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Application of Low Copy Number DNA Profiling

Peter Gill

Forensic Science Service, Trident Court, Birmingham, UK

Low copy number (LCN) DNA profiling is a technique sensitive enough to analyze just a few cells. When this kind of analysis is carried out, special considerations are needed to interpret the results. In particular, it is important to consider the implications of allele dropout and the possibility of contamination from a laboratory source. A rationale for interpreting LCN DNA is described.

Key words: *alleles; DNA; forensic medicine; gene amplification; gene dosage; polymerase chain reaction; short tandem repeat*

Generally, the lower limits of sensitivity recommended by manufacturers of short tandem repeat (STR) multiplex systems are in the region of 250 pg. Multiplexes usually work at their optimum efficiency when 1 ng of DNA is analyzed (1,2) and not more than 28-30 cycles of amplification are carried out. Interpretation of DNA profiles is assisted by the use of systems that are not too sensitive. This is important because the scientist often needs to associate the presence of a bloodstain (or other evidence) with the DNA profile itself. A highly sensitive system that may reveal DNA from sources other than the body fluid analyzed would require careful consideration when the evidence was interpreted. For this reason, validation exercises often include studies on the effect of rough handling, coughing, or sneezing onto garments to determine if it is possible to transfer casually DNA to evidential material.

Nevertheless, forensic scientists always seek to increase the sensitivity of their methods and the easiest way to do this is simply to raise the number of polymerase chain reaction (PCR) amplification cycles. Findlay et al (3) demonstrated that single (buccal) cells could be analyzed when 34 cycles were used with second generation multiplex (SGM) system. Interpretation was not straightforward – additional alleles were observed, the sizes of stutters were enhanced, and allele drop out was common. However, such profiles may be interpreted if robust guidelines are used. Subsequently, increasing the sensitivity of PCR by raising the number of cycles has been used to increase the range of evidence types analyzed. For example, Wiegand and Kleiber (4) and Wiegand et al (5) analyzed epithelial cells transferred from an assailant after strangulation using 30-31 cycles of PCR. Van Hoofstat et al (6) analyzed fingerprints from grips of tools with 28-40 cycles. Barbaro et al (7) reported analysis of STRs from hair shafts in the absence of the root using 35-43 cycles.

Increased PCR cycles are routinely used by anthropologists and forensic scientists to identify ancient DNA from bones. We (8) used 38-43 cycles to analyze STRs from 70-year-old bone from the Romanov family, Schmerer et al (9) and Burger et al (10) analyzed STRs from bone thousands of years old (60 and 50 PCR cycles, respectively), whereas some other authors used modified PCR methods, for example, a nested primer PCR strategy (11). The nested primer PCR strategy used a first round amplification with 40 cycles, with subsequent analysis of a portion with further 20-30 cycles. This method was used to analyze DNA from charred human remains and minute amounts of blood.

Comparison of both different methods available to analyze DNA in amounts less than 100 pg and varying cycling conditions between 28-60 cycles showed that the optimum for both SGM and AMP*fl*STR®SGM Plus™ systems (Applied Biosystems, Foster City, CA, USA) was 34 cycles (12). There was little to be gained by increasing the cycle number further, since it did not result in increased sensitivity, but encouraged artefact production. The extreme sensitivity of the method suggested that analysis should only be attempted in a sterile environment to reduce the possibility of contamination from personnel within the laboratory itself.

Nevertheless, all methods used to analyze low copy number (LCN) DNA suffer from several disadvantages that are primarily derived from stochastic variation. When present in low copy number, a molecule that is amplified by chance during the early rounds of the PCR is likely to be preferentially ampli-

fied. There are, therefore, several consequences that cannot be avoided:

a) Allele drop out may occur because one allele of a heterozygote locus can be preferentially amplified;

b) Stutters may be preferentially analyzed – these are sometimes known as false alleles; and

c) The method is prone to sporadic contamination – amplifying alleles that are unassociated with the crime stain, or sample.

This means that different DNA profiles may be observed after replicate PCR analyses. Tarbelet et al (13) suggested a method of replicated analyses that comprised a rule that an allele could only be scored if observed at least twice in replicate samples. This theory was expanded by us (12), who adopted Tarbelet's duplication rule and demonstrated that it was conservative in relation to a new likelihood ratio (LR) method that assessed DNA profiles in relation to sporadic allelic contaminants, stutters, and allelic drop-out. Provided that the level of sporadic contamination was not high (<30% per locus), the duplication method was demonstrated to be conservative relative to the likelihood ratio method.

Interpretation of LCN DNA Profiles

In conjunction with the increased use of DNA profiling, there has been a parallel development in interpretation methodology. In particular, Cooke et al (14,15) and Evett (16) introduced the notion of the "hierarchy of propositions". This has led to a much deeper understanding of the interpretative process. However, there is currently considerable lack of understanding about issues of transfer and persistence, and further work is being undertaken in this area.

Hierarchy of Propositions

The hierarchy of propositions takes as its premise that scientific evidence may only be interpreted if at least two competing propositions are considered. The top level, level III, of the hierarchy represents the *offense* level. These are the propositions that are most usually seen to be the province of the jury. For example:

a) The suspect is the offender.

b) The suspect is unconnected with the incident.

These embody the assumption that an offense has indeed been committed and, in general, this would seem to be a level that scientists would prefer to avoid.

The second level, level II, represents the *activity* level. These would be pairs of propositions that the scientist may feel qualified to address, given adequate information about the case circumstances. For example:

a) The suspect broke the window at the scene.

b) The suspect is unconnected with the incident.

In general, such propositions invoke the classic forensic considerations of transference and persistence.

The third level, level I, is the *source* level, which comprises propositions that relate to the origin of recovered material. For example:

a) The bloodstain came from the suspect.

b) The bloodstain came from some unknown person.

Such propositions would be appropriate when the circumstances are such that the scientist is unable to express an opinion with regard to particular actions or activities. Inevitably, the lower the level of the propositions, the more of the interpretation is left to the jury.

When samples comprise <100 pg of DNA, even level I propositions may be inappropriate. We may have a DNA profile from the crime sample but because of several uncertainties we may not be justified in inferring that it came from the stain that was sampled. Because of this, we have introduced a new idea – that of "sub-level I propositions". For example:

a) The DNA profile came from the suspect.

b) The DNA profile came from some unknown person.

Addressing propositions at this level means that the scientist is unable to express a substantive opinion of how the DNA arrived at the site from which it was recovered, or even whether it came from the stain that may have been the reason that sampling was carried out. All considerations between the sub-level I propositions and the level III propositions that the jury must ultimately address must be left to the court, though the scientist has a clear duty to advise the court on the issues that are relevant.

Association of the DNA Profile with the Evidential Material Analyzed

There are two broad categories of evidence types – discrete (eg, bone, hair) and non-discrete (eg, blood stains). When using LCN, it is generally easier to associate a DNA profile with a discrete evidence type. This is because the analysis of bone samples is not attempted without removing the outermost layer by physical methods (eg, sandpaper) to minimize the possible contamination from modern DNA. Similarly, hair shafts can be washed in a detergent solution to remove adhering DNA. This cannot be done with evidence types that are not discrete, eg, blood stained cloth, hence the chance is increased that a DNA profile may not be directly associated with the evidential body fluid that is "apparently" analyzed. Because there is a serious possibility of transferring LCN DNA from a modern source, to either minimize the chance of contamination or to identify an occurrence, we use the following guidelines:

1. DNA extractions and setting up PCR reactions are carried out in a dedicated laboratory.

2. Personnel wear disposable laboratory coats, gloves, and face masks.

3. Benches and equipment are frequently treated with bleach (or equivalent) and irradiated with UV light.

4. PCR amplification is carried out in a separate laboratory or laboratory area.

5. Negative controls are used with every test to demonstrate absence of contamination.

6. PCR tests are duplicated wherever possible.

All results are compared against a staff database.

A database to eliminate investigators of crime scenes as potential contributors is also under preparation.

Defining when DNA Transfer Can Occur

Before and after a crime event, there is the potential for adventitious transfer of cells. Note that the term contamination is reserved for transfer of DNA *after* the crime event. Adventitious transfer and laboratory contamination usually involves low levels of DNA.

The association of body fluid and the DNA profile is not implicit. If the body fluid giving a positive presumptive test is small or degraded then the DNA profile may have originated from an alternative source. For example, a fresh saliva stain, the latter solely contributing to the observed result, may mask a small-degraded blood-spot. The scientist cannot infer either the type of cell donating the DNA or the time when the cells were deposited.

An estimate of the quantity of DNA is useful to assist in the interpretation of the relevance of a DNA profile. For example, if a visible fresh bloodstain yields several micrograms of DNA, it is not unreasonable to associate the DNA profile with the bloodstain according to level I proposition. However, the association is uncertain if the bloodstain is minute, old, and yields just a few picograms of DNA. It may be appropriate to use a sub-level I proposition. Inevitably, there is a direct relationship between the quantity of DNA present and the relevance of the evidence.

The interpretation of the case can only follow after an assessment of all the available evidence, taking into consideration the scenarios offered by prosecution and defense lawyers.

Assessment of Contamination Risks

DNA can be transferred at any time before, during, and after the crime. The foregoing discussion has covered the possibility of adventitious transfer at a period before the crime and it is implicit that the DNA profile matches a suspect. If the DNA profile does not match the suspect, then post-crime transfer must be considered. Contamination is transfer of DNA after the crime event. Potential sources of contamination are:

a) Investigative officers, pathologists, etc, at the crime scene;

b) Laboratory staff;

c) Cross contamination from samples processed in the laboratory, e.g., by aerosol; and

d) Plastic-ware contamination (may be contaminated at the manufacturing source).

Whereas a) and b) can be covered by reference to staff databases, and databases of investigating officers, c) and d) are more difficult to detect but are minimized by good laboratory design, staff wearing anti-contamination clothing and face masks, and UV sterilization of plastic-ware.

Transfer of DNA by individuals unassociated with the crime before the crime event itself is defined as adventitious transfer. When a DNA profile does *not* match the suspect, the following possibilities apply:

a) The suspect is innocent and the perpetrator profile has been visualized.

b) Cells have been transferred by an innocent individual before the crime (perpetrator has not shed cells) – *'adventitious transfer'*.

c) Cells have been transferred by an investigator after the crime event (perpetrator has not shed cells) – *'contamination'*.

Note that mixtures may show DNA profiles arising from a combination of the three different events listed.

The circumstances of the victim leading up to the crime event is unknown to the scientist, hence the possibilities of adventitious transfer cannot be directly ascertained. Once the crime has been discovered, the scene and the associated evidence enter a controlled environment, where the risk of contamination is minimized by the adoption of good laboratory and investigative practice.

The primary risk of contamination is wrongful exclusion – particularly if the contaminant masks the perpetrator's profile. For the converse to apply – wrongful inclusion, either tube mix-up or gross contamination (e.g., use of pipette tips contaminated in the laboratory – e.g., used twice) would be required. Good laboratory practice renders this a virtual impossibility and is not considered further here.

Current Reporting of Sub-level I Propositions in Statements

Because of uncertainties that surround persistence and transfer, the statements are written to reflect this and interpretation usually proceeds according to sub-level I principles. Examples of the wording used in statements are given below. Interpretation is dependent upon a full analysis of the circumstances of the crime and based on a careful consideration of all of the non-DNA evidence.

Observation of Mixtures

With LCN, mixtures are commonly encountered. It cannot be determined whether recovered DNA profiles are associated with a crime event. An example statement follows:

"The observation of mixed DNA STR profiles (ie, from more than one individual) can be anticipated. For example, from past experience it is not unusual to detect DNA profiles on items that match the profile of an individual who has habitually worn that item. However, currently we have no information to assist

with questions of transfer and persistence of low levels of DNA on items such as XXXXXX. Thus consideration should be given as to how the DNA detected has been transferred to that item, and consequently to the relevance of finding profiles matching the individuals in the case." In the following, two alternatives are considered. No reference is made about the origin of the body fluid type – it is simply stated that DNA was recovered from the item. "*Either* the majority of the DNA originated from Mr. X; *or* the majority of the DNA originated from someone other than and unrelated to Mr. X. If this DNA had, in fact, originated from Mr. X, then I would expect to obtain matching profiles."

In the summary section the following paragraphs are included – this statement was specifically written for a case where DNA from a watchband matched a suspect.

When very small amounts of DNA are analyzed, special considerations arise as follows:

a) Although a DNA profile has been obtained, it is not possible to identify the type of cells from which the DNA originated, neither is it possible to state *when* the cells were deposited.

b) It is not possible to make any conclusion about transfer and persistence of DNA in this case. It is not possible to estimate when the suspect last wore the watch, if it is his DNA.

c) Because the DNA test is very sensitive, it is not unexpected to find mixtures. If the potential origins of DNA profiles cannot be identified, it does not necessarily follow that they are relevant to this case, since transfer of cells can occur as a result of casual contact.

Effectively, the strength of the LCN DNA evidence is decreased compared to conventional DNA analysis. This inevitably arises from uncertainties relating to the method of transfer of DNA to a surface and uncertainties relating to when the DNA was transferred. It is emphasized that the relevance of the DNA evidence in a case can only be assessed by a concurrent consideration of all the non-DNA evidence. Research is currently being undertaken to devise a probabilistic Bayesian method that encapsulates the DNA and non-DNA evidence.

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Correspondence to:
Peter Gill
Forensic Science Service
Trident Court
2960 Solihull Parkway
Birmingham B37 7YN, UK
101642.3373@compuserve.com