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Comparison between automated DNA extraction employing the EZ1 platform and manual methods using real forensic samples

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Abstract

In the last 30 years, advances in Molecular Biology techniques have allowed its application as an essential tool in the criminal investigation, with a particularly relevant application in the determination of authorship of crimes and the identification of missing persons and unknown corpses. However, with the popularization of Forensic Genetics and the growth of violence, there has been a significant increase in the demand for these tests, which has led Forensic Genetics Laboratories to search for more efficient and safe methods of processing this increasing volume of samples. In this sense, automation was one of the adopted solutions, allowing the processing of the samples more accurately and with the minimum of human interference, reducing the risks, and being a more economical alternative when used in large scale. This paper aims to present the results obtained concerning the standardization of the use of an automated DNA extraction platform and to evaluate the advantages of this method in relation to the manual methods in the extraction of forensic samples from the Laboratory of Forensic Genetics of POLITEC-AP. For the realization of the experiments, 500 samples of DNA were extracted using manual methods (Organic and Chelex 100®) and using the automated extraction equipment EZ1 Advanced XL® from Qiagen ©. The automated extraction using the EZ1® following the manufacturer's protocol or after the adaptation of the protocol presented qualitative and quantitative results superior to those obtained using manual methods, both for reference saliva samples and unknown samples of unknown cadavers (teeth and bones) and secretions collected from victims of sexual violence.

***Keywords*:** DNA extraction; EZ1; Automated extraction; Forensic genetics; DNA forensics.

Resumo

Nos últimos 30 anos, os avanços nas técnicas de Biologia Molecular têm permitido a sua aplicação como ferramenta essencial na investigação criminal, com aplicação particularmente relevante na determinação da autoria de crimes e na identificação de pessoas desaparecidas e cadáveres desconhecidos. No entanto, com a popularização da Genética Forense e o crescimento da violência, houve um aumento significativo da demanda por esses exames, o que levou os Laboratórios de Genética Forense a buscar métodos mais eficientes e seguros de processamento desse crescente volume de amostras. Nesse sentido, a automação foi uma das soluções adotadas, permitindo o processamento das amostras com maior precisão e com o mínimo de interferência humana, reduzindo os riscos e sendo uma alternativa mais econômica quando utilizada em larga escala. Este trabalho tem como objetivo apresentar os resultados obtidos quanto à padronização do uso de uma plataforma automatizada de extração de DNA e avaliar as vantagens deste método em relação aos métodos manuais na extração de amostras forenses do Laboratório de Genética Forense da POLITEC-AP . Para a realização dos experimentos, 500 amostras de DNA foram extraídas por métodos manuais (Extração orgânica e Chelex 100®) e utilizando o equipamento de extração automatizado *EZ1 Advanced XL® da Qiagen ©*. A extração automatizada com o *EZ1®* seguindo protocolo do fabricante ou após adaptação do protocolo apresentou resultados qualitativamente e quantitativamente superiores aos obtidos por métodos manuais, tanto para amostras de saliva de referência quanto para amostras de cadavers desconhecidas (dentes e ossos) e secreções coletadas vítimas de violência sexual.

Palavras-chave: extração de DNA; EZ1; Extração automatizada; Genética forense; Análise forense de DNA.

1. INTRODUTION

The use of Genetics and Molecular Biology with the Forensic purpose is a reality and a sector of high relevance in the current expert units around the world, contributing decisively to the materialization of the technical proof and indication of criminal authorship or identification of missing persons. The pioneering use of this innovative tool can be credited to Sir Allec Jeffreys and his colleagues who in the early 1980s applied the DNA minisatellites analysis in paternity investigation and identification in criminal cases. The polymorphisms observed in the minisatellites allow the differentiation of individuals by evaluating the number of repetitions in a sequence that each person presents, depending on the number of systems used, this tool makes the DNA of each person something profoundly unique. From these findings, Jeffreys et al. developed the fingerprint DNA that through the southern blot technique generates a single band pattern for each person [1].

After the development of PCR (Polymerase Chain Reaction) samples with minimal amounts of biological material were successfully processed in the first laboratories of Forensic Genetics. These Laboratories quickly spread to the expertise units around the world, making it possible for these jointly employed techniques to revolutionize the forensic sciences with the ability to link suspects to crime scenes, not only by pointing out likely culprits but also by avoiding the conviction of innocents, a robust tool for the judicial system [2].

To use the information contained in DNA molecules in forensic investigations, it is necessary to separate the DNA from the other elements contained in the cell, such as proteins, lipids, carbohydrates, etc. in addition to external agents such as sand, sludge, pigments and other products, many of which can act as PCR inhibitors, preventing or hindering DNA amplification. The methods of extracting DNA are very varied, each with advantages and disadvantages according to the type of sample [2].

Several factors affect the ability to obtain a DNA profile. The first issue is sample quantity. The sensitivity of polymerase chain reaction-based (PCR) DNA typing methods is noteworthy but still limited. The second concern is sample degradation. Prolonged exposure of even a large blood stain to the environment or bacterial contamination can degrade the DNA and render it unsuitable for further analysis. The third consideration is sample purity. Most DNA typing methods are robust, and dirt, grease, some dyes in fabrics, and other substances can severely compromise the DNA typing process. Environmental insults will not change DNA allele “A” into allele “B,” but they can adversely affect the ability of the scientist to obtain a complete DNA profile from the sample [3].

Methods for extraction and purification of DNA from biological samples collected in vivo are well established and routine [3]. Sources may include blood, hair, hair roots, saliva, semen and so forth. Samples collected from post-mortem remains may also be analyzed, although they may sometimes be problematic. DNA break-down or diagenesis followed after death may advance rapidly in a time and environment dependent process. In many cases, the collection of peripheral blood samples is impaired, and viable material may be restricted to the soft and hard tissues. In hot and humid climates, skeletonization can occur in little as a few days. DNA may be fragmented and chemically modified. In such conditions, ‘low-template’ DNA analysis may be necessary [4].

The quantity of DNA recovered varies according to the source. In peripheral blood, for example, 20,000 to 40,000 ng/ml of DNA may be present and in semen 150,000 to 300,000 ng/ml. As sperm cells contain haploid DNA—having unpaired chromosomes, semen samples generally contain half the number of copies of a particular genetic target compared with their diploid counterparts found in most other cells of the body [4].

Hair and bone contain much smaller amounts of DNA. Plucked hair routes may yield 750 ng of DNA, whereas naturally shed roots may yield only 1 to 12 ng. Bone may yield 3 to 10 ng/mg of DNA depending on the bone condition, which under a range of conditions may be sufficiently weak that little or no DNA may be detectable—typically this amount will be below a threshold of about 1 ng of DNA [4].

Forensic DNA laboratories are experiencing a demand for processing an increasing number of cases and evidence samples due to the successful application of DNA technology to evidence collected from a wide variety of crimes. This demand has often resulted in large case backlogs that forensic laboratories have difficulty to manage it. To suitable the rising need for DNA analyses, the laboratories have sought methods to increase throughput. Robotic sequencers are routinely used to analyze amplified samples, and some forensic laboratories have implemented the use of liquid handlers for more efficient sample management. Also, the use of largescale automated DNA extraction instruments is becoming common in databasing laboratories and even some larger casework laboratories [5].

DNA extraction consists of chemically treating the biological sample to break the cell, purifying the DNA present in the sample by washing, removing the impurities and finally isolating the purified DNA. The correct handling and the methodology applied in this step have a direct influence on the quantity and quality of the DNA extracted [6,7].

According to Butler (2009), the organic method of DNA extraction consists of cell lysis through SDS (sodium dodecyl sulfate), DTT (Dithiothreitol) or EDTA

(Ethylene diamine tetra acetic acid) together with proteinase K. DNA purification through phenol and chloroform solvents in addition to the isoamyl alcohol. Finally, the purified DNA is recovered and hydrated by the addition of TE buffer or ultralow water. The organic method is one of the most used in the purification of DNA because of its high efficiency of delivering the well-purified and high molecular weight DNA, ideal for analysis of polymorphisms and also for PCR [8].

Despite the positives, the organic method uses many chemicals that are toxic, causing risks to those who perform them, the method also has many steps to follow and requires that the sample tubes be changed during the process, which makes it a time-consuming procedure with high chances of contamination or exchange of samples [8,9].

Through organic extraction, it is possible to perform differential extraction, which separates epithelial cells from sperm cells, a method that is very useful in cases of sexual violence, since it isolates the male and female DNA from the sample, which in most cases is mixed in these cases. The procedure is initially performed by rupturing the cell and nuclear membranes of female cells by incubating them in a mixture of proteinase K and SDS, while the sperm fraction is subsequently lysed through a mixture of SDS, proteinase K and DTT [10].

The differentiation occurs because the sperm cells do not undergo lysis without the presence of the DTT, which breaks the cell's nuclear membrane. Thus the female DNA is extracted first. It is worth noting that the differentiated extraction does not work in cases where the suspect underwent vasectomy because there were no spermatozoa [10].

Another popular manual method of DNA extraction is the CHELEX 100® protocol, where sterile water and proteinase K are used for cell lysis. Addition of a chelating resin called CHELEX at five percent and incubation at a temperature of 56°C for purification and centrifugation to occur DNA suspension, followed by incubation at 100°C to destroy the cellular proteins, after a last centrifugation the CHELEX and the residues cells are deposited at the bottom of the tube, leaving only the DNA in the supernatant [11].

CHELEX is composed of styrene-divinylbenzene copolymers containing iminodiacetate ions that bind to magnesium ions and hold it. This causes DNA-disrupting nucleases to be inactivated. It is a relatively inexpensive method and consists of a few steps and requires only one tube per sample; it is also a fast method that provides fewer chances of contamination or exchange between samples. However, if the concentration of the sample from which the DNA is to be extracted is high, the presence of PCR inhibitors may occur, and since the CHELEX method promotes DNA denaturation, resulting in only single strands of DNA, its use is indicated for PCR execution only [11,12].

FTA paper extraction is a practical method used for extracting DNA from blood or saliva samples. FTA paper is a particular paper, which was developed by Lee Burgoyne in the 1980s to store DNA. It is an absorbent paper that contains reagents that promote cell lysis, denaturation of cellular proteins and protects the DNA from external agents such as oxidation, UV rays and prevents the growth of bacteria. FTA extraction consists only of adding a drop of blood to the paper and allowing it to dry, cell lysis occurs at the time of contact with the paper, and the DNA is stored in the matrix of this paper, a cut of the paper is washed with solvent to removal of the PCR inhibitors and the supernatant can be sent directly to PCR. FTA paper is an advantageous option because it is easy to apply, the same paper can be subjected to more than one procedure (amplification and typing) and can be stored at room temperature for an extended period. Despite the advantages, it is not recommended to use the dry paper inside the tubes because of the static, which means that the papers do not remain inside the tubes [13-15].

Automation in the forensic laboratory routine is essential for fast and efficient processing of samples, as well as contributing to the elimination of errors. Dedicated compact instruments for low-medium labor demand are an excellent choice for laboratories with small spaces or with limited physical structure, without the need to redesign the department [5,14,16,17].

Many laboratories have developed technologies to automate various procedures in work with DNA, one of these technologies is the extraction of a solid phase of DNA, which has the concept of capturing DNA while performing several washes for its purification. In general, this method is fast, requires minimal contact with the sample and performs high yield extractions. The extraction of solid phase allowed the automation of DNA extraction using robotized pipettes and trays capable of working simultaneously with some samples ranging from 12-16 to 96 samples, combining this with the small number of steps for the extraction, the result is a gain in the execution time of the extraction. The automated extraction makes almost constant contact between the extraction person and the sample unnecessary, reducing the risk of contamination of the sample [5,14,16,18,19].

The most commonly used material for extraction in this method is silica. In the silica column method, a chaotropic salt breaks the hydrogen bonds of the DNA molecule in water and renders the denatured proteins and thermostable nucleic acids capturing the acids by the difference in charge between them and the silica. In acidic pH, the silica column can absorb amounts of DNA around 95%, and the impurities will be removed by several washing processes guaranteeing the absence of inhibitors

in the final result, with the alkaline pH the DNA elution takes place. The silica column method does not use carcinogenic compounds, which makes it a safe option for the manipulator [14,18].

Another method used in automation is paramagnetic resin, which is silica-coated magnetic beads. The cells undergo lysis through a buffer and at acidic pH small magnetic resin beads bond to the DNA, and a magnet draws them to the side or base of the tube to separate the DNA from the impurities. The impurities are withdrawn by extracting the remaining liquid from the beads, and the magnetic particles undergo further washing to purify the DNA better. DNA is released by heating the particles [2,18,19].

There are companies that have developed specific kits for extracting DNA from bones (one of the most challenging DNA sources because of the concentration of minerals, inhibitors, and presence of fungal and bacterial DNA from these samples spend a good deal of time in nature until they are found and collected) for use with machines employing magnetic particle technology [20].

Extraction by the automated method is a quick procedure because it does not use centrifugation or vacuum filtration, and the amount of DNA extracted depends on the capacity of the particles used [8,20]. The Qiagen Corporation has developed an automated method for DNA extraction involving the BioRobot EZ1 workstation and magnetic bead technology. The BioRobot EZ1 workstation is a small, rapid, and reliable extraction instrument that functions using pre-programmed extraction protocols and single-use reagent cartridges. The BioRobot EZ1 is capable of extracting high DNA quality from up to six samples in as few as 20 min using the chaotropic extraction with paramagnetic silica bead purification [5,18].

DNA extractions on the BioRobot EZ1 employ guanidine thiocyanate (GuSCN) guanidine hydrochloride (GuHCl) extraction method. These chaotropic agents lyse cells, denatured proteins, and inhibitory nucleases as well as promote the binding of DNA to the paramagnetic-silica beads. On the BioRobot EZ1, the binding of DNA to the silica beads and the wash steps occur within the barrier pipette tip. DNA bound to the silica beads is eluted in a solution of low ionic strength. [5].

This experiment aims to evaluate the efficiency, speed, convenience, and safety of DNA extraction from forensic samples using the EZ1 Advanced XL automated system with the EZ1 DNA Investigator Kit, from the evaluation of the results obtained from different samples using the quantification of DNA by real-time PCR and capillary electrophoresis. A protocol adapted for the use of the EZ1AdvancedXL® was also developed for the processing of samples of cases of sexual violence.

2. MATERIAL AND METHODS

The research was carried out in the Laboratory of Forensic Genetics (LGF) of the Technical-Scientific Police of Amapá (POLITEC-AP), which presents and uses in its routine of forensic analysis the equipment and reagents mentioned in this study.

Samples

A total of 500 real forensic samples were used, which were housed in the Biological Evidence File (BEF) of the Laboratory of Forensic Genetics of POLITEC-AP, consisting of 108 samples from cases of sexual violence (vaginal or anal secretion), 36 spots on swabs collected at a crime scene or instruments used for perpetrating crimes, 306 reference samples of blood or saliva collected with swab and 50 samples consisting of bones and teeth related to unidentified corpses.

Extraction of DNA

DNA extraction from the 500 samples was performed, 157 using the automatic method using Qiagen® EZ1 Advanced XL®, 71 by an organic method and 272 by CHELEX®. The option for real forensic samples allows us to evaluate how the different extraction methods recover DNA from potentially critical samples, with reduced amounts of DNA or degraded, which occurs daily in the Forensic Genetics laboratories of the expert units.

For swabs containing blood or saliva stains, the CHELEX 100® method was used, where the tip of the sampling swab was incubated for 30 minutes at room temperature in 1 mL of sterile water, after which the tip of the swab was manually pressed against the bottom of the tube for two minutes to loosen the cells, the tubes were centrifuged for 3 minutes at 12,000 rpm, then 975 µL of the solution was removed and the remainder lightly mixed, 200 µL of Chelex was added at five percent of proteinase K, this solution was mixed carefully with the pipette and incubated at 56 ° C for 30 minutes, after incubation the tube went to the vortex for five seconds and then centrifuged for 10 seconds at 12,000 rpm, at the end of the centrifugation the tube was incubated in a dry bath at 100° C for exactly eight minutes, after incubation the tube passed through the vortex for five seconds and finally centrifuged at 12,000 rpm for three minutes.

For samples of sexual violence (vaginal secretions and/or anal content) that were positively sorted for semen, the differential organic extraction method with DNA concentration on Microcon 100® membranes were used, the procedure was done by inserting the end with swab cotton in a microtube with 800 µL of Differential Lysis Buffer and 10 µL of proteinase K and incubating in a

water bath at 56° C for at least 2 h. After that, the tube was lightly homogenized in the vortex and centrifuged for one minute at 14,000 rpm. After centrifugation, the cotton swab end was pressed against the side of the microtube to recover the impregnated liquid. The supernatant (FNE - No Sperm fraction) was transferred to identified microtubes and stored in the refrigerator until step with chlorophane. At the bottom of the original tube, 500µL of Differential Lysis solution was added to the pellet (FE - Sperm fraction), lightly resuspended pellet and then centrifuged at 12,000 rpm for 5 min. After discarding the supernatant (repeat up to a total of 3 washes), it was added 500 µL of Differential Lysis Solution, 10 µL of Proteinase K and 20 µL of 0.39 µM DTT. The samples were then vortexed and incubated at 56 ° C for at least 2 hours.

After this second incubation, 400 µL of Chlorophane was added to the FE and FNE fractions of each sample and vortexed. Centrifugation at 13,000rpm for 3 min. Transfer of the aqueous phase to the concentrator unit (Microcon 100), containing 100 µL of TE. New centrifugation at 10,000 rpm until the volume has been filtered. Then 400 µl of ultrapure water was added to the concentrator unit and centrifuged at 10,000 G until the entire volume be filtered (repeat in total two washes). In the end, 50 µL of TE was added to recover the extracted DNA (Note: At this time change the microtube, vortex and turn the concentrating membrane). After centrifugation for 5 minutes at 1000 g, the DNA is extracted.

With the method using the EZ1 Advanced XL® different pre-treatments were carried out for the following samples:

I - A 200 mg fragment of material was used for the bone samples, each fragment was placed in a 2 mL microtube containing 600 µL of 0.5 M EDTA and incubated for 24 hours at 37° C. Then 20 µl of proteinase K was added and incubated at 56° C for three hours. The tubes were centrifuged at 6000 rpm for four minutes, 200 µl of the sample pre-treated was transferred to the EZ1® sample tube. The "trace" protocol was selected to the equipment.

II - The other samples using the DNA recovery using EZ1 were transferred to a 2 mL tube of the kit, 290 µL of the G2 buffer and 10 µL of proteinase K (both composed of the kit) were mixed in vortex for 10 seconds, the tubes were incubated at 56° C for 15 minutes in thermoblock and then were inserted into EZ1 following the "trace" protocol which is already predefined in the equipment.

III - For the cases of sexual violence that had samples extracted by the automated method with the EZ1, in addition to the protocol indicated above by the manufacturer, an adapted protocol was also used that uses the pre-treatment already described for the extraction of Differential DNA followed by isolation and purification of DNA by EZ1 (see Supplementary material).

DNA quantification

After DNA extraction from the samples, they were quantified using the Real-Time PCR technique, which is performed by adding 2 µL of DNA with 10 µL of the Plexor Master Mix®, 1 µL of the Plexor Primer Mix® and 7 µL of sterile water. The samples were amplified using BIORAD's IQ 5 Real-Time PCR equipment, the fluorescences used were FAM, CO560, CR610, and iC5 and the amounts of DNA were inferred using PROMEGA's Plexor Analysis Software program (insert reference).

Amplification by PCR

For PCR, 1 µl DNA, 2.5 µL Master Mix, 1.25 µL Primer Mix and 7.75 µL water were used. The optimum DNA concentration for amplification is 1ng/µL. With the results of the quantification, it is possible to know the amount of DNA of each sample. Samples with less than ideal DNA were concentrated, using 8.75 µL of DNA and adding no water to the PCR mix, samples with DNA above the ideal were diluted in sterile water. This adjustment in the concentration is essential for the normalization of the samples allowing obtaining a cleaner electropherogram, and that enables the identification of all alleles in all systems, saving Drop out for example. Autosomal genetic markers (D3S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, Vwa, D8s1179, TPOX, FGA) in addition to the sex indicating amelogenin loci were co-amplified by the PCR method using the Powerplex16 HS kit (Promega).

Capillary electrophoresis

The amplification products were subjected to capillary electrophoresis in ABI 3130 AvantMR (Applied BiosystemsMR) genetic analyzer and the genetic profiles obtained were analyzed with ABI Prism 3100 Avant Data Collection v2.0, 3100 and GeneMapper ID v3.2 software after reading the fluorescence.

For the capillary electrophoresis step, 1 µL of DNA plus a solution of 9 µL of a solution containing formamide, ILS and fluorophores were used.

It was determined that the lowest concentration of DNA needed to obtain a genetic profile was 0.02ng / µL, in which case 8.7 µL of the sample was used in the reaction, which would result in 0.174 ng of DNA in the PCR.

Statistical analysis

To compare the yield between the different DNA extraction methods, chi-square tests were performed by LxC contingency table and LxC partition with Yates correction using BioEstat 5.0 software [21].

3. RESULTS AND DISCUSSION

In this study, a total of 500 samples were used, divided into 157 samples extracted by automatic methods and 343 by manual methods. Out of the 157 samples extracted with EZ1 Advanced XL, 122 of them contained the minimum concentration (0.02 ng/µL) of DNA required to obtain a genetic profile and out of the 343 of the samples extracted by the manual methods, 264 presented sufficient DNA concentrations to obtain a genetic profile (Figure 01).

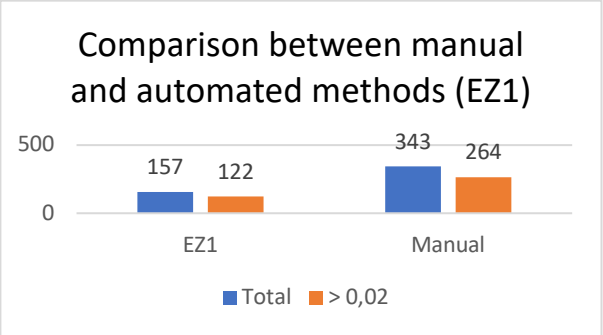

Figure 01. *Total samples extracted by each method and the number of samples from which it was possible to extract enough DNA to obtain a genetic profile (more than 0.02 ng / µL).*

Concerning the samples from cases of sexual violence, from a total of 108 samples, 24 were processed using the EZ1 platform following the manufacturer's protocol, with the use of 8 (33%) samples with minimum concentrations to obtain a genetic profile. In 48 samples the automated method was used with EZ1 but with the adapted protocol, resulting in 45 samples with DNA amounts above the minimum necessary for the amplification, yield of 93.75%. In 36 samples that were processed using the organic extraction method, 26 (72%) presented minimum concentrations to obtain a genetic profile. These results indicated a statistically excellent use of the automated method using the EZ1 platform with an adapted protocol regarding the other methods used. Comparing the results obtained by the chi-square test with Yates correction between the extraction by the automated method with the EZ1 following the manufacturer's protocol and by the automated method with the EZ1 and an adapted protocol, it was observed that the differences were statistically significant ($\chi 2 = 4.417$, FD = 1, p = 0.0356) (Figure 02).

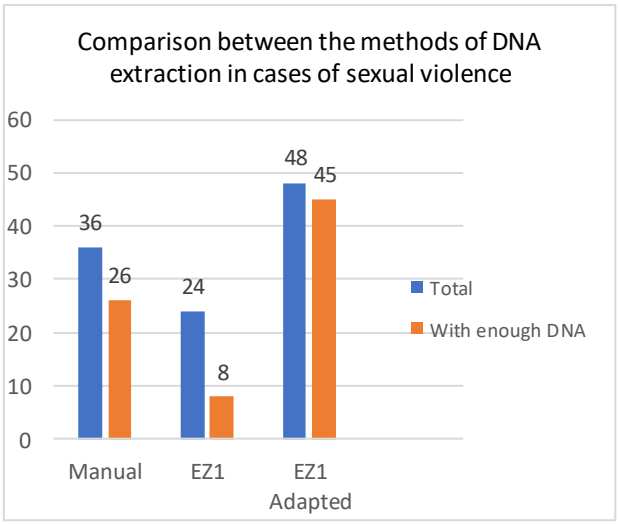

Figure 02. *Comparison of the DNA extraction yield in cases of sexual violence by three methods.*

Samples from stains found at a crime scene totaled 36 samples. 21 samples were submitted to extraction by the automated EZ1 platform, and it was possible to extract enough DNA from 14 of these samples. 15 spot sampes passed through the organic method of phenol/chloroform and concentration in microcon 100 membranes, 12 of them had enough DNA to obtain a genetic profile. Here, the applied manual method was 80%, against 67% of EZ1 (Figure 03).

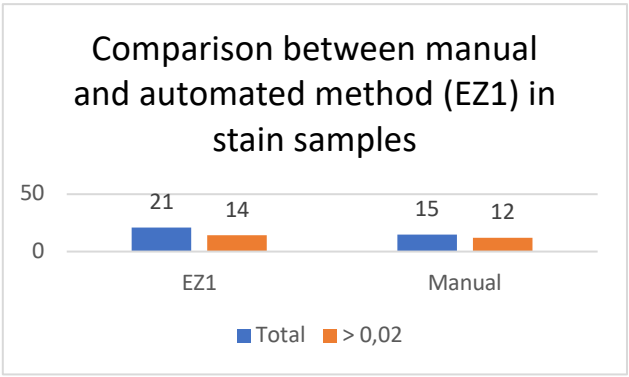

Figure 03. *Samples from stains found at crime scenes from which enough DNA was extracted to obtain a genetic profile (more than 0.02 ng / µL).*

The results of the extraction of DNA in the samples of cases of sexual violence and bloodstains by automated method using the EZ1 platform following the manufacturer's protocol presented lower results than those observed through the manual method, this result can be partially explained in function of that the "Trace", "Tip

Dance" and "Large Volume" protocols, which are preset extraction protocols employed in EZ1®, uses sample volumes between 200 and 400 µL, while the organic method uses large sample volumes, reaching 800 µL or more. This limitation in the volume of the sample may lead to reduced use of the DNA present in the sample and lower results in the concentrations obtained by the automated method.

The low utilization in extracting DNA from samples of sexual violence using EZ1 using the manufacturer's protocol had already been described by Carey et al. (2011) who compared three different automated extraction platforms, including EZ1 and although not observed significant differences in the performance of the three devices in most of the samples, observed a low use of EZ1 in the extraction of a semen sample. According to the authors, a sample called type 9 (Semen), the EZ1 Advanced XL yielded an average DNA concentration of 0.14 ng/µl compared to the AutoMate Express at 1.40 ng/µl and the Maxwell 16 at 1.53 ng/µl. The DNA Investigator handbook does not contain a specific protocol for neat semen. Instead, the "sperm fraction" portion of the differential extraction protocol was used. The absence of an optimized protocol for processing neat semen or semen stains with the EZ1 Advanced XL may explain this difference and could be remedied with the development of a dedicated protocol [22].

In an attempt to improve the performance of DNA extraction in cases of sexual violence using the automated system EZ1, the technique described by the manufacturer was modified by changing the pretreatment. Instead of employing the reagents indicated in the protocol, extraction was performed employing the differential method using differential lysis buffer (Sperm Wash Buffer: 10mM Tris HCl, 10mM EDTA, 50mM NaCl, 2% SDS, pH 7.5), proteinase K, SDS and DTT, which initially promotes the differential rupture of the female cells and subsequently, after washes to remove the female DNA present in the sample, promotes the breakdown of sperm cells under the action of DTT. In the original manual protocol, after this pretreatment, the sperm and non-sperm fractions would have their DNA purified using a step of organic extraction with phenol-chloroform-isoamyl alcohol (chlorophane) followed by concentration in membranes type microcon-100.

In the automated method using the adapted EZ1 platform, instead of following organic extraction and membrane concentration, after pretreatment, the samples were then extracted using the EZ1 DNA Investigator KIT on the Qiagen® EZ1 Advanced XL® automated platform. The results obtained using this automated extraction method named EZ1 with adapted protocol were significantly superior to the manual methods (differential extraction) and automated with EZ1 following the manufacturer's protocol, as can be seen in figure 03. The protocols for the preparation of the "Sperm Wash Buffer "as well as, the procedure for differential DNA extraction is attached (Supplementary Figures 01-02).

Also, 306 reference samples were used. 34 were extracted by EZ1, which was able to extract enough DNA from 31 samples. 272 of these samples were extracted with Chelex 100®; sufficient DNA could be obtained in 212 samples. With reference samples, the manual method yeild was 78%, while EZ1 was successful with 91% of samples (Figure 04).

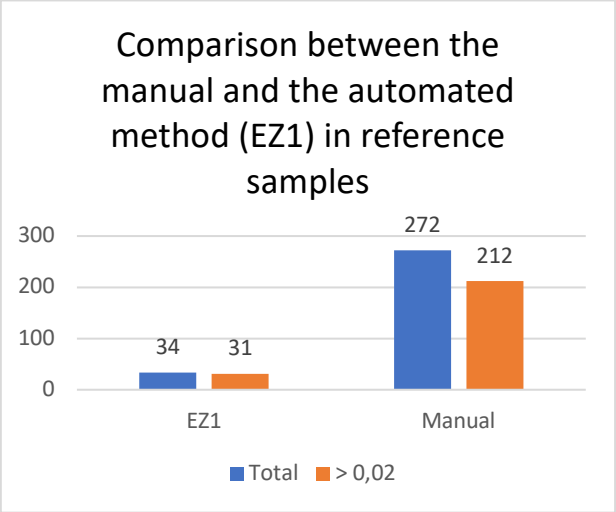

Figure 04. *Representative graph of the reference samples from which it was possible to extract enough DNA to obtain a genetic profile (more than 0.02 ng/µL).*

As can be seen in the above graph, the automated method of extracting DNA using EZ1 was significantly superior to the CHELEX manual method for DNA recovery from reference samples. Although the average amount of DNA obtained is higher in samples using CHELEX, the use of samples extracted by the automated method (EZ1), given the greater homogeneity of the DNA concentrations obtained. This quantitative normalization of the DNA recovered by the automated method described is an additional advantage that may even dispense with the need for DNA quantification before PCR, thus reducing the cost of the examination.

Fifty samples from bone and teeth were used, of which 30 were extracted using the automated protocol indicated by the manufacturer using the EZ1 platform, resulting in 24 samples with minimal amounts of DNA capable of producing a genetic profile. 20 of these samples were submitted to organic extraction, where it was possible to obtain profiles from 14 of them. In this case, we have the automated method with 80% of use against 70% of the organic method.

It is noteworthy that the bone and tooth samples used in this experiment came from real cases, some of which resulted from exhumation or material filed in the Forensic Anthropology sector of POLITEC-AP for many years, which would naturally bring additional complications to the recovery of a genetic profile from these samples (Figure 05).

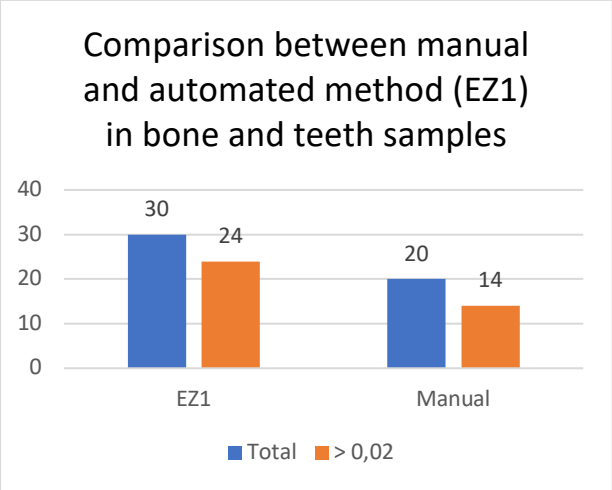

Figure 05: *Representative chart of samples from teeth and bones from which it was possible to obtain a genetic profile (more than 0.02 ng / µL).*

Regarding the bone and tooth samples, although the extraction using the automated method using the EZ1 platform, presented a higher percentage of recovery of the recovered DNA, these results were not statistically significant. On the other hand, the automated method was significantly superior concerning the speed and safety of the protocol used, besides the fact to eliminate the use of chlorophane that entails environmental risks and to the health of the professional who is processing the material.

In the experiment conducted by Ip et al. (2015), which aimed to compare the results of different extraction methods, among them the Chelex 100® method and automated extraction methods, the methods using silica column were able to extract higher concentrations of DNA than the other methods. In the experiments carried out in the POLITEC-AP laboratory, the total DNA average obtained with EZ1 was 1.59 ng / µL whereas that of the manual methods (chelex® and organic with microcon®) was 9.3 ng / µL. We believe that the difference in concentration between the two methods is due to the way the EZ1 extracts, which is using magnetic beads, where it is necessary the availability of free area on the beads surface so that the DNA molecule binding occurs, that is, if the beads are already saturated, the additional DNA present in the sample will be discarded [23].

The ideal DNA concentration of the samples is 1ng/µL for amplification and genotyping. Concentrations far above the ideal can generate an electropherogram with many noises and leakage of fluorescence, while concentrations far below may make it impossible to identify some alleles in the electropherogram (Drop out) as a function of preferential amplification resulting from stochastic effect. Therefore, when comparing the DNA averages extracted by the two methods, we can say that EZ1 also has an advantage in this aspect, since the average concentration obtained was 1.59 ng / µL against 9.2 ng / µL and 9.4 ng / µL of the methods by Chelex 100® and organic extraction respectively.

Still according to Ip et al. (2015), the magnetic particle extraction method, which is the same as that employed by the EZ1 Advanced XL, is advantageous for the time saving it offers in relation to methods such as the organic, because it contains a small number of steps and the possibility of working with several samples at a time. This fact was verified in the laboratory of POLITEC-AP, since after the pretreatment of the samples it was possible to obtain DNA extraction from 14 samples in the time of 20 minutes, while the steps of the organic method can take up to more than one day for the extraction of DNA from the same number of samples [23].

According to Cândido (2013), magnetic particle-based DNA extraction platforms are efficient for extracting DNA from teeth and bone samples, confirming the results observed in the present study that recovered an average of 2.3 ng / µL of DNA from these samples, a result more than one hundred times higher than the minimum indicated as capable of obtaining a genetic profile [20] (Supplementary Figure 03).

The manual extraction methods, under normal conditions, consume longer time. The CHELEX 100® method takes about two hours while the organic method takes between 5 to 24 hours to obtain the purified DNA. In contrast, the automatic extraction using the EZ1® platform takes around 20 minutes in the purification phase. In the three methods mentioned above, samples may be subjected to pretreatment ranging from 15 minutes to 24 hours (Tables 01 – 02).

CONCLUSION

The automated method used following the manufacturer's protocol or following protocol adaptation showed superior quantitative and qualitative results than those obtained using manual methods, particularly in reference samples, teeth, and bone samples, and cases of sexual violence requiring lysis to separate the male DNA from the female DNA.

In some manual methods, it is necessary to handle toxic agents, as in the case of organic extraction, in which case the automated method employing EZ1 is also advantageous. The fact that the automated method presents fewer steps that require the direct contact of the expert/technician with the samples allows a significant reduction of the risks of some exchange, contamination or intoxication.

In this way, the extraction of DNA using Qiagen © EZ1 Advanced XL® proved to be efficient, easy to process and safe for both the operator and the reliability of the obtained results. One of the great advantages is minimal human interference during most of the procedure. Without considering the pre-treatment of the samples that is variable, the DNA purification processing employing the EZ1 requires around 20 minutes for 14 samples, which proved to be much faster than the other methods tested.

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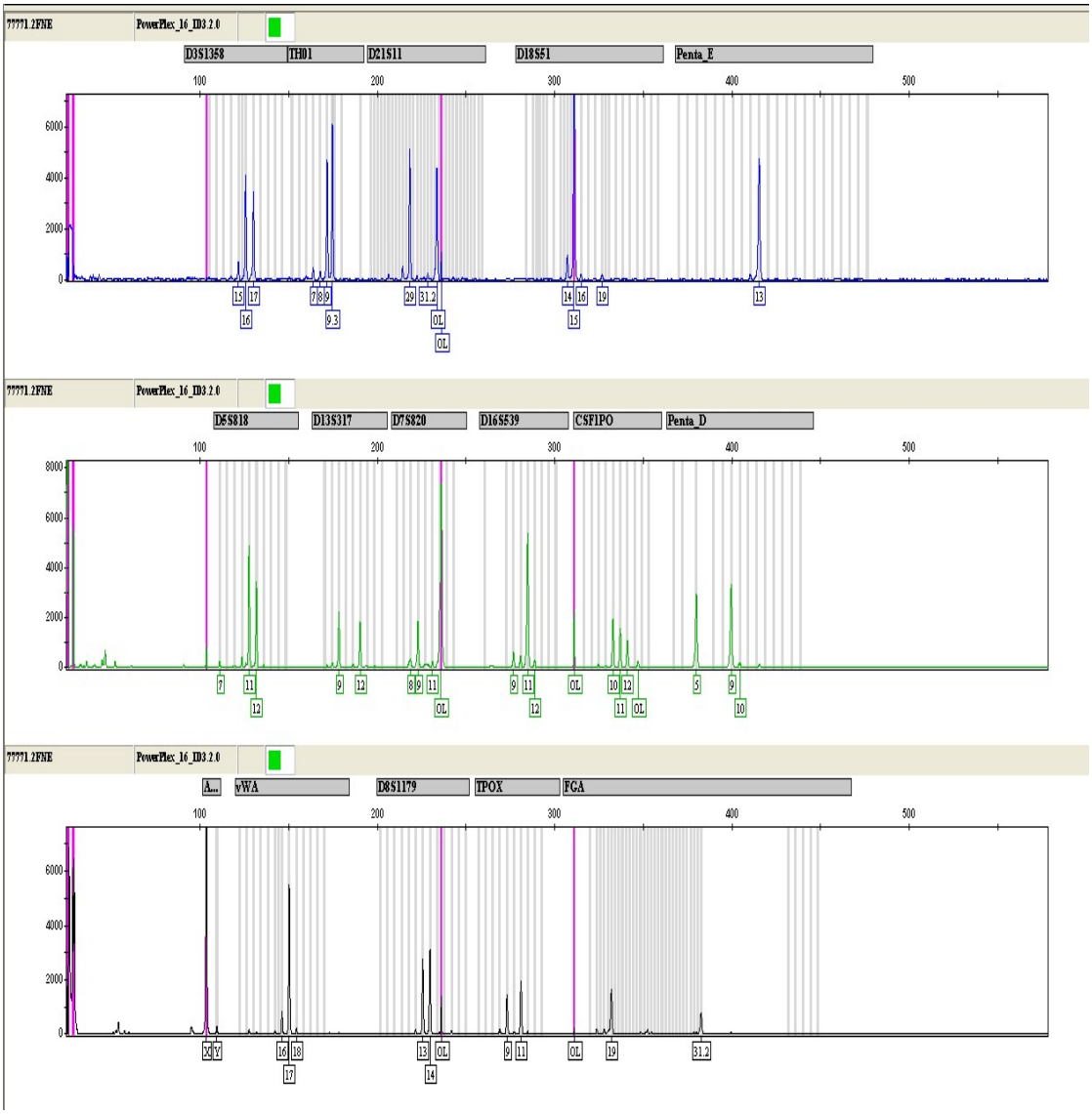
Table 01. *Table containing the number of samples used, and their subdivision as to the origin of the sample, types of extraction used and the number of samples that delivered enough DNA to obtain a genetic profile.*

Concentration of recovered DNA	ANALYZED SAMPLES								
	Stain		Sexual Violence			Reference		Bones and Teeth	
	Chelex	EZ1	Organic	EZ1	EZ1*	Chelex	EZ1	Organic	EZ1
>0.02 ng/µL	12	14	26	8	45	212	31	14	24
<0.02 ng/µL	3	7	10	16	3	60	3	6	6
TOTAL	15	21	36	24	48	272	34	20	30
	36		108			306		50	

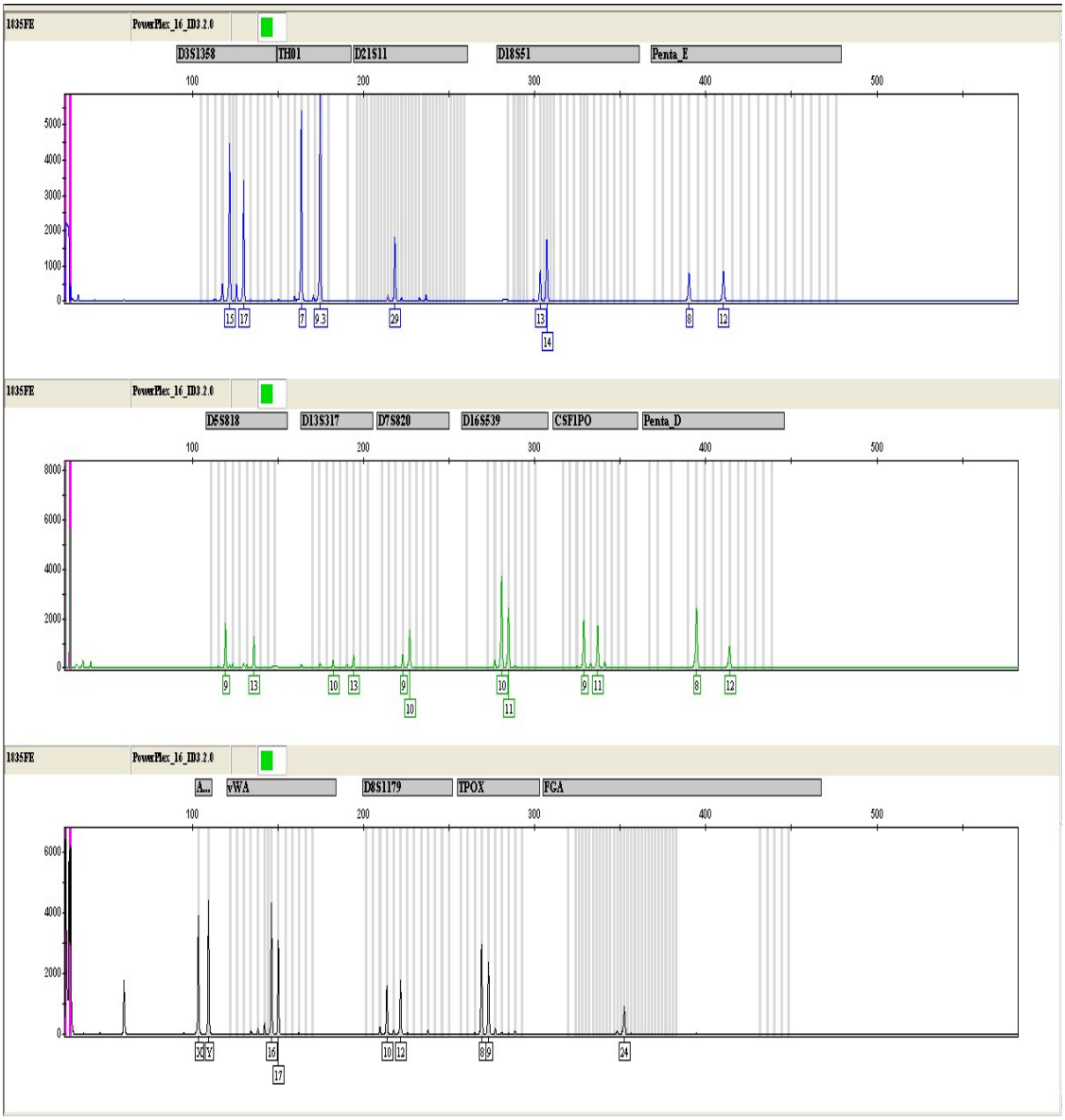
** EZ1 platform with adapted protocol.*

Table 02. *Comparative table of the general aspects of extraction methods.*

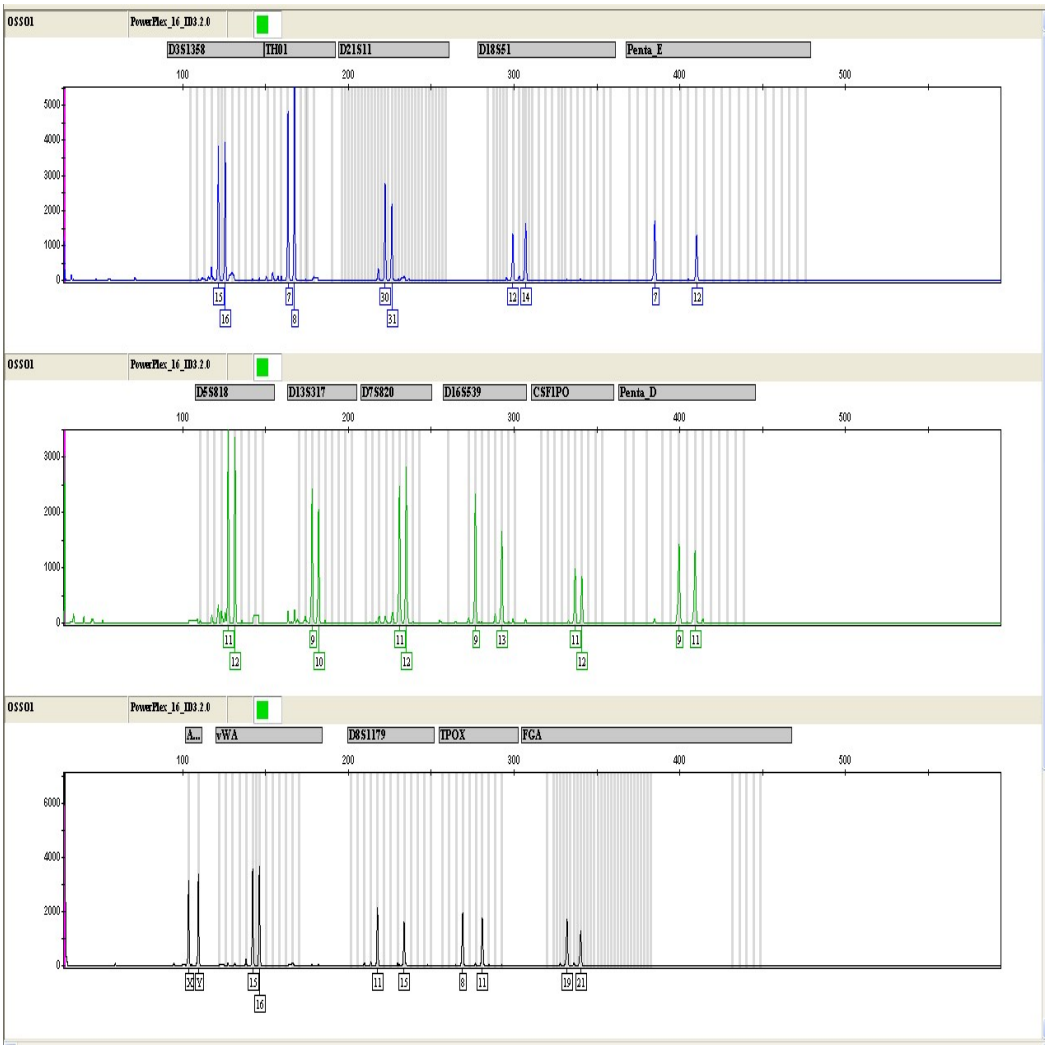
COMPARATIVE	EZ1	CHELEX 100®	ORGANIC
Pretreatment	15 minutes to 48 hours		
Extraction time (min.)	20	90	240 ~ 300
Differential extraction	Yes	No	Yes
Cost per sample (R$)	60	0,25	55
Health risk	Low	Low	High
Risk of contamination	Low	Medium	High
Exchange risk	Low	Medium	High
Concentration (ng/µL)	1,59 (Medium)	9,2 (High)	9,4 (High)



Supplementary Figure 01. *Electropherogram of DNA extracted from a sample of sexual violence using EZ1®.*



Supplementary Figure 02. *Electropherogram of DNA extracted from a sample of sexual violence using organic methods.*



Supplementary Figure 03. *Electropherogram of DNA extracted from the bone samples using EZ1.*