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Epigenetic discrimination of Lebanese monozygotic and dizygotic twins: Summarized notes in a single paper

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ABSTRACT

Monozygotic twins' differentiation is worldwide limited due to the fact that they share the same genotype. For forensic purposes, many studies involving monozygotic twins were accomplished. Comparative studies including dizygotic twins were also performed. In Lebanon, the use of rapidly mutating Y-STRs was unsuccessful in differentiating the male monozygotic twins. Only the epigenetic treatment using the bisulfite reaction and the HRMC technique was helpful in twins' discrimination. The wide range of data we needed in our publications was hardly obtained by different kinds of resources. It is an effort and time-consuming to search for information in many research articles, reviews, short communication. The aim of this paper is to summarize many notes in order to minimize the time spent by researchers interested in twins' discrimination research.

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INTRODUCTION

Standard forensic genetics is characterized by its power of discrimination (Stewart et al. 2015). Yet, it cannot distinguish individuals within a monozygotic pair (MZ) due to the fact that they share the same DNA profile (Vidaki & Kayser, 2018). The researchers recruited twenty-eight unrelated pairs of Lebanese male and female MZ twin pairs to study the discriminatory power of different approaches. The first study focused on rapidly mutating Y-STRs and no discrimination between individual members of male Lebanese MZs pairs was achieved (Romanos & Borjac, 2018). However, in the second study using DNA methylation, we were able to differentiate individuals within 71.42% of the Lebanese MZs twin pairs regardless of gender (Romanos & Borjac, 2021). It is assumed that epigenetic discrimination is the result of exposure to different environmental stimuli (Stewart et al. 2015).

This distinction was determined by using bisulfite conversion of Cytosine in Alu-Sp & Alu-E2F3 epigenetic biomarkers followed by real-time polymerase chain reaction (PCR) and high resolution melting curve (HRMC) analysis. Bisulfite conversion is the "gold standard" for DNA methylation analysis. To determine methylation pattern, bisulfite conversion is commonly used where unmethylated cytosine is converted by sodium bisulfite to uracil, while 5mC remains unconverted. Concerning HRMC, it is a novel, rapid, and sensitive technique. The melting behavior of the amplified DNA sequences can be well characterized by the HRMC technique. HRMC post-amplification detects different melting temperatures (Tm) due to the different GC content obtained by the bisulfite treatment (Fraga et al. 2005, Mccarthy et al. 2013, Fitarelli-Kiehl et al. 2016, Piovesan et al. 2019). The third study, involving epigenetic discrimination of fourteen unrelated pairs of Lebanese dizygotic (DZs) pairs, was accomplished for a comparative analysis (Romanos & Borjac, 2021). Both technical, as well as analytical notes, are considered as the basis of any successful experiment. Yet, to assure the well-performing of each experiment step, we encountered huge challenges concerning collecting its important notes.

OBJECTIVE OF THE STUDY

In our studies, we had to deal with a huge number of research articles and reviews, to sum up, the needed notes of the applied techniques such as the bisulfite treatment and the HRMC analysis. Since this task consumed a lot of our time and effort, we tend to summarize many critical notes in this paper.

DATA DESCRIPTION

The primary note consists of the DNA quantification importance. Both pre and post-bisulfite treatment DNA quantification is needed (Vidaki et al. 2018). The second note involves the use of consistent protocols for all the samples starting from the DNA collection method ending with the real-time PCR steps (Butler & Willis, 2020). The choice of an appropriate sample type for the epigenetic study is needed also. Usually, reference samples such as saliva and blood are the best choice for any epigenetic test (Holliday, 2006). Good quality and quantity of DNA are necessary. The choice of an appropriate epigenetic kit is essential as well mainly preferred to contain a DNA protect buffer. Concerning DNA quality, the harsh conditions of high salt and temperature and low pH during the bisulfite treatment lead to DNA fragmentation to less than 500 bp. Thus, the choice of a suitable epigenetic kit minimizes this DNA yield loss and prevent its fragmentation. The DNA protect buffer included in the epigenetic kit assures a high complete cytosine conversion rate of 99% (Patterson et al. 2011, Kurdyukov & Bullock, 2016). Regarding the DNA quantity, normalization before each step is necessary to provide sample to sample uniformity. Usually, the starting amount of DNA is crucial for bisulfite conversion (usually 400 ng is needed); the sample should not contain too much DNA to prevent incomplete conversion, and should not contain too low DNA to prevent not having conversion at all (Li et al. 2011).

The HRMC analysis is applicable after bisulfite conversion in case the researcher seeks a single base-pair resolution and a relatively small final amplicon size of less than 300 bp. Normalization before HRMC is important to assure comparable amplification because the difference in samples cycles threshold (Ct) must be no more than 3 due to the fact that difference of 3 Ct indicates $2^3 = 8$ times more DNA template so some samples will start amplification before other ones. In addition, the added HRMC steps at the endpoint of real-time PCR is preferable

to have a final temperature around 60º C to assure a complete DNA fragments reassociation before data acquisition took place during the increase to the denaturation temperature of 95 º C (Stewart et al. 2015, Vidaki & Kayser, 2018). As for the HRMC used PCR kit, it is better to include EvaGreen® saturating dye rather than SYBRGreen® since the high EvaGreen® concentration will give higher fluorescence data without inhibiting the PCR amplification. Concerning the primer design, the researcher should be aware that once the DNA is bisulfite converted then it would be single-stranded since strands are no more complimentary. Only one set of forward or reverse primers, usually consisting of only A, T, and C nucleobases, will bind to the treated DNA. After rounds of amplification, this specific set will be the template to the other primers set permitting its binding (Fraga et al. 2005, Hernandez et al. 2013). Both controls and error rates emphasize the hypothesis that results are not due to the chance (Kloosterman et al. 2014). This test requires at least two types of controls. The first type indicates the biological and technical variations through the untreated samples. Tm of two different MZ pairs is compared to detect the biological variation and it must be found different. Meanwhile, the Tm of two individuals within the same MZ pair is compared to detect the technical variation and must be found similar. The technique is considered consistent once the obtained biological variation is higher than the obtained technical variation.

These findings lead to the fact that the detected MZ twins' differentiation is confirmed not to be due to the chance (Martino et al. 2013). The bisulfite converted, methylated and unmethylated, epitect controls consist of the second type of controls provided by the manufacturers. It will indicate the well-performing of the designed primers once showing that the Tm of the methylated samples is higher than that of the unmethylated ones (Huang et al. 2015). On the other hand, error rates involve both the individual and the assay standard deviations, the latter one defined to be around 0.5º C in the PCR-HRM technique (Stewart et al. 2015). In addition, Bonferroni correction would be helpful while having more than two tests in the same study (Carracedo, 2013). The final note is about what statistical test should be used. The best statistical test in such an experiment would be the paired t-test permitting to compare the average Tm between individuals within the same MZ pair; the non-independence of the three technical replicates/individual in our study makes the independent t-test not valid (Martino et al. 2013).

CONCLUSION

This single paper provides an organized summary that would be easy and quick access of many notes for any researcher concerning the sample and epigenetic kit type choices, normalization, HRMC steps, HRMC used PCR kit, primer design, controls, error rates, and the statistical test choice. We hope that this paper will help any researcher that will use similar techniques as we used in our studies to save time and effort.

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