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Avoiding Errors and Pitfalls in Evidence Sampling for Forensic Genetics

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1. Introduction

DNA fingerprinting or DNA profiling (as it is now known) was first developed by Alec Jeffreys in 1985 (Jeffreys et al., 1985), who found that in the human genome, some regions contained DNA sequences that were repeated over and over again, next to each other. He also discovered that the number of repeated unit could differ from individual to individual allowing human identity testing. Since that time, DNA typing methods has been commonly used in criminal cases (to identify a suspect or a victim or to absolve an innocent individual) as well as in the identification of missing persons or in paternity testing. Today, the most commonly used DNA repeat regions used are microsatellites also known as Short Tandem Repeats (STR). These loci in which the repeat unit is at least two bases but no more than seven in length, are amplified by PCR (Polymerase Chain Reaction) in a multiplex fashion (multiple primers) reducing sample consumption. Today, for the majority of forensic cases where DNA of preserved quality is available, the identification procedures of biological samples are performed by commercially well-validated kits incorporating 15-16 highly variable STR loci (plus amelogenin) such as PowerPlex[®] (Promega) and AmpF/STR[®] (Applied Biosystems). With highly automated equipment, STR profiling can process hundreds of samples each day and became the cornerstone of forensic DNA testing, including national DNA databases with STR-profiles of convicted felons. Nevertheless, it is of great importance to make the distinction between the samples containing large quantities of high quality DNA and those containing minute amounts of DNA and/or poor quality molecules. If for the first type of samples, the occurrences of errors or pitfall are rare, in the second type, the interpretation of the allelic profiles should be done with care and caution.

In this article, the authors will focus on the analysis of challenging samples, in other words, samples containing either (i) minute amount of DNA or (ii) degraded DNA or (iii) mixture of DNA or (iv) DNA polymerase inhibitors or (v) contaminating DNA molecules. Indeed, DNA is stable and remains intact when stored in a dry or frozen state but will be degraded when stored under inappropriate or bacterially contaminated conditions. Two types of damage are mainly likely to affect DNA over time: hydrolytic and oxidative damage. Hydrolytic damage results in deamination of bases and in depurination and depyrimidination, whereas oxidative damage results in modified bases (Lindahl, 1993). Both mechanisms reduce the number as well as the size of the fragments that can be amplified by PCR. Failure to amplify DNA may also result from the presence of inhibitors that interfere

with the PCR such as low-molecular-weight compounds, supposedly derived from the crime scene environment, which coextract with the target DNA molecules and potentially inhibit the activity of the DNA polymerase (Keyser-Tracqui C. and Ludes B., 2005). Contamination by DNA coming from outside the case represents one of the major limitations to DNA analysis. The authors will describe the strategies developed to overcome the difficulties which begin with the biological sample collection.

2. Biological sample collection

2.1 Samples

Various kinds of samples can be typed with the PCR-based methodologies such as:

- Blood samples and blood stains
- Cigarette butts (Hochmeister et al., 1991)
- Human hairs with a special mention of the possibility of analysis of single hair (Higuchi et al., 1991)
- Urine samples and urine stains (Brinkmann et al., 1992)
- Fingernail scraping (Wiegand et al., 1993)
- Bite marks (Sweet et al., 1997)
- All kinds of touched objects (Van Oorschot and Jones, 1997) such as tools, clothing, firearms, parts of vehicle, food, condoms, glass, bottles, lip cosmetics, wallets, jewellery, paper, cables, stones and construction material (Van Hoofstat et al., 1999; Webb et al., 2001; Wickenheiser, 2002; Ruddy, 2002; Polley et al., 2006; Petricevic et al. 2006; Sewell et al., 2008; Horsman-Hall et al., 2009)
- FTA cards can be used to collect blood or saliva in order to assure a better preservation of the DNA molecules by the specific fixation on the treated card paper
- Teeth and bone tissues as well as burnt tissues

Touched objects provide a wide scope for revealing the offender's DNA profile in investigations of offences including theft, burglary, vehicle crimes, street robbery, drug cases, homicide, rape and sex offences, clandestine laboratories, armed robbery, assaults, crime. The positive DNA identification from those samples allowed the creation of national offender databases (Harbison et al., 2001; Gunn, 2003; Walsh and Buckleton, 2005; Gill et al., 2000; Whitaker et al. 2001) to identify serial offenders and criminals.

2.2 Collecting methodologies

One of the best methods to collect trace samples is the use of swabs after having identified as precisely as possible the areas to target. The first step is to swab the hole defined surface by one or several moistened swab multiple times with some pressure and rotation given to the swabs. The second step is to complete the swabbing by the application of dry swabs to recapture the moisture containing hydrated cells. Co-extraction of these swabs to enhance overall retrieval of DNA is recommended (Castella and Mangin, 2008; Sweet et al., 1997; Pang and Cheung, 2007).

The moistening agent can be sterile water, 0, 01% sodium dodecyl sulphate (Wickenheiser, 2002) or isopropanol (Hansson et al., 2009). The quantities of cellules retrieved depend also of the physical characteristics of the surface (Wickenheiser, 2002) and the use of different moistening agents for different surfaces may facilitate collection. The quality of the swabs is also important, the quality should be DNA-free; cotton swabs are the most frequently used but other types such as foam may also be considered (Wickenheiser, 2002; Hansson et al.,

2009; 57, 111, 112). It has been shown that the yield of DNA from moist or frozen swabs are higher than from dried swabs. After collecting the biological material from a surface it is recommended to process the swab in the laboratory. If these conditions are not available, the swabs must be frozen immediately after collection.

According to some authors, tape is the best way to retrieve DNA containing material from worn clothing or from touched surfaces without collecting in the same time inhibitory factors present on this material (staining chemicals and/or color denim). By pressing a strip of tape multiple times over a target area, the most recently deposited material, with fewer inhibitory factors, are collected. In our experience, this method is not often used and should be replaced by the easiest way to collect DNA such as cutting away stain fragment samples.

To isolate relevant target cells from other over-whelming cell types, laser microdissection techniques were used. The different cell types can be recognized by morphological characteristics, various chemical staining or fluorescence labeling techniques. These methods allow to establish a clear DNA profile from few cells present in a mixture samples that otherwise had not be detected while swabbed by the major component and not detectable in the profile (Elliott et al., 2003; Anslinger et al., 2005; Anoruo et al., 2007; Sanders et al., 2006). With laser micro dissection techniques (Anslinger et al., 2007; Vandewoestyne et al., 2009), it has been shown that cells derived from a male contributor can be analyzed separately from those derived from a female contributor after morphological or fluorescent labeling identification. For this method, coated glass slides are required and a sample must be transferred from the collection material to the slide. As cells could be lost during this transfer, it would be preferable to use actually laser microdissection methodology is directly used on the initial collection material.

3. DNA analyses

3.1 DNA extraction

The classical ways of DNA extraction from forensic routine case work were the organic methods and sometimes the use of resin like Chelex 100R Bio-Rad (Walsh et al., 1991) which may induce the molecule degradation during long storage periods. Actually, in cases of degraded samples or when only minute amounts of DNA are available, the use of silica-coated magnetic beads to capture the molecules from the rest of the lysed cells is recommended. These extraction procedures are also performed in some laboratories by robotic systems (Greenspoon et al., 2004; Frégeau et al., 2010). The loss of DNA during the extraction step could be linked to the substrate sustaining the sample. Nevertheless, this loss is principally linked to the used methodologies namely the organic extraction techniques. The majority of samples submitted for analyses contain relatively large amounts of DNA, above the 0.1-0.5ng minimum required by most common STR profiling systems. Below this amount, specific methods like those used by molecular anthropologists on ancient DNA samples must be developed.

The optimization of the extraction methods involves:

- The extraction of all the available DNA;
- To remove all amplification inhibiting elements without the loss of DNA;
- To amplify all the extracted molecules with adding the amplification reagents to the device containing the DNA rather to add the DNA to the amplification tube and to loose molecules in pipette tips or on the tube walls ;

3.2 DNA quantitation

It seems not necessary to quantitate all the samples in particular highly degraded samples or trace samples given the expected low concentration of DNA. The only advantage lay in having an indication of the approximate quantity present in order to prevent repeat analyses of over-amplified samples and when interpreting the profile. It must be emphasized that a negative quantitation result should not prevent to process the samples. With the real-time quantitation method applied on low template samples, the results should be taken as an indication of the concentration and not as an absolute measurement as with higher DNA amounts. In criminal cases, it is of common practice to retain a certain amount of the samples for the future further typing by a second laboratory as a cross examination.

3.3 DNA amplification

For samples containing enough DNA of high molecular weight, the classical technics of DNA extraction can be performed without pitfall, appropriate technologies were developed to increase the chance to obtain useful profiles from very minute DNA samples such as the low copy number (LCN) procedure with extra cycles or low template DNA (LTDNA) methods. Minute samples or trace DNA refers to samples where only 100pg to 200pg of DNA could be extracted according to different authors. These methods increased the possibility to amplify successfully DNA from trace scene samples (McCartney, 2009; Budowle et al., 2009). Difficulties can be raised in the interpretation of those profiles where the peak heights may be below a validated threshold level.

During this step, the exponential amplification of DNA results in the production of billions of copies of the template molecule. So every DNA contamination will be also amplified and can false the result and on the other hand the excess of DNA produced by the PCR will be present either on the machines used but also in the surrounding environment such as the air and the work surfaces. To avoid these contaminations, all the steps of the analyses (pre-PCR, PCR itself, post-PCR) must be performed in physically separated laboratories.

The step of amplification is a very critical one and was optimized for low level template amounts. Amplification is the main field where the biologists must have control of the quality of the molecule. To enhance the success of trace DNA amplification, it was proposed to increase the number of cycles (Gill et al., 2000). The number of cycles used during the PCR of the STR loci is increased to 34 compared to the standard 28 cycle reactions. In molecular anthropology and in ancient DNA work, the number of cycles could be increased up to 60 in order to maximize the success of amplification (Rameckers et al., 1997). Numerous authors have described the efficacy of increasing cycle numbers ((Gill et al., 2000; Whitaker et al., 2001; Kloosterman et Kersbergen, 2003). Complete profiles with substantial increases in peak heights have been described (Gill et al., 2000) but contaminating DNA may also be amplified through enhancing the number of cycles. When the sensitivity is increased, more sporadic contamination will be detected and the laboratories must enhance the stringency of contamination prevention. "Mini-STR" kits were developed containing redesigned primers which had significantly higher success rates with degraded DNA due to smaller amplicons. The minifiler STR kit[®] produced by Applied Biosystem showed a higher success rate with degraded or inhibited DNA than the classical kits and requires also a lower template input approximately 0.125 ng compared to 0.5ng (Mulero et al., 2008). The optimization of the multiplex with the increased priming and amplification efficiency of the new primers can explain the better sensitivity of the amplification.

The efficiency of the amplification reaction can also be increased by the addition of chemical adjuvants such as bovine serum albumin (BSA). BSA is known to prevent the inhibition of the activity of Taq polymerase by sequestering phenolic compounds which otherwise scavenge the polymerase (Kreader, 1996).

3.4 Detection of amplified product

To increase the detection of amplified product, methods have been developed to purify the PCR amplicons, to remove salts, ions and unused dNTPs and primers from the reaction by using filtration (Microcon filter columns), silica gel membranes (Quiagen MinElute) or enzyme hydrolysis (ExoSAP-IT) (Forster et al., 2008; Petricevic et al., 2010; Smith and Ballantyne, 2007)). This purification step is performed to remove negative ions such as Cl⁻ which prevents inter-molecular competition occurring during electrokinetic injection allowing a maximum amount of DNA to be injected into the capillary of the sequencer. To enhance the quantity of DNA available for the detection, it is also possible to concentrate the PCR product during the purification process.

3.5 Difficulties of the typing of trace DNA

The side effect of increasing the ability to amplify the DNA molecule and in particular minutes amounts of material is the increased likelihood of contamination being detected and of artifacts of the amplification process due to stochastic effects.

Four major cases of interpretation difficulties can be summarized:

- Allele drop-out is due to a preferential amplification of one allele at one or more heterozygous loci. This kind of pitfall is relatively frequent when very low quantities of DNA are amplified (Whitaker et al., 2001; Gill et al., 2000; Gill et al., 2005; Lucy et al., 2007). The interpretation of profiles obtained from minutes amounts of DNA must in each case take in account the possibility of an allele drop out.
- Allele drop in, this occurrence is due to amplification artifacts such as stutter. This artifact may be also frequently seen in the analyses of trace DNA amounts (Whitaker et al., 2001). When stutter alleles are present in a STR profile it is rather difficult or impossible to characterize the number of individuals having their DNA in the sample and assigning of alleles within a mixture.
- Allele drop is due to sporadic contamination occurring from various origins such as crime scene, sampling, non DNA free material or at the laboratory work.
- A decreased heterozygote allele balance within a locus and between loci. In this feature, the peak height imbalance within and between loci are due to the same amplification effects that cause drop-out. In those cases, the evaluation of the zygosity at a particular loci may be extremely difficult.

No methods can actually eliminate completely artifact product during the amplification step in particular when the DNA is degraded or present in minute amounts but their occurrence should be statistically evaluated. To be able to develop such an approach it is of importance to understand the factors that may cause each type of artifact and the accurate data regarding the frequency and scale of their occurrence. Benschop et al. (2010) present one of the first large-scale efforts to characterize artifacts generated by different trace DNA amplifications. These authors showed also their investigations to highlight an effective method to generate a useful consensus profile.

3.6 Pitfall at the interpretation step

For each profile interpretation, the sampling of biological material found at the crime scene must be replaced into context and the possibility of pitfalls should be taken into account such as the possibilities of material transfer, the difficulties of the amplification process and the possibility of artifacts affecting the true result. This interpretation carefulness is of particular importance when the analyses are performed on degraded or very low quantities of DNA and has to consider imperatively the four most common features which can occur in those cases: allele drop-out, allele drop-in, stutter bands, contamination and decreased heterozygote balance. Strict interpretation guidelines can give reliable and robust result and minimize these pitfalls.

The introduction of detection thresholds may give a reliability of DNA profiles interpretations in particular for degraded DNA or minutes amounts of DNA. The background noise is generally eliminated by the establishing a threshold of 50 RFU. In order to avoid false homozygote by allelic drop-out, separate thresholds were established referred to as the low-template DNA threshold T , the match interpretation threshold (Budowle et al., 2009), the limit of quantitation (Gilder et al., 2007) is set at 150-200 RFU. The allele peaks should be above this limit to be sure that it is a true homozygous but even the respect of this limit may not prevent allele drop-out in all cases. Other authors (Gill and Buckleton, 2010) have recommended that instead of thresholds, a more continuous measure should be used which is modeled on the risk of dropout based on peak heights.

One of the most used methods to eliminate incorrect genotypes is to replicate the amplifications reactions and to generate consensus profiles (Whitaker et al., 2001; Gill et al., 2000; Benschop et al., 2010; Taberlet et al., 1996). But currently, no consensus has been found on either the minimum number of replicates needed or how frequently one needs to observe an allele within the number of replicates conducted to be sure that the found allele is a true one. Benschop et al., (2010) consider that four replicates for degraded or very low amounts of DNA may be the most appropriate rules for considering a profile as a true one.

Gill et al. (2000) proposed a statistical model, mentioned by other authors (Balding and Buckleton, 2009; Gill and Buckleton, 2010; Curran, 2005), which provides the necessary probabilistic methods where the probability of observing the evidence profile can be combined with prior knowledge regarding dropout, the number of potential contributors, the possibility of contamination and other factors (Van Oorschot et al., 2010).

3.7 Mixture interpretation

A particular mention must be made for DNA mixture interpretation. In fact mixed samples are by definition composed of one or more major contributors with high quantities of DNA and with a minor contributor present only at trace levels, in other cases, the contributors are all present at trace levels. A profile can be falsely identified as a false mixed samples when high stutter peaks are present indicating that the DNA is coming from multiple individuals although it truly derive from a single source. In mixed samples, the high probability of drop-in, drop-out and increased stutter bands avoid the precise determination of the number of contributors and the separation of the genotypes at any given locus. This is frequently the case in degraded DNA or when the DNA is present in very few amounts (Walsh et al., 1996; LeClair et al., 2004; Gibb et Huell, 2009).

In such cases, the amplification reaction is also source of bias and pitfalls in over-amplification of some alleles and allowing a dropping-out of minor contributor's alleles at some loci.

Recommendations were published by the International Society of Forensic Genetics on mixture sample interpretation (Gill et al, 2006). A likelihood ratio (LR) approach was proposed for the interpretation for low template level mixture with the incorporation of an assessment of the probability of allele drop-in and drop-out in such cases.

Bright et al. (2010) proposed the use of the heterozygote balance and average peak heights at each locus to calculate the mixture ratio and distinguish among the contributors' genotypes (Van Oorschot et al., 2010).

For all these reasons, interpretation of mixture samples must be done very carefully particularly in cases where DNA is degraded or present in few quantities.

4. Contaminations issues

Contaminations are the major pitfall in the analyses of DNA in the forensic field either in producing valuable profiles or in accurate interpretation of the results. This is a major issue when the samples are degraded or when the DNA molecules are present in minute amounts. Contaminations may appear in every step of the analysis process from the sampling on the crime scene to the laboratory work. Ruttly and Graham (2005) highlight that the contaminations can occur on the body itself or during the sampling of the evidences, at the scene of the crime, during the transportation of the body to the mortuary, at the autopsy room and after, of course, during the laboratory procedures.

At the crime scene, one of the more frequent situation where contaminations of the crime scene can occur if the individuals who entered the scene speak or caught and handle evidences over the corps before the arrival of the forensic investigative team. Ruttly and Graham (2005) described airborne DNA contamination in mortuaries.

Methods were described in order to avoid the possibility of contaminations:

- To perform analyses about the persistence of DNA on different kinds of surfaces in various environmental conditions (Toothman et al., 2008; Ruttly et al., 2003; Cook et Dixon, 2007);
- To improve and standardize the sample collection methodologies in order to improve the targeting of the samples and to decrease unwanted underlying DNA;
- To collect the profiles of all the persons involved in the collecting and laboratory steps to recognize a contamination coming from these professionals;
- Some laboratories require samples from the area immediately adjacent to the target area to have a so called "blank sample".

The operating procedures on the crime scene must be precisely fixed to minimize the possibility of contaminations (Ruttly et al., 2003):

- To avoid breathing, talking and of course coughing during the sampling step in restricting the access of non specialist investigators to the scene;
- The use full-body scene suit (to avoid contamination by cell shedding coming from exposed areas of skin), hood, hair net, gloves and mouth masks by all the investigators in charge of the sampling step;
- To avoid direct touching of the evidences containing the DNA and changing gloves and masks regularly at the crime scene and obviously in the laboratories;
- All the results are compared against the database containing the DNA profiles of all the persons who were involved in all the steps of the sampling and laboratory processing of the evidences in order to detect contaminations coming from them;

- To use DNA-free disposable equipment to collect the DNA on the target surfaces (Van Oorschot et al., 2005), and to systematically decontaminate thoroughly all the devices which would be in physical contact with the sample.
- For victims taken to a hospital in attempt to seek treatment, the different surfaces (stretcher, hospital beds, tables), the instruments which will be used (scissors to cut away the clothing, electrocardiogram leads, other medical equipment).

Methods to minimize the possibility of contamination in the laboratory have been largely developed. Some of the guidelines are:

- Use of DNA-free plastic ware and consumables, recommendations for manufacturers and laboratories were made by several scientific societies (Gill et al., 2010), Scientific Working Group on DNA Analysis Methods [SWGAM], European Network of Forensic Science Institutes [ENFSI], Biology Specialist Advisory Group [BSAG];
- Shortwave (254 nm) UV exposition of the working surfaces when nobody is working and frequent and thorough cleaning of work areas within laboratories. The top of doors of each room are also equipped with UV source. All appliances, containers, pipets, racks, laboratory coats and work areas (laminar airflow surfaces, PCR box) are cleaned and irradiated by UV during the non-working hours (Keyser-Tracqui et Ludes, 2005).
- Periodic assessment of the level and location of DNA within the work place and on relevant tools;
- All the different steps of the analysis process going from the sample examination step to the extraction procedure, the DNA amplification reaction and at the end, the interpretation of the profiles must be conducted in dedicated laboratory rooms. The analyses of traces samples are also performed a part of the high DNA quality and quantity DNA samples. A “one-way traffic” rule is also observed in the laboratory, once the technician has entered the PCR or the post-PCR rooms, they are not allowed to return to the extraction or pre-PCR rooms until the next day or a complete cloth changing in order to prevent contamination by aerosol particles. All general equipment and apparatus, pipets as well as reagents are dedicated to the analysis area (Extraction, pre-PCR, post-PCR rooms) ;
- Cross comparison of results obtained from different cases (having recorded at which locations the analyses were performed by whom and at what time) to detect unexpected contaminations;
- Analysis of reference samples and extraction (blank) as well as amplification controls at each step of the procedure are a major help to highlight inter-case contamination. The extraction control checks the purity of the extraction reagents and the amplification control indicates the purity of the PCR reagents with no DNA added.

The possibility of the presence of contaminations should be taken in mind at every profile interpretations in particular in cases of degraded DNA or if the molecule is present in very few quantities. As described before the difficulty of the interpretation of a mixed sample must be emphasized, in fact the profile can contain background DNA, crime-related DNA, post-crime contamination.

5. Conclusions

Since the method of DNA fingerprints has been described two majors goals have been followed, first to obtain highly discriminating genetic profiles from minute amounts of DNA and for highly degraded samples, second to avoid the possibility of contaminations due to the crime scene work, the sampling step or the laboratories procedures.

Swabbing and taping a touched area for retrieval of DNA seems simple but experience in case works showed how easy it is to get wrong. The scene crime technicians should be trained and wear appropriate scene clothing to protect the crime scene and its environment. The interpretation of the results should take in account these contamination possibilities by a LR framework incorporating the criminal aspects of DNA evidence (Raymond et al., 2008).

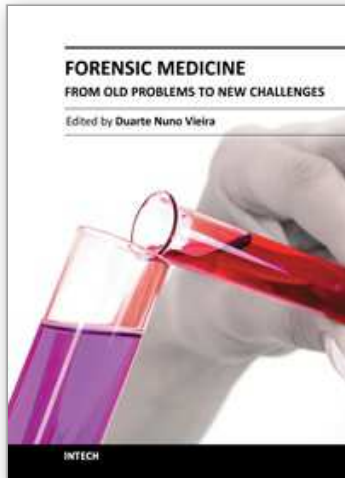
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Forensic medicine is a continuously evolving science that is constantly being updated and improved, not only as a result of technological and scientific advances (which bring almost immediate repercussions) but also because of developments in the social and legal spheres. This book contains innovative perspectives and approaches to classic topics and problems in forensic medicine, offering reflections about the potential and limits of emerging areas in forensic expert research; it transmits the experience of some countries in the domain of cutting-edge expert intervention, and shows how research in other fields of knowledge may have very relevant implications for this practice.

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