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Setting Interpretation Thresholds and Results with Low-Level DNA Analysis

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Copy of poster available:
http://www.cstl.nist.gov/biotech/strbase/pub_pres/Promega2009_Hill.pdf

The term Low Copy Number (LCN) DNA is typically used when there is less than 100 - 125 pg of genomic DNA present in a sample [1,2]. Efforts to enhance signal include longer injection with capillary electrophoresis, salt removal from the amplified product, reduced volume polymerase chain reaction (PCR), increasing the number of PCR cycles, and increasing the enzyme concentration. More and more labs are attempting to process lower amounts of DNA, many without realizing the consequences of doing so. STR typing kits will generally fail to amplify all of the loci present or even one or both alleles present within a locus at these low levels of DNA. Partial incorrect profiles are generated that can be misleading without taking additional precautions including replicate testing [3,4]. In these cases, there are too few copies of the DNA template to provide reliable (PCR) amplicons, causing preferential amplification to occur [5]. Next generation manufacturers' kits are being made more sensitive with improved PCR master mixes and more robust DNA polymerases. This can potentially lead to labs pushing the envelope and getting results that may not represent the true DNA profile of the originating source due to stochastic effects including allele dropout or drop-in.

We have performed multiple LCN experiments to evaluate two different samples that are heterozygous at every locus in the AmpFlSTR Identifiler™ (Applied Biosystems, Foster City, CA) and PowerPlex® 16 HS (Promega Corporation, Madison, WI) PCR amplification kits. Completely heterozygous samples were used in order to evaluate peak height ratios and potential imbalance due to stochastic effects (as compared to 9947A which is often used but has many homozygous loci). Each sample was tested with 10 replicates at multiple concentrations, including several considered to be LCN amounts (1 ng, 100 pg, 30 pg, and 10 pg) and at different PCR ranging from 28 to 34 cycles [3,6]. They were tested with 10 replicates to determine the consensus profile, where an allele cannot be scored (considered real) unless it is present at least twice in the replicate samples [2,3,6,7]. The heterozygote peak height ratios (PHR) were calculated and compared at different concentrations and PCR cycling [2,4]. In addition, 1:3 and 3:1 mixture samples at LCN total DNA amounts (100 pg) were evaluated and compared in 10 replicates. Results are shown with different multiplex kits. Thoughts on setting interpretation thresholds to avoid stochastic effects will be described. The value of anchoring DNA quantitation results to a calibrated reference material will also be discussed.

References

[1] Gill, P. (2001) Application of low copy number DNA profiling. *Croatian Med. J.* 42(3): 229-232.

[2] Gill, P., Whitaker, J., Flaxman, C., Brown, N., and Buckleton, J. (200) An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. *Forensic Sci. Int.* 112(1): 17-40.

[3] Kloosterman, A.D. and Kersbergen, P. (2003) Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci. *J. Soc. Biol.* 197(4): 351-359.

[4] Whitaker, J.P., Cotton, E.A., and Gill, P. (2001) A comparison of the characteristics of profiles produced with the AMPFISTR SGM Plus multiplex system for both standard and low copy number (LCN) STR DNA analysis. *Forensic Sci. Int.* 123: 215-223.

[5] Walsh, P.S., Erlich, H.A., and Higuchi, R. (1992) Preferential PCR amplification of alleles: Mechanisms and solutions. *PCR Meth. Appl.* 1: 241-250.

[6] Gill, P. (2002) Role of short tandem repeat DNA in forensic casework in the UK—past, present, and future perspectives. *BioTechniques* 32(2): 366-385.

[7] Caragine, T., Mikulasovich, R., Tamariz, J., Bajda, E., Sebestyen, J., Baum, H., and Prinz, M. (2009) Validation of Testing and Interpretation Protocols for Low Template DNA Samples Using AmpFlSTR Identifiler®. *Croatian Med. J.* 50: 250-67.

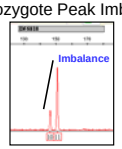
Issues with Low Template DNA Analysis:

Definition of Low Template DNA

- Working with **<100 pg genomic DNA** (~15-17 diploid copies of nuclear DNA markers such as STRs)
- Data below the stochastic threshold level where PCR amplification is not as reliable (determined by each laboratory; typically 150-250 RFUs)
- Enhancing the sensitivity of detection (34 cycles instead of 28 cycles)
- Having too few copies of DNA template to ensure reliable PCR amplification
- Often the minor component of mixture samples consists of low DNA template amounts

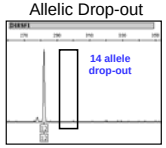
Some issues with LCN DNA

Heterozygote Peak Imbalance



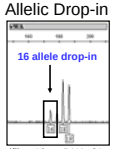
Identifiler, 30 pg DNA, 31 cycles

Allelic Drop-out



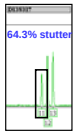
Identifiler, 30 pg DNA, 31 cycles

Allelic Drop-in



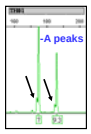
Identifiler, 10 pg DNA, 31 cycles

High Stutter



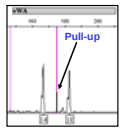
Identifiler, 10 pg DNA, 31 cycles

-A peaks



Identifiler, 100 pg DNA, 31 cycles

Bleed Through/Spikes

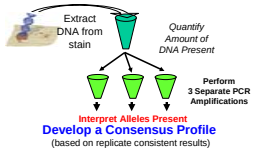


Identifiler, 100 pg DNA, 31 cycles

*These scenarios occur with higher amounts of DNA

Consensus Profiles for Optimal Results

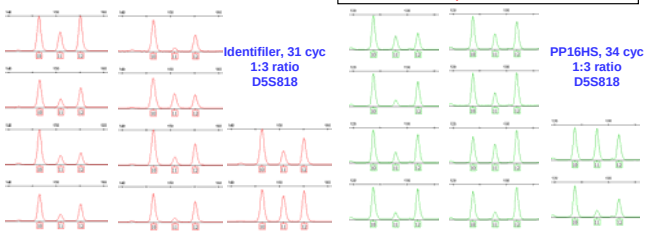
- Typically 2 - 3 PCR amplifications from the same DNA extract
- An allele is usually not scored (considered real) unless it is present at least twice in replicate samples



Challenges of Low Level DNA Mixtures

- 2 samples that are heterozygous at all loci were mixed in 1:3 and 3:1 ratios with a 100 pg total amount of DNA and were tested with Identifiler (31 cyc) and PP16HS (31 and 34 cyc) in 10 replicates

*The variation in replicates is evident in both kits



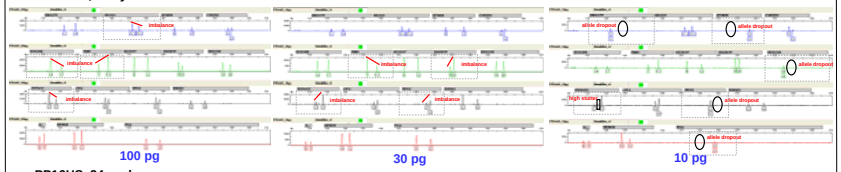
LCN Sensitivity Data and Results with Identifiler™ and PowerPlex® 16 HS:

Experimental Design

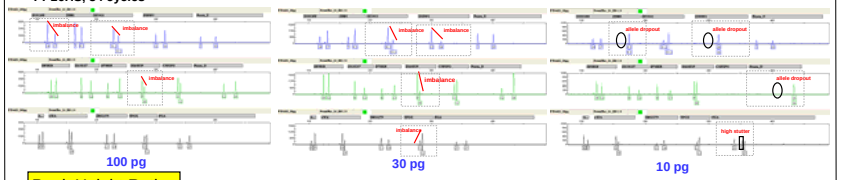
- Pristine DNA Samples
  - 2 single-source samples (and mixtures created from these)
  - **heterozygous for all loci tested** (permits peak height ratio studies
- Low DNA Template Amounts
  - Dilutions made after DNA quantitation against NIST SRM 2372
  - **100 pg, 30 pg, and 10 pg** (1 ng tested for comparison purposes)
- Replicates
  - **10 separate PCR reactions** for each sample
- STR Kits
  - **Identifiler and PowerPlex 16 HS** (half-reactions)
- Increased Cycle Number
  - Identifiler (**28 cycles and 31 cycles**; 28 for 1 ng)
  - PowerPlex 16 HS (**31 cycles and 34 cycles**; 30 for 1 ng)

Example Profiles

Identifiler, 31 cycles

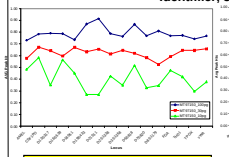


PP16HS, 34 cycles

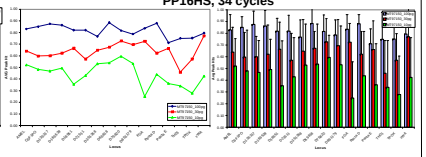


Peak Height Ratios

Identifiler, 31 cycles



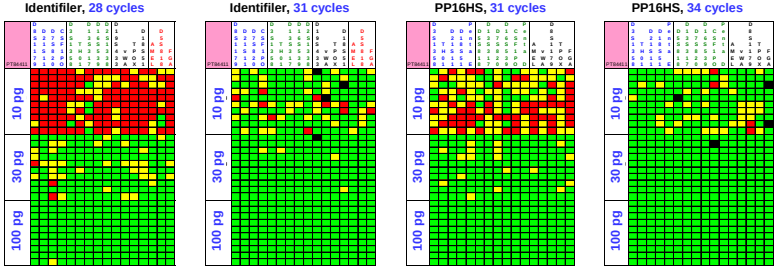
PP16HS, 34 cycles



Sensitivity Comparisons

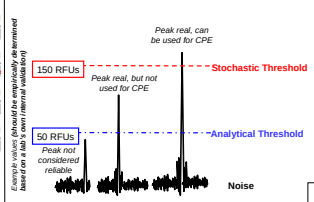
Loci contained within each kit are depicted across the X-axes, and input DNA amount in ng and replicate results is shown on the Y-axes

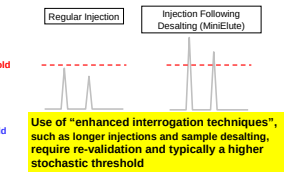
Green = full (correct) type
Yellow = allele dropout
Red = locus dropout
Black = drop-in



Stochastic Thresholds for Interpretation

Two thresholds are often set for analysis of STR typing data. The **analytical threshold** (typically set at around 50 RFU) reflects the instrument sensitivity, which is impacted by baseline noise in collected data. The analytical threshold helps determine what is a true peak signal—and thus a potential STR allele. The **stochastic threshold** (typically set at 150-200 RFU) helps determine what is reliable PCR data and is impacted by assay and CE injection parameters. When a peak is above a stochastic threshold, a high degree of certainty exists that if the sample is heterozygous at the tested STR locus, then the sister allele will be seen above the analytical threshold. When all peaks at a locus are above the stochastic threshold in a mixed sample, then the assumption can be made that all alleles in the tested sample are accounted for and thus the locus can be used in combined probability of exclusion (CPE) calculations.



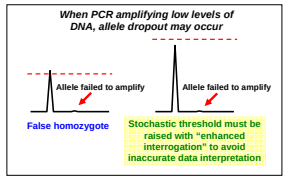


Use of "enhanced interrogation techniques", such as longer injections and sample desalting, require re-validation and typically a higher stochastic threshold

Use of an "enhanced interrogation technique", such as QIAGEN MiniElute*, reduces salt levels in samples causing more DNA to be injected

The stochastic threshold must be raised to account for the increased sensitivity

*Smith, P.J. and Ballantyne, J. (2007) Simplified low-copy-number DNA analysis by post-PCR purification. *J. Forensic Sci.* 52: 820-829



For some recent suggestions on determining a Stochastic Threshold, see:

Gill, P., Puch-Solis, R., Curran, J. (2009) The *low-template-DNA* (stochastic) threshold—its determination relative to risk analysis for national DNA databases. *Forensic Sci. Int. Genetics* 3: 104-111.

Tvedebrink, T., Eriksen, P.S., Mogensen, H.S., Morling, N. (2009) Estimating the probability of allelic drop-out of STR alleles in forensic genetics. *Forensic Sci. Int. Genetics* 3: 222-226

Summary

- Low-template DNA (LT-DNA), often referred to as low-copy number (LCN), is often defined as <100-200 pg input DNA.
- In order to improve sensitivity, the number of PCR cycles is often increased (e.g., 31 or 34 cycles instead of 28 cycles) when amplify DNA with conventional STR kits.
- While increasing the assay sensitivity enables lower amounts of DNA to be detected, these "enhanced interrogation techniques" are prone to stochastic amplification effects that are exhibited in the form of allele drop-out and drop-in.
- To improve result reliability, replicate amplifications are typically compared from low-level DNA samples and consensus profiles developed. Cautious data interpretation rules are also applied based on validation studies.
- Identifiler with 31 cycles and PowerPlex 16 HS with 34 cycles were comparable in performance with low-level DNA analysis.

Acknowledgments and Disclaimer

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