How Low Can You Go?

An Evaluation of Low Copy Number (LCN) DNA Testing

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Outline of Topics to Discuss

- Introduction to Low Copy Number (LCN) DNA: What is LCN DNA?
- DNA concentration of samples: How low can you go?
- Challenges and limitations with LCN DNA testing
- LCN data and Peak Height Ratios (PHR)
- Other methods for higher sensitivity and signal enhancements

Introduction to Low Copy Number (LCN) DNA

Some Definitions of Low-Copy Number (LCN)

- Work with **<100 pg genomic DNA** (~15-17 diploid copies of nuclear DNA markers such as STRs)
- Below stochastic threshold level where PCR amplification is not as reliable (determined by each laboratory; typically 150-250 RFUs)
- Enhancing sensitivity of detection (34 cycles instead of 28 cycles)
- Too few copies of DNA template to ensure reliable PCR amplification
- Other terms for LCN:
  - Low-level DNA
  - Trace DNA
  - Touch DNA

LCN is dependent on the amount of DNA present NOT the number of PCR cycles performed; LCN conditions may exist with 28 or 34 cycles

Why attempt LCN? …

- Improved success rates with high sensitivity DNA testing vs. standard procedures
- Volume crime samples (burglary)
- Bone samples to provide improved matching statistics over mtDNA analysis

DNA Concentration in Samples: How Low Can You Go?

Amounts of DNA Required

1985-1995 **RFLP/VNTRs** **50 ng – 1000 ng**

↓

1991-present (kits since 1996) **PCR/STRs** **0.5 – 2 ng**

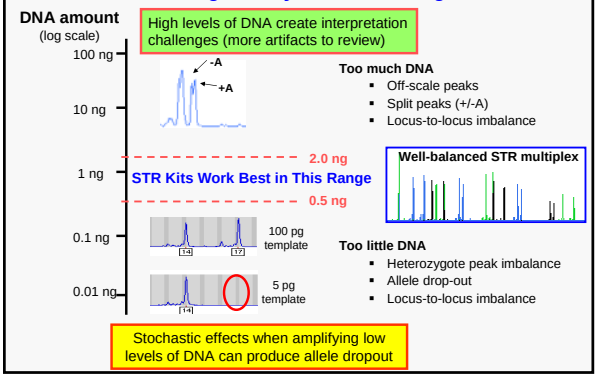
↓

1999-present **LCN/STRs** **<0.1 ng**

LCN extends the range of samples that may be attempted with DNA testing

Impact of DNA Amount into Multiplex PCR Reaction

We generally aim for 0.5-2 ng



Where does low copy number start?

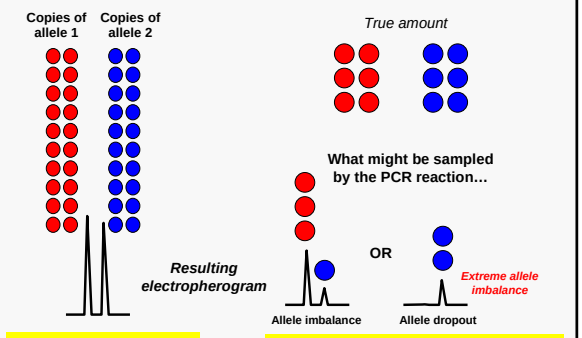
<100 pg template DNA

(Butler, 2001, Fregeau & Fourney 1993, Kimpton *et al* 1994)

Amount of DNA	~ # of cells
1 ng	152
0.5 ng	76
0.25 ng	38
0.125 ng	19
0.0625 ng	10

Robin Cotton, AAFS 2003 LCN Workshop
"Are we already doing low copy number (LCN) DNA analysis?"

Stochastic Statistical Sampling



>20 copies per allele

6 copies copies per allele **(LCN)**

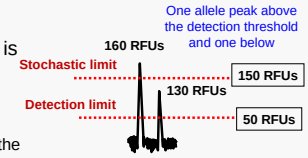
Stochastic Effect

- Sometimes called "preferential amplification" – not really a correct term since either allele may be amplified if the other drops-out…not related to allele size
- Stutter product amounts may go up…
  - If in an early cycle of PCR, the stutter product is amplified more (due to sampling effect)
- Contaminating DNA can also be amplified giving rise to allele "drop-in" or a mixture

Types of Results at Low Signal Intensity
(Stochastic amplification potential)

Straddle Data

- Only one allele in a pair is above the laboratory stochastic threshold



At low levels of input DNA, the potential for straddle data is high.

The issue is best avoided by re-amplifying the sample at higher input DNA

Otherwise **straddle data makes locus inconclusive**

Straddle data may be caused by degradation, inhibition and low copy issues.

Issues with Data Below the Stochastic Threshold

- PCR artifacts and stutter become prevalent
- Low levels of bleed through are possible
- Instrument spikes are more numerous
- -A peaks may appear
- Dye blobs become more significant in overall e-gram
- Low level 2nd contributors may show peaks

Challenges and limitations with LCN DNA testing

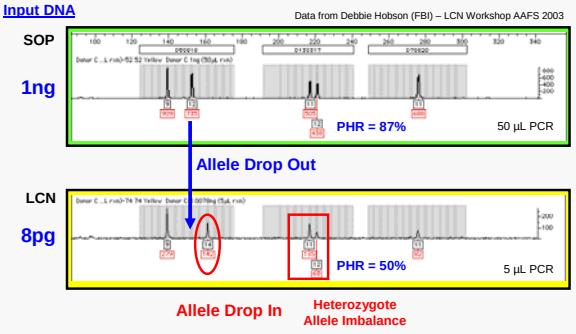
Challenges of LCN

Gill, P. (2001) *Croatian Med. J.* 42(3): 229-232

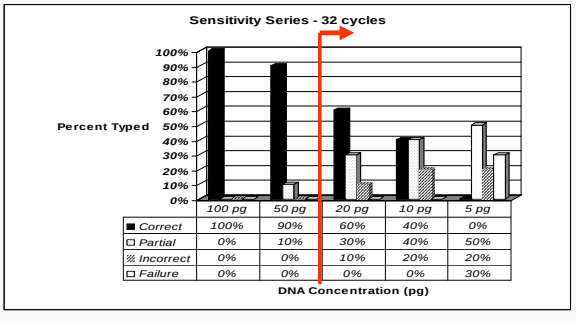
- Increased chance for contamination (want a sterile lab environment to reduce staff contamination)
- Data interpretation is more complicated (due to stochastic variation during PCR amplification):
  - Allele drop-out
  - Allele drop-in
  - Increased stutter products
  - Heterozygote peak imbalance

Comparison of STR Kit Amplification SOP with LCN

Using the Same DNA Donor



Problems with Obtaining Correct Allele Calls at Low DNA Levels



	100 pg	50 pg	20 pg	10 pg	5 pg
Correct	100%	90%	60%	40%	0%
Partial	0%	10%	30%	40%	50%
Incorrect	0%	0%	10%	20%	20%
Failure	0%	0%	0%	0%	30%

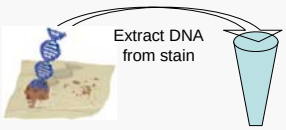
Coble, M.D. and Butler, J.M. (2005) *J. Forensic Sci.* 50: 43-53

Suggestions for Optimal Results with LCN

- At least two* PCR amplifications from the same DNA extract (if enough DNA is present to do more than 4-5 amplifications, then most likely a single aliquot would be run under standard STR typing conditions)
- An allele cannot be scored (considered real) unless it is present at least twice in replicate samples
- Extremely sterile environment is required for PCR setup to avoid contamination from laboratory personnel or other sources

*five is better; results are typically viewed as investigative

Typical LCN Procedure



Extract DNA from stain

Quantify Amount of DNA Present

Perform 3 Separate PCR Amplifications

Interpret Alleles Present

Develop a Consensus Profile
(based on replicate consistent results)

New Interpretation Rules Required for LCN

ELSEVIER

Forensic Science International
112 (2000) 17–40

Forensic Science International

www.elsevier.com/locate/forsciint

An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA

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Replicate LCN Test Results from FSS

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.

Table 2. Results of Six Replicate PCR Tests of a Sample Under Low Copy Number Analysis Conditions Compared to the Control Sample

	Amelo	D19	D3	D8	THO	VWA	D21	FGA	D16	D18	D2
CONTROL	X X	14,14	18,18	15,15	7 9.3	19,19	28 32.2	20,23	9,12	12,16	17,23
Sample											
1	--	14 F'	--	15 F'	--	--	28 32.2	20 F'	--	16 F'	--
2	X F'	--	18 F'	15 F'	--	19 F'	--	--	12 F'	--	--
3	X F'	--	--	15 F'	--	--	--	--	--	--	17 F'
4	X F'	14 F'	18 F'	--	--	--	--	--	9 12	--	--
5	X F'	--	18 F'	--	--	18 F'	--	--	--	--	--
6	X F'	14 F'	--	--	--	19 F'	28 32.2	20 F'	--	12 F'	--
Consensus	X F'	14 F'	18 F'	15 F'	--	19 F'	28 32.2	20 F'	12 F'	--	--

The consensus result is reported, provided that an allele is observed at least twice. If only one allele is observed, then an F' designation is given to denote the possibility of allele drop-out.

F' used to designate that allele drop-out of a second allele cannot be discounted when only a single allele is observed (OCME uses "Z")

Example LCN Data

Experimental Design

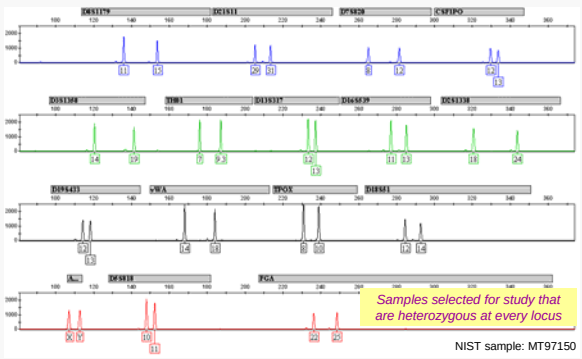
- 3 samples (Caucasian, African American, and Hispanic) that are heterozygous for all loci tested (2 peaks for each locus)
- DNA templates tested: 100 pg, 50 pg, and 10 pg
- Tested in triplicate
- Identifiler kit was used (1/2 reactions)
- Tested with 2 different cycles: 31 and 34

Data Analysis

- Determining consensus profile – 2 out of 3 times the allele is observed
- Concordance analysis with samples run with "normal" parameters (non-LCN conditions – 28 cycles) and higher concentrations (at 1 ng DNA)
- Summarizing incorrect allele calls, heterozygote peak imbalance, allele drop-out, locus drop-out, stutter percentages, and non-specific artifacts

1 ng template DNA, 28 cycles

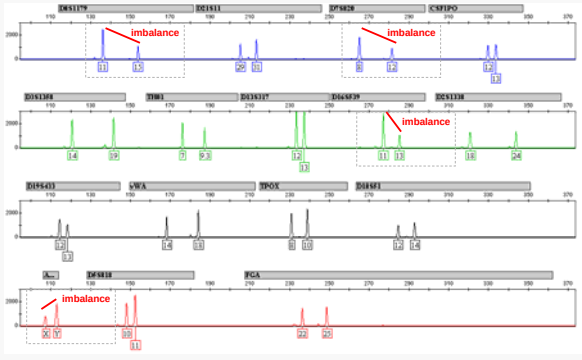
Identifiler data (full profile)



Peak Height Ratios (PHRs) all >0.80

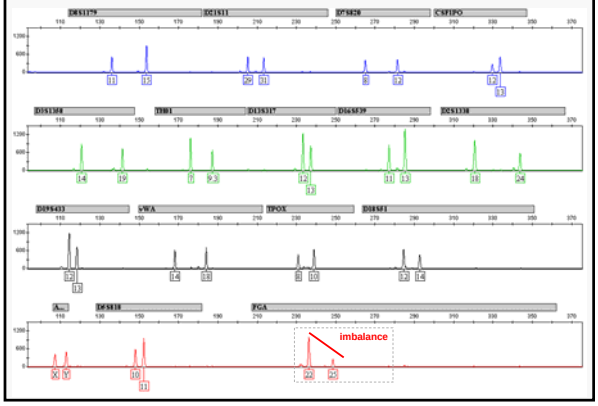
100 pg template DNA with 31 cycles of PCR

Identifiler data (full profile)



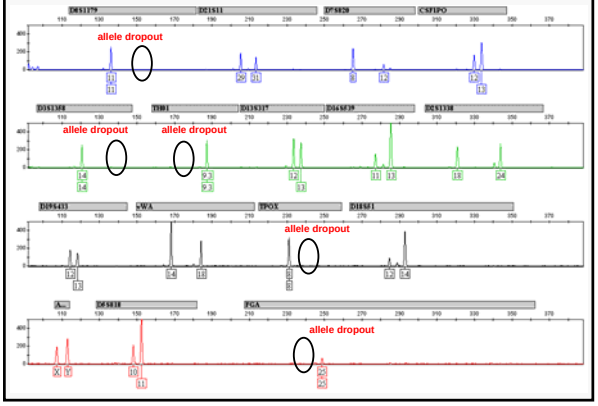
50 pg template DNA with 31 cycles of PCR

Identifiler data (full profile)



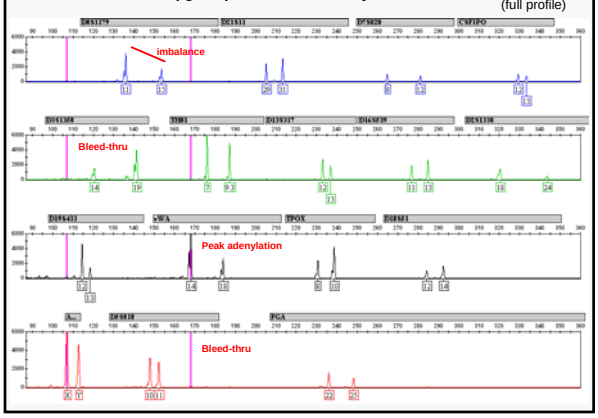
10 pg template DNA with 31 cycles of PCR

Identifiler data (full profile)



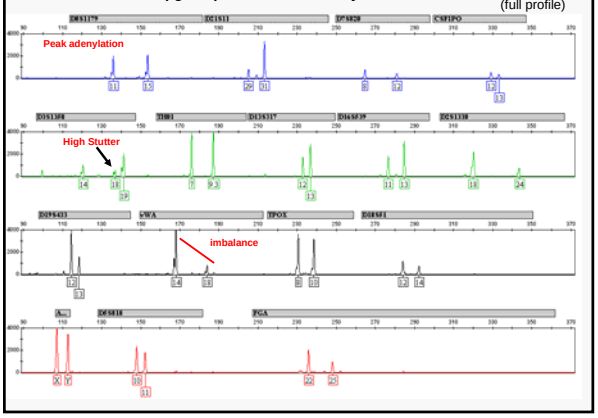
100 pg template DNA with 34 cycles of PCR

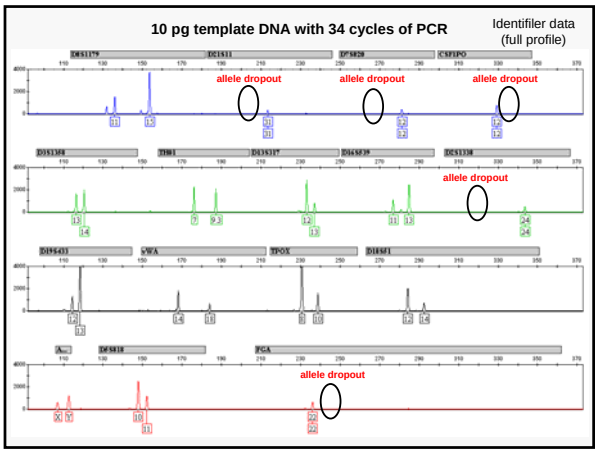
Identifiler data (full profile)



50 pg template DNA with 34 cycles of PCR

Identifiler data (full profile)





Peak Height Ratio Measurements

Identifiler STR Kit – only FGA shown

Signal aided with 31 PCR cycles

		Peak Heights (RFUs) FGA-22	FGA-25	PHR	Average PHR
100 pg	(1)	1692	1517	0.90	0.69 (±0.23)
	(2)	1915	864	0.45	
	(3)	1239	909	0.73	
50 pg	(1)	992	260	0.26	0.49 (±0.36)
	(2)	1422	419	0.29	
	(3)	895	805	0.90	
10 pg	(1)	--	66	0	0.37 (±0.32)
	(2)	54	107	0.50	
	(3)	130	219	0.59	

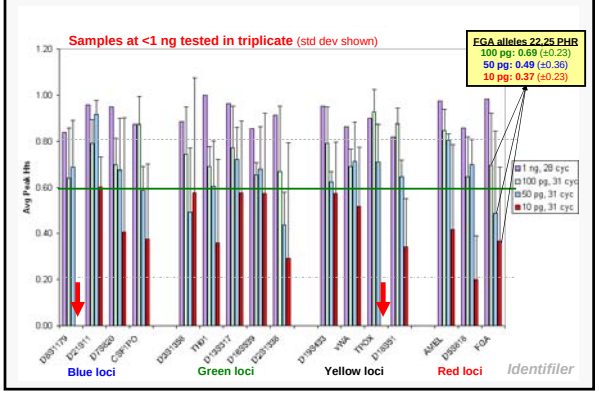
Pretty good balance

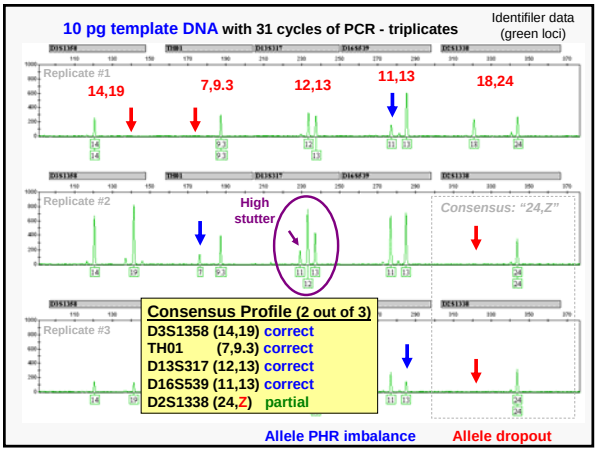
Severe imbalance

allele dropout

All levels performed in triplicate...

Peak Height Ratio Comparisons



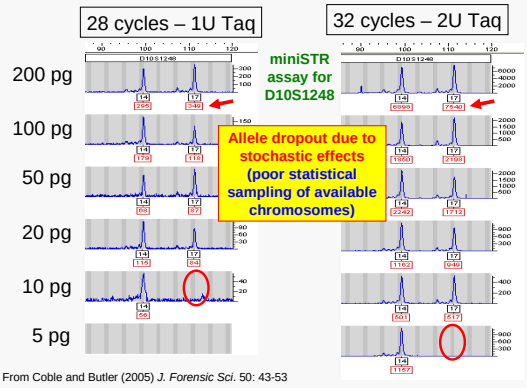


Other methods for higher sensitivity and signal enhancements

Improving Sensitivity

- Improved recovery of biological material and DNA extraction
- Longer injection on CE
- Salt removal from CE sample – enhances electrokinetic injection
- Reduced volume PCR – concentrates amplicon
- Increase number of cycles in PCR and/or TaqGold concentration
- Use miniSTRs – shorter amplicons amplify better; **MiniFiler**
- Use mtDNA – higher copy number per cell

Higher Sensitivity with More Polymerase and Cycle Numbers



From Coble and Butler (2005) *J. Forensic Sci.* 50: 43-53

Modifications in DNA Analysis Process to Improve LCN Success Rates

- **Collection** – better swabs for DNA recovery
- **DNA Extraction** – into smaller volumes
- **DNA Quantitation** – qPCR helps with low DNA amounts
- **PCR Amplification** – increased number of cycles
- **CE Detection** – longer electrokinetic injection; more sensitive fluorescent dyes
- **Interpretation** – composite profile from replicate analyses with at least duplicate results for each reported locus
- **Match** – is it even relevant to the case?

LCN Summary

- LCN often defined as <100-200 pg input DNA
- Typically involves increasing the number of PCR cycles when performing multiplex PCR to amplify DNA with conventional STR kits (e.g., 34 cycles instead of 28 cycles)
- Enables lower amounts of DNA to be detected with STR markers but is prone to contamination
- Cautious data interpretation rules must be adopted as allele drop-out and drop-in may occur due to stochastic amplification effects

Thank you for your attention…

Our team publications and presentations are available at:
http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm

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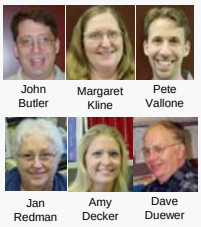

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