LOW COPY NUMBER - CONSIDERATION AND CAUTION

Bruce Budowle, Deborah L. Hobson, Jill B. Smerick, and Jenifer A.L. Smith

Laboratory Division, FBI, Washington, DC

DEFINITION

The wide acceptance of DNA typing in forensic science is due to its sensitivity of detection and discrimination. The use of the polymerase chain reaction (PCR) affords enhanced sensitivity of detection, and the ability to amplify multiple loci simultaneously increases the power of the assay. Because of the successes encountered with STR typing, it was inevitable that some individuals would endeavor to type samples containing very minute amounts of DNA (1-8).

The approach that amplifies less than typical minimum amounts of DNA template and then attempts to interpret the results has been termed low copy number (LCN) typing. LCN typing has been defined by Gill, et al. (4,5) as typing samples containing less than 100pg of DNA. However, because of variation in estimates of DNA quantity within and between methods of quantitation, LCN typing is better defined as the analysis of any results below the stochastic threshold for normal interpretation. When few copies of DNA template are present, stochastic amplification may occur, resulting in either a substantial imbalance of two alleles at a given heterozygous locus or allelic dropout. Typically, minimum amounts of DNA template are utilized in a PCR so that stochastic effects can be reduced. In the reverse dot blot systems (AmpliType PM and DQA1+PM, PE Biosystems), S or C dots, respectively, were used to evaluate whether a DNA template used in the PCR was above a level at which stochastic effects may have an impact on the relative balanced yield of two alleles at a given heterozygous locus. Similarly, for STR typing, peak height (or area) serves as a stochastic control. An effective stochastic interpretation threshold does not exist for LCN typing. Results are evaluated where stochastic effects during PCR are prevalent.

LCN TYPING

Current multiplex STR typing strategies are sufficiently sensitive to detect alleles in the LCN range, without further modification. Any peaks, or bands, residing below the stochastic threshold (i.e., the interpretation threshold) and above the detection threshold are from LCN samples. Thus, LCN typing can be performed with routine methods. Nonetheless, enhancing the sensitivity of detection for LCN typing has been sought. The typical strategy to enable better detection of LCN DNA alleles is to increase the number of PCR cycles, for example from 28 to 34 (for SGM Plus Kit, Applied Biosystems) (4,5). Similarly, Kloosterman and Kersbergen (9) suggested amplifying the DNA using the standard 28 cycles for the SGM Plus kit and typing the sample following standard operating protocols; if a low level result is obtained, then more Taq polymerase is added to the remaining PCR and 6 more cycles of the PCR are performed to carry out LCN typing. Alternatively, with current instrumentation (and particularly with capillary electrophoresis), one need not increase the cycle number to achieve similar results with LCN typing. A few approaches that can be used include: 1) reducing the PCR volume; 2) microcon filtration of the amplicons to remove ions that compete with DNA when being injected into the capillary; 3) use of a formamide with a lower conductivity; 4) adding more amplified product to the denaturant formamide; and 5) increasing injection time. Yet, the same concerns (e.g., allele drop-out, allele drop-in (defined below), heterozygote imbalance, increased stutter) that can increase sensitivity of detection persist with these other approaches. Figure 1 shows that signal can be increased using the same quantity of template and reducing the PCR volume. The overall yield of amplification product is not substantially different with different PCR volumes; however, the concentration of product increases with reduced PCR volumes. This experiment does not demonstrate allele drop-out, because the initial template of 125 pg may be sufficient to minimize stochastic effects. Figure 2 shows that alleles can be detected in a sample containing approximately one cell equivalent of DNA using the standard number of cycles of PCR (28 cycles for Profiler Plus kit, Applied Biosystems) by 1) increasing the amount of sample injected into the capillary; 2) using formamide with lower conductivity (i.e., HiDi); 3) washing the sample to remove ions (by microcon dialysis); and 4) increasing injection time to 10 seconds. Enhanced sensitivity with this approach is based on the fact that molecules are loaded into

the capillary in a competitive fashion, biased towards high charge-to-mass ratio ions. Removing more of these ions, by dialysis or by using more pure formamide, allows more DNA to be injected into the capillary. However, at these low levels of template DNA, alleles (and loci) can drop-out (Figure 3). At such low levels, allele drop-out is expected, and the alleles that drop-out may vary between replicate samples. However, allele drop-in (i.e., sporadic contamination) also occurs (Figure 3). Allele drop-in does not appear to be systematic, because the observation of allele drop-in did not occur in both replicate analyses shown in Figure 3, and the negative control did not show contamination (Figure 4). However, detectable contamination that cannot be accounted for limits the utility of LCN typing. Gill, et al. (5) assume that the occurrence of alleles dropping-in is assessed based on population allele frequencies; perhaps drop-in alleles should be monitored and recorded over time to confirm their assumption.

The stutter product for an STR allele with "n" tetranucleotide repeats is four bases shorter (n – 4) than the true allele and is inherent to the amplification of STR loci. Under current forensic analyses, stutter products are usually less than 14% in height or area than the true allele. When analyzing LCN DNA, the percent stutter increases and can be greater than the true allele (4,5). The degree of stutter is not predictable.

The presence of three or more alleles at a given locus is indicative of a mixed sample. Also, the presence of two alleles at multiple loci, with substantially different peak heights, may be indicative of a mixed sample. Mixture analyses and confirmation of a mixture are not reliable with LCN typing, because of imbalance of heterozygote alleles, increased production of stutter products, and allele drop-in can occur.

LIMITATIONS AND CONSIDERATIONS

Philosophically, LCN typing has some differences compared with general DNA typing that should be considered. In a typical forensic DNA analysis, attempts are made to exclude the suspect (or victim), and only after failing to exclude, inferences are made about the rarity of the DNA profile. Because of the extreme sensitivity of detection, background level DNA and DNA from casual contact may and will be detected. Thus, LCN typing cannot be used for exculpatory purposes. Thus, the paradigm for the use of LCN typing differs fundamentally from general DNA typing regarding the capacity to exculpate individuals. Moreover, in contrast to attempting to concentrate the sample to obtain a quantity of DNA that would yield data closer to an acceptable stochastic threshold, the sample often is diluted to utilize redundancy for allele interpretation.

However, there may be some limited applications where LCN analysis is warranted, such as some investigative lead situations. The consequence of its use should be considered, because such analyses may still end up in court, and one may have to explain the limitations of LCN typing and the presence of alleles due to the vagaries of LCN typing. In contrast, samples that can be cleansed of exogenous DNA may be more suitable for LCN typing. These include: bones, teeth, and hair shafts (10,11). LCN typing of these single source samples then can be used for exculpatory, as well as inculpatory, purposes. One may consider the typing of human remains that contain little intact DNA appropriate for LCN typing (usually the typing is not used for identification of perpetrator(s) of a crime(s)). These samples are still subject to allele drop-out and sporadic low level background contamination from laboratory chemical reagents, but exogenous DNA from casual contact or secondary transfer can be minimized or eliminated.

Consistency and reproducibility requirements of STR typing must be reconsidered for LCN typing. Amplification of DNA samples with LCN often will be subjected to stochastic effects, as has been described above. Thus, it is difficult to validate LCN typing, because results often are not reproducible and reagents are usually not subjected to quality control (QC) at the conditions prescribed for LCN typing. Reproducibility of results, however, must be invoked by requiring alleles to be present in multiple amplifications of the same sample. The number of times an allele should be seen in a number of separate amplifications from a sample is still open to debate and will depend on the type of sample. Single source samples may not require independent multiple amplifications for analysis. Moreover, to achieve typing of multiple PCRs of the same sample, the sample usually has to be diluted so it can be divided into two or more amplification reactions. Diluting, in lieu of concentrating, such that the most available template is not placed into one PCR, is contrary to current effective practices (although necessary for LCN typing).

Perhaps before undertaking LCN typing, limitations of the analysis should be disclosed to all involved, including other lab personnel, supervisors, police, lawyers, the court, and even the public. Publicizing the potential of the application of LCN typing without describing its limitations may cause misunderstanding. Considerations include:

1) the basic philosophy of forensic DNA analyses to attempt to exclude does not apply;

2) in contrast to attempting to concentrate the sample to obtain a quantity of DNA that would yield data closer to an acceptable stochastic threshold, the sample often is diluted to utilize allele redundancy for interpretation;

3) if redundancy is used, the number of controls should be defined (and be at least equal to the number of LCN amplifications);

4) there is no minimum threshold for guidance and confidence in interpretation. The operational interpretation thresholds currently in use (for example 150 or 200 RFUs for Profiler Plus and Cofiler Kits and the ABI 310, Applied Biosystems) do not apply with LCN modified procedures. In fact, the current stochastic thresholds are invalid for LCN typing;

5) contamination is a greater concern which can result in such issues as allele drop-in. Practices for reducing contamination should be at least equivalent to those employed for mitochondrial DNA typing (10,11);

6) because of enhanced sensitivity, casual contact must be considered. If the victim and the accused had any previous contact, the result may have no relation to the case at hand;

7) heterozygous peak imbalance is augmented;

8) the relative proportion of stutter can be increased;

9) it is difficult to determine if a true mixture is apparent and not possible to separate contributors in a mixture;

10) kits and reagents may not have been subjected to QC under the conditions they are being used;

11) statistical interpretations should be modified to better represent the uncertainty associated with LCN typing;

12) the data should not be entered into CODIS;

13) LCN typing should not be applied to post-conviction analyses and examination of old cases without substantial consideration. It can not be used for exculpatory purposes, and LCN contamination from handling may have occurred;

14) because of the small sample size, the tissue source of the DNA cannot be inferred.

Lastly, one can only evaluate the results within the context of each case, which problematically increases the interpretation or speculation on events by the analyst.

CONCLUSIONS

Caution should be undertaken when attempting LCN typing. The success rate is low; often the results cannot be interpreted or are meaningless for the case. The method cannot be used for exculpatory purposes. However, for single source samples where exogenous DNA can be removed and for typing of human remains, LCN typing may be applicable. While there have been some dedicated efforts to exploit LCN typing, primary efforts still should be to reduce stochastic effects on minute evidence samples. Research possibly could include: 1) improving extraction efficiency to obtain greater quantities of template DNA, such as using tape lifts to recover more DNA from the substrate or using carrier DNA during extraction; 2) designing shorter amplicons for greater PCR efficiency on degraded samples; 3) improving the rate of heat transfer and reducing the diffusion coefficient in the PCR; and 4) fragmenting the DNA prior to PCR to augment denaturation and thus increase the amplification efficiency of minimal DNA template.

ACKNOWLEDGMENT

This is publication number 01-26 of the Laboratory Division of the Federal Bureau of Investigation. Names of commercial manufacturers are provided for identification only, and inclusion does not imply endorsement by the Federal Bureau of Investigation.

REFERENCES

1.van Oorschot RA, Jones MK (1997) DNA fingerprints from fingerprints. Nature 387:767.

2. Barbaro A, Falcone G, Barbaro A (2000) DNA typing from hair shaft. Progress in Forensic Genetics 8:523-525.

3. Findlay I, Frazier R, Taylor A, Urquhart A (1997) Single cell DNA fingerprinting for forensic applications. Nature 389:555-556.

4. Gill P (2001) Application of low copy number DNA profiling. Croatian Medical Journal 42:229-232.

5. Gill P, Whitaker J, Flaxman C, Brown N, Buckleton J (2000) An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. Forensic Science International 112:17-40.

6. Ladd C, Adamowicz MS, Bourke MT, Scherczinger CA, Lee HC (1999) A systematic analysis of secondary transfer. Journal of Forensic Sciences 44:1270-1272.

7. Wiegand P, Kleiber M (1997) DNA typing of epithelial cells after strangulation. International Journal of Legal Medicine 110:181-183.

8. Wiegand P, Trubner K, Kleiber M (2000) STR typing of biological stains on strangulation tools. Progress in Forensic Genetics 8:508-513.

9. Kloosterman A, Kersbergen P (2001) Efficiency and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR STR loci. Presented at 19th Congress of the International Society of Forensic Genetics, Munster, Germany.

10. Budowle B, Smith JA, Moretti T, DiZinno J (2000) DNA Typing Protocols: Molecular Biology and Forensic Analysis, BioTechniques Books, BioForensic Sciences Series, Eaton Publishing, Natick, MA.

11. Wilson MR, Polanskey D, Butler J, DiZinno JA, Replogle J, Budowle B (1995) Extraction, PCR amplification, and sequencing of mitochondrial DNA from human hair shafts. BioTechniques 18:662-669.

Figure 1. A display of the effect of reducing the volume of the PCR. 125 pg template was used in each PCR. The top panel was a 50 µl reaction; the middle panel was a 25 µl reaction; and the bottom panel was a 5 µl reaction. The loci are D5S818, D13S317, and D7S820 from the Profiler Plus kit.

Figure 2. The top panel displays results using 1 ng of template and standard operating procedures (SOP) (10). The bottom panel displays results from the same individual using 7.8 pg of template, microcon dialysis of the PCR product, 4 µl of PCR product mixed with formamide (HiDi), and a 10 second injection into the capillary. The loci are D3S1358, vWA, and FGA from the Profiler Plus kit.

Figure 3. The top panel displays results using 250 pg of template and SOP (10). The loci are D3S1358, vWA, and FGA from the Profiler Plus kit. The loci vWA and FGA are circled for comparison reference with the other panels. The middle panel displays results from the same individual using 7.8 pg of template, microcon dialysis of the PCR product, 4 µl of PCR product mixed with formamide (HiDi), and a 10 second injection into the capillary. Note that there is complete drop-out of the vWA locus and allele drop-out at the FGA locus. The bottom panel displays a replicate analysis under the same conditions as in the middle panel. Note that there is allele drop-in of the vWA 18 allele.

Figure 4. A display of the amplification blank using microcon dialysis of the PCR product, 4 µl of PCR product mixed with formamide(HiDi), and a 10 second injection into the capillary. The Profiler Plus kit was used.