rate of 0.5 mL/min. The quantity and quality of saliva produced, however, is influenced by several pathological and physiological conditions which includes, taste and smell stimulation, hereditary factors, hormonal status, and oral hygiene [7].

A perpetrator can be identified by the material containing DNA that he/she leaves at a crime scene. DNA can be found in the blood, sperm, hair and saliva at the crime scene. Samples taken from trace evidence items are subjected to DNA isolation, and subsequently the generation of Short Tandem Repeat (STR) profile which is then compared to the saliva sample obtained from a suspect or compared to a DNA Database. If there is a match, a probability of identity is calculated [3].

About 2 to 160 ng of DNA extracted from cigarette butts is adequate for typing [8]. DNA samples recovered from a crime scene are frequently exposed to damaging environmental conditions such as light, heat and bacterial decomposition before they are collected for analysis. Hence, generating an evidentially valuable profile from these quality-compromised samples is a great

challenge to the forensic scientist [9]. No study have been reported in this context where the cigarette butts are exposed to outdoor or indoor conditions up to a period of 1 week, especially in Malaysia. However, specific studies pertaining to this area have indicated that the purity of DNA extracts from saliva being lower than the optimal range [10] and cigarette butt samples are notably difficult to analyse as they contain DNA that are contaminated with PCR inhibitors such as phenolics, and tars from the flavour additives, paper additives as well as the smoke or may contain DNA that are degraded [11]. In this context, this study serves to evaluate the purity and concentration of the DNA from cigarette butt samples exposed to the outdoor and indoor at varying conditions as well as durations of exposure that may be useful in elucidating this aspect in the court of law.

Materials and methods

Chemical and Samples

The reagents used in this study were Chelex from Sigma Aldrich (USA) and Proteinase K (20 mg/mL) from Promega (USA). The samples used in this study were Marlboro (original) cigarettes purchased from a local store at Taman Universiti, Johor Bahru from the supplier Philip Morris (Malaysia) Sdn. Bhd.

Laboratory Equipment

The laboratory equipments used in this study were: Data Logger, KKI Instruments (M) Sdn. Bhd.; Deep freezer (-20^{o}C), Model YCD-EL259, Remi Scientific Sdn. Bhd.; E-pure (Ultrapure (Type 1) Water), Biopak® Polisher, Merck Millipore Corporation, Germany; Fumehood, Micromec Technologies Sdn. Bhd.; Microcentrifuge, Model 5430 R, Eppendorf, Medigene Sdn. Bhd.; Mini-centrifuge, GeneReach Biotechnology Corp, Taiwan; Nanodrop 1000 Spectrophotometer, Thermo Fisher Scientific Inc.; Oven, incubator, Memmert, Germany; Rain Gauge, Weather, Atmosphere; Refrigerator, LG Electronics Inc., Korea; Vortex Mixer, Jeio Tech, Korea; Water-bath, Memmert, Germany; and weighing balance, Model HR-250AZ, A&D Company Limited, Japan.

Sampling

Ten individuals, each smoked seven cigarettes, providing a total of 70 cigarette butts for this study were collected, exposed to outdoors (open field) and indoors (in the laboratory) for one day, three days and seven days prior to DNA extraction. One cigarette butt from each individual was smoked and served as a control sample. The temperature, humidity and rainfall (for outdoor samples) were recorded using a data logger, humidity meter and a rain gauge, respectively.

DNA Extraction

Cigarette butts were handled with forceps at all times. Using a sterile scalpel blade, approximately 5 mm wide strip from the paper covering the cigarette butt in the area contacted with the mouth was sliced. The paper slice from the cigarette butt was pulled, cut into smaller pieces and placed in a sterile, 1.5 mL microcentrifuge tube. An aliquot of 400 µL of 10% (w/v) Chelex and 20 µL Proteinase K (20 mg/mL) was added to the tubes and vortexed for 30 seconds. The tubes containing the mixture were then incubated at 56°C overnight. The tubes were then vortexed for 30 seconds and subsequently incubated in a boiling waterbath at 100°C for 8 minutes. The tubes were then vortexed for 30 seconds and spun in a microcentrifuge at a maximum speed (13,000 rpm) for 3 minutes. The supernatant was transferred to a new microcentrifuge tube and the concentration of DNA was measured [12].

Quantification of DNA

The successfully extracted DNA was quantified using Thermo Scientific Nanodrop 1000 Spectrophotometer according to the guidelines provided in the operational manual to give an absorbance ratio ($A_{260/280}$) which indicates the DNA purity and concentration (ng/µL) [13].

Amplification and Typing

The amplification reactions (12.5 µL primers + 12.5 µL master mix + 3 µL test sample) were used with thermal cycling parameters recommended by the manufacturer [14]. The parameters include an initial hold at 95°C for 11 minutes, followed by a 28 cycles of denaturation step at 94°C for 20 seconds, an annealing step at 59°C for two minutes, an

extension step at 72°C for one minute, subsequently a final elongation step at 60°C for 25 minutes and the samples were held at 4°C after amplification. The samples were amplified on the GeneAmp® PCR system 9700 thermal cycler by Applied Biosystems. The 3500xL Genetic Analyzer was used to perform capillary electrophoresis. Then, 10 µL of reaction mixture (1 µL of PCR product or Allelic Ladder + 9 µL of the formamide:size standard mixture) was prepared, heated in thermal cycler for 3 minutes at 95°C and subsequently placed on ice for 3 minutes. The samples were then transferred into the capillary using electrokinetic injection with initial injection conditions of 1.2 kV for 24 sec and electrophoresis run was started. The GeneMapper® ID-X Software v1.4 was used to analyse the data. The AmpFlSTR® Identifiler® Direct Allelic Ladder was used to genotype the analysed samples. The AmpFlSTR® Identifiler® Direct Control DNA 9947A was used as a positive control to evaluate the efficiency of amplification step and confirm the ability of kit reagents to produce a profile of genotype expected [15].

Results and Discussion

DNA Purity and Concentration

A good quality DNA sample should have a $A_{260/280}$ ratio of 1.7-2.0 which is the optimal purity range [16]. The mean $A_{260/280}$ absorbance ratio of butt samples exposed outdoors was 1.65 ± 0.073 after one day exposure, 1.49 ± 0.096 after 3 days exposure and 1.26 ± 0.083 after one week exposure to the environment, hence showing a gradually decreasing trend of purity over the period of 1 week (Table 1). In contrast, samples exposed indoors showed a mean $A_{260/280}$ absorbance ratio of 1.80 ± 0.15 after one day exposure, 1.91 ± 0.11 after three days exposure and 1.76 ± 0.086 after one week exposure to the environment, which within the optimal range. Hence, the findings in this study showed that the mean purity of DNA recovered from saliva traces on cigarette butts exposed outdoor was lower than that exposed indoors. The $A_{260/230}$ ratio for cigarette butts both exposed outdoors and indoors was in the range of 0.44 to 0.93. The $A_{260/230}$ value for a "pure" nucleic acid is 1.8 – 2.2. Hence, since the $A_{260/230}$ ratio of the extracts is lower than the accepted target, this may indicate the presence of carbohydrates, magnetic beads, residual phenol or proteins [13]. This was consistent with the fact that the cigarette butt filter paper is made up of cellulose acetate, a plant origin which interferes with the purity of extracted DNA. Some salivary samples itself may contain impurities such as peptides, carbohydrates, aromatic compounds, buffer salts, or extracellular proteins such as mucin in saliva, all of which could contaminate and compromise the quality of DNA, hence causing the purity of DNA extract to fall below the optimal range [10].

The results in this study showed that the concentration of DNA extracted from the saliva traces on cigarette butt samples exposed outdoors ranged from 271.39 ng/µL to 1306.34 ng/µL with an average yield of 377.99 ± 282.08 ng/µL after one day exposure, 279.67 ng/µL to 2088.5 ng/µL with an average yield of 585.83 ± 470.62 ng/µL after three days of exposure and 320.71 ng/µL to 539.25 ng/µL with an average yield of 390.99 ± 127.47 ng/µL after one week of exposure to the environment. In contrast, the results also showed that the DNA extracted from the saliva traces on cigarette butt samples exposed indoors ranged from 148.92 ng/µL to 358.02 ng/µL with an average yield of 266.38 ± 70.47 ng/µL after one day exposure, 221.73 ng/µL to 316.77 ng/µL with an average yield of 290.18 ± 69.05 ng/µL after three days of exposure and 216.35 ng/µL to 381.68 ng/µL with an average yield of 274.11 ± 83.97 ng/µL after one week of exposure to the environment (Table 1).

Table 1: Purity and Concentration of DNA from Smoked Cigarette Butt Samples Exposed to the Indoor and Outdoor Environments.

Environment	Day	Purity		Concentration (ng/µL)	Mean Ambient Temperature ± SD	Humidity
		$A_{260/280}$ ± SD	$A_{260/230}$ ± SD			
Outdoor	1	1.65 ± 0.073 (1.55 – 1.78)	0.60 ± 0.086 (0.47 – 0.74)	377.99 ± 282.08 (271.39 – 1306.34)	25.4 – 36.8°C ± 3.97	55% to 99% * Heavy rains (1.5 mm, 8.3 mm, and 15.9 mm) were recorded on days-4, 6 and 7 respectively
	3	1.49 ± 0.096 (1.26 – 1.59)	0.60 ± 0.091 (0.48 – 0.73)	585.83 ± 470.62 (279.67 – 2088.5)	24.4 – 36.8°C ± 3.98	
	7	1.26 ± 0.083 (1.12 – 1.35)	0.71 ± 0.094 (0.60 – 0.93)	390.99 ± 127.47 (320.71 – 539.25)	24.4 – 36.8°C ± 3.63	
Indoor	1	1.80 ± 0.15 (1.60 – 2.02)	0.53 ± 0.048 (0.44 – 0.58)	266.38 ± 70.47 (148.92 – 358.02)	24.2 – 27.4°C ± 1.38	85% to 99%
	3	1.91 ± 0.11 (1.71 – 2.11)	0.54 ± 0.035 (0.49 – 0.61)	290.18 ± 69.05 (221.73 – 316.77)	21.7 – 27.4°C ± 1.35	
	7	1.76 ± 0.086 (1.60 – 1.91)	0.55 ± 0.057 (0.49 – 0.65)	274.11 ± 83.97 (216.35 – 381.68)	21.7 – 27.6°C ± 1.15	

The DNA concentration and purities from the control sample (smoked, unexposed butts) was 206.48 ± 17.75 ng/µL and $A_{260/280}$ of 1.77 ± 0.053 and $A_{260/230}$ of 0.47 ± 0.029, respectively.

Kruskal-Wallis pairwise comparisons using Mann-Whitney test (P = 0.05) was used for determining the significant differences among groups for the DNA concentration and purity. The data for the DNA concentration are presented as median ± interquartile range.

Kruskal-Wallis pairwise comparisons using Mann-Whitney test (P = 0.05) was also used to determine the differences within groups for the concentration (ng/µL) of DNA on cigarette butts exposed outdoors and indoors. ANOVA with Bonferroni post hoc test (P = 0.05) was used for determining the differences within groups for the purity ($A_{260/280}$) of DNA on cigarette butts exposed outdoors and indoors.

The amount of saliva excreted by the individual differs could be varied due to several factors such as activity of smoker, mood and intake of psychoactive drugs [17]. Beside that, the amount of DNA retained on the exposed butts in various environments could be different. DNA extracted from exposed cigarette butt samples may contain exogenous DNA such as that from insects, animals, fecal matter, plants, soil, and microbial DNA besides human, which contributes largely to the total DNA yield [18]. For the butts exposed outdoors, the average concentration of DNA recovered is observed to be higher after three days exposure compared to after one day exposure, consistent to the fact that the longer the butts exposed to the environments, there may be more exogenous DNA, contaminants and microorganisms. However, the average amount of DNA recovered after seven days exposure outdoors is observed to be lower than that after 3 days exposure. This may be due to the high rainfall of 8.3 mm and 15.9 mm on the 4^{th} and 5^{th} day after exposure respectively, which possibly might have washed away some DNA from the butts.

Overall, the findings in this study showed that the purity of DNA recovered from the saliva traces on cigarette butts exposed outdoors and over a longer period of exposure time are lower than that exposed indoors and over a shorter period of exposure time. On the other hand, the concentration of DNA recovered from the saliva traces on cigarette butts exposed outdoors could be relatively variable compared to that exposed indoors, attributable to the differences in humidity and temperature.

Short Tandem Repeat (STR) Typing

Selected cigarette butt samples were submitted for STR typing to the PDRM forensic laboratory, Cheras. Table 2 summarises the STR typing results for representative samples (*i.e.*, high purity, intermediate purity and low purity samples). Full profiles were obtained from DNA extract samples with optimal and near optimal purity range. A partial profile was obtained in DNA extract sample with a

low purity range and no profile was obtained in DNA extract sample with very low purity.

Table 2: AmpFlSTR® Identifiler® Direct Amplification Results for Butt Samples Exposed Outdoors and Indoors.

Sample	Purity ($A_{260/280}$)	#Loci Detected	Detected STR Alleles	Amplification Results
OCB2-01	1.67	16/16	27	Full Profile
OCB5-01	1.68	16/16	23	Full Profile
OCB6-03	1.26	5/16	5	Partial Profile
OCB5-07	1.12	0/16	0	No Profile
ICB5-03	2.11	16/16	23	Full Profile

Overall, the quality of DNA obtained was a primary determinant of the STR profile obtained. Also, since the Identifiler® Direct Kit specifically detects primate alleles, only the human species amplifies for loci tested [15]. Hence, human DNA can still be recovered from saliva traces of cigarette butts exposed to the different environmental conditions up to 1 week. In this study, while the purity reported remains between low to high, DNA profiling was still successfully performed for samples with high and intermediate DNA purities. This proved that although the DNA purity could be lower than the optimal range, the possibility of identifying the perpetrator using DNA evidence recovered from cigarette butt samples may still be possible. Figure 1 shows the electropherograms results of the AmpFlSTR® Identifiler® Direct amplification of DNA extract from the exposed cigarette butts samples.

Conclusions

The results from this study showed that the purity of DNA recovered from cigarette butts in indoor experiment were higher than the purities of outdoor experiment. The purity was ranged from low to high, and samples with intermediate to high purity was proven to enable DNA profiling, hence could be useful in human identification.

Great variability from the concentration of DNA found on the saliva traces on cigarette butts from the outdoor experimental set-up was obtained compared to indoor samples. This could be attributable to the differences in humidity as well as temperature. However, since the concentration of DNA reported could be constituted of human as well as non-human DNA, hence, the interpretation of the DNA concentration in this aspect may not be the best means for elucidating its potential value in forensic aspect. The fact that the purity of DNA revealed in this study can explain the exposure of DNA, this may be the parameter to look for in the future.

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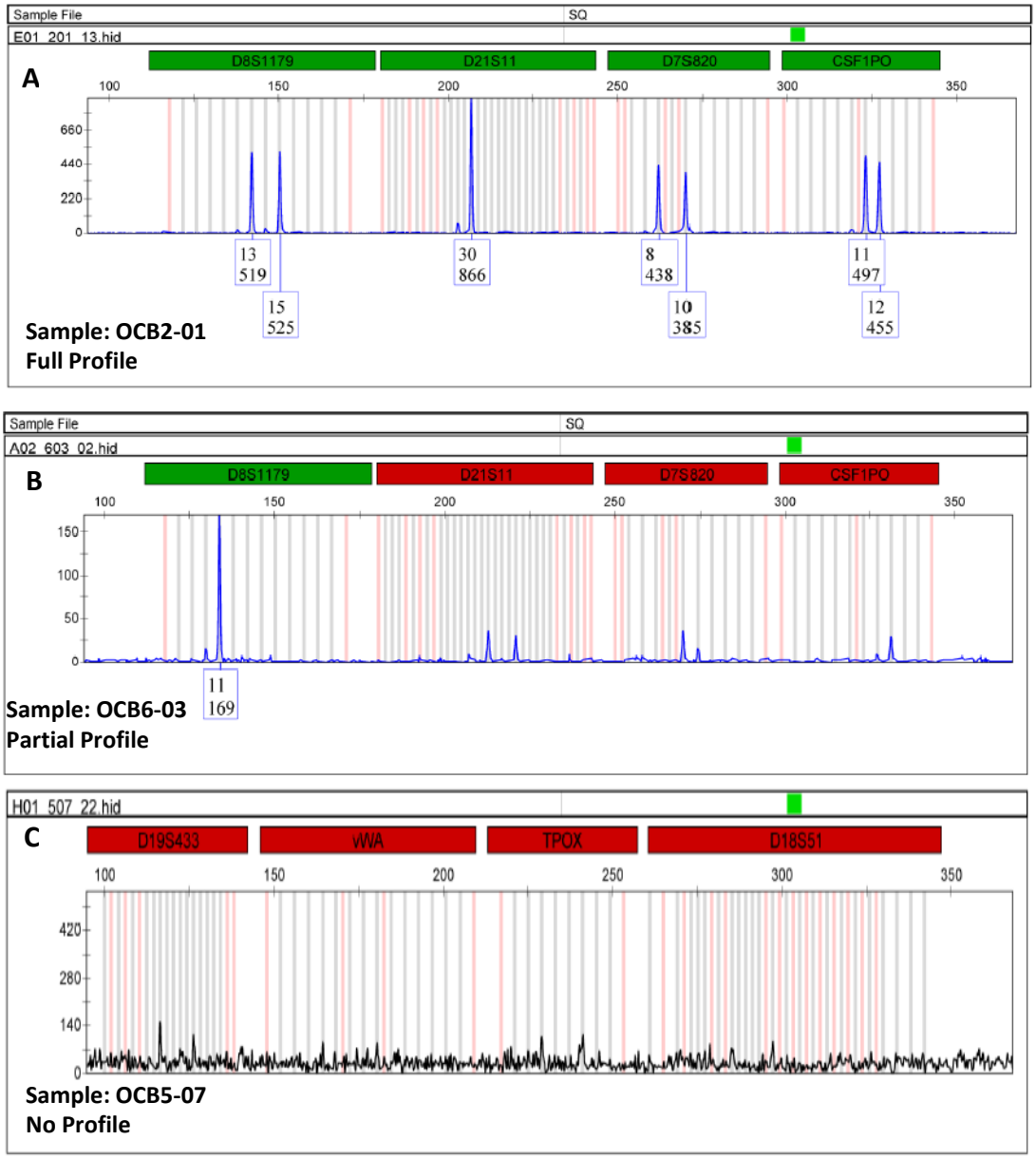

Fig. 1: Electropherograms showing Ampflstr® Identifiler® direct amplification of DNA extract from cigarette butts exposed to outdoors A) one day after exposure (a260/280: 1.67); b) 3 days after exposure (a260/280: 1.26); and c) one week after exposure (a260/280: 1.12).

References

1. Oleski, K., Campbell, C., Patel, R. and Welker, J. (2010). DNA Extraction for obtaining DNA from Cigarette Filter Paper Collected in Paris and East Lansing for PCR Amplification Detecting p53 Mutations. Retrieved on 12th February 2016 from Ace Learning Company. Inc website: 35.9.122.184/p53mutations.pdf
2. Axelrod, A., Antinozzi, G. and Axelrod, A. (2007). *The Complete Idiot's Guide to Forensics*. New York: Alpha Books.
3. Dettmeyer, R. (2013). *Forensic Medicine*. Berlin: Springer-Verlag Berlin and Heidelberg GmbH & Co KG.
4. Yudianto, A. (2009). Effectiveness of Cigarette Butts as an Alternative Material for Forensic DNA Identification with Polymerase Chain Reaction (PCR) in Short Tandem Repeat (STR) Loci. *Folia Medica Indonesiana*. 45(2), 112-114.
5. Linch, C. A. and Prahlow, J. A. (2008). Microscopical Examination of Particles on Smoked Cigarette Filters. *Forensic*

Science, Medicine and Pathology. 4(4), 228-233. Humana Press Inc.
6. Sharma, R. (2008). *Concise Textbook Of Forensic Medicine & Toxicology*. (2nd ed.). New Delhi: Elsevier
7. Roberts, K. A. and Johnson, D. J. (2012). *Investigations on the Use of Sample Matrix to Stabilize Crime Scene Biological Samples for Optimized Analysis and Room Temperature Storage*. Unpublished note, National Criminal Justice Reference Service, U.S.
8. Hochmeister, M. N., Budowle, B. and Jung, J. (1991). PCR - Based Typing of DNA Extracted from Cigarette Butt. *International Journal of Legal Medicine*. 104, 229-233.
9. Thacker, C. R., Oguzturun, C., Ball, K. M. and Court, D. S. (2006). An Investigation into Methods to Produce Artificially Degraded DNA. *Elsevier*. International Congress Series 1288, 592-594.
10. Hedman, J. (2011). DNA Analysis of PCR-Inhibitory Forensic Samples. Sweden: Media-Tryck, Lund.
11. Shepherd, C., Harbison, S. and Saul, D. J. (2012). Extraction of DNA from cigarette butts using forensicGEM® Cigarette. *ZyGEM Application Note 102*. New Zealand: ZyGEM Corporation Ltd.
12. Andrew, L. R. (1999). *Chelex DNA Extraction*. Polis Diraja Malaysia (PDRM) Forensic Laboratory. Method No. 6A, Version 3.
13. Thermo Fisher Scientific. (2010). *Nucleic Acid Thermo Scientific NanoDrop Spectrophotometers.* [Brochure]. USA: Thermo Fisher Scientific Inc.
14. Thermo Fisher Scientific (2015). *AmpFlSTR® Identifiler® Direct PCR Amplification Kit* [Brochure]. USA: Life Technologies.
15. Applied Biosystems. (2012). *AmpFlSTR® Identifiler® Direct PCR Amplification Kit*. Life Technologies Corporation
16. Oswald, N. (2010). Determining DNA Concentration and Purity. *Bitesize Bio*. Retrieved October 26, 2015, from http://bitesizebio.com/13501/dna-concentration-purity/
17. Zacny, J. P. and Stitzer, M. L. (2012). *Human Smoking Patterns*. Monograph 7. National Cancer Institute.
18. Bali, L. E., Diman, A., Bernard, A., Roosens, N. H. C. and De Keersmaecker, S. C. J. (2014). Comparative Study of Seven Commercial Kits for Human DNA Extraction from Urine Samples Suitable for DNA Biomarker-Based Public Health Studies. *Journal of Biomolecular Techniques*. 25(4), 96–110. National Center for Biotechnology Information, U.S.

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