MAAFS DNA Workshop

Introduction to Low Copy Number (LCN) DNA Testing Issues

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Presentation Outline

- LCN – what is it and why attempt it?
- DNA quantity in samples and qPCR
- Stochastic PCR amplification
- Caution & challenges with LCN
- Literature summary: DNA from fingerprints, single cells
- Consensus profiles and LCN interpretation rules
- Contamination and efforts to avoid it
- Secondary transfer and variable shedding
- Whole genome amplification – is it a solution to LCN?
- Other methods for higher sensitivity and signal enhancements
- Summary and final thoughts

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 pg)
- 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

LCN is not a "new" technique…

- 1996 – Taberlet *et al.* describe "reliable genotyping of samples with very low DNA quantities using PCR"
- 1997 - single cell STR analysis reported
- 1999 – Forensic Science Service begins LCN casework in UK (as an alternative to mtDNA)
- 2001 – Budowle and FBI co-authors urge caution with using LCN

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

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

LCN in the News…

"New tool for DNA testing" (Feb 4, 2006)
by Jo McKenzie-McLean in Fairfax **New Zealand** Limited

"The new [LCN] technique, which involves amplifying the DNA through 34 cycles instead of the usual 28, [will] be a powerful tool for New Zealand police, judging by its success in Britain"

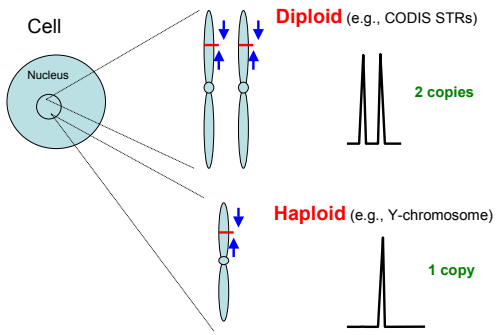
"British forensic scientists have had between a **70 per cent and 90% success rate** getting profiles from cold cases where previous techniques had failed"

"The new technique was about **10 times more sensitive than traditional procedures** and could be used to test minute samples"

http://www.stuff.co.nz/stuff/print/0,1478,3561521a11,00.html

DNA quantity in samples

Diploid vs. Haploid



Calculation of the Quantity of DNA in a Cell

1. **Molecular Weight of a DNA Base Pair = 618 g/mol**

   A = 313 g/mol; T = 304 g/mol; A-T base pairs = 617 g/mol
   G = 329 g/mol; C = 289 g/mol; G-C base pairs = 618 g/mol

2. **Molecular Weight of DNA = 1.98 x10^{12} g/mol**

   There are **3.2 billion base pairs** in a haploid cell ~3.2 x 10^9 bp
   (~3.2 x 10^9 bp) x (618 g/mol/bp) = 1.98 x 10^{12} g/mol

3. **Quantity of DNA in a Haploid Cell = 3 picograms**

   1 mole = 6.02 x 10^{23} molecules
   (1.98 x 10^{12} g/mol) x (1 mole/6.02 x 10^{23} molecules)
   = 3.3 x 10^{-12} g = 3.3 picograms (pg)
   A diploid human cell contains ~6.6 pg genomic DNA

4. **One ng of human DNA comes from ~152 diploid cells**

   1 ng genomic DNA (1000 pg)/6.6pg/cell = **~303 copies of each locus**
   (2 per 152 diploid genomes)

Adapted from D.N.A. Box 3.3, J.M. Butler (2005) Forensic DNA Typing, 2nd Edition (Elsevier Academic Press), p. 56

At the 2003 AAFS LCN Workshop (Chicago,IL), **Robin Cotton** from Orchid Cellmark presented a talk entitled "Are we already doing low copy number (LCN) DNA analysis?"

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

Values for # of cells adjusted to reflect updated DNA quantitation numbers

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

Assume sample is from a **single source**:

Amount of DNA	Total Cells in sample	~ # of copies of each allele if het.
1 ng	152	152
0.5 ng	76	76
0.25 ng	38	38
0.125 ng	19	19
0.0625 ng	10	10

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

Assume sample is a **1:1 mixture** of two sources:

Amount of DNA	Total Cells in sample	~ # of cells from each component
1 ng	152	76
0.5 ng	76	38
0.25 ng	38	19
0.125 ng	19	10
0.0625 ng	10	5

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

Assume sample is a **1:3 mixture** of two sources:

Amount of DNA	~ # of cells from **major component**	~ # of cells from **minor component**
1 ng	114	38
0.5 ng	57	19
0.25 ng	28	10
0.125 ng	14	5
0.0625 ng	7	2

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

Assume sample is a **1:9 mixture** of two sources:

Amount of DNA	~ # of cells from **major component**	~ # of cells from **minor component**
1ng	137	15
0.5ng	68	8
0.25ng	34	4
0.125ng	17	2
0.0625ng	9	1

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

Low copy number situations exist in many samples

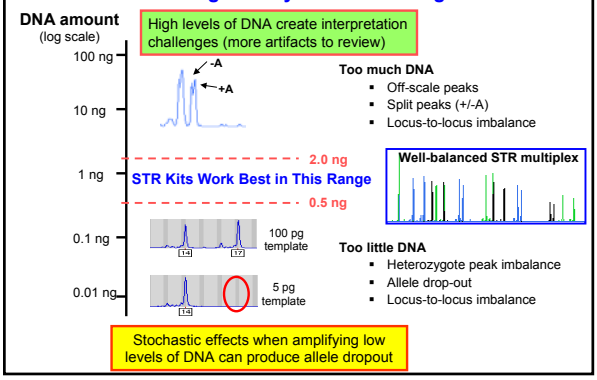
- In a 1:1 mixture, each DNA source is at LCN when the total amount of DNA in the amplification reaction is ~ 0.125 ng.
- In a 1:9 mixture, the minor component could be at LCN **even when the total amount of DNA in the amplification is 1 ng**.

Two different amplifications would be useful with a 1:9 mixture situation:
Normal level of total DNA (e.g., 1 ng) so that major component is on-scale
High level of total DNA (e.g., 5 ng) so that minor (e.g., ~500 pg) is out of LCN realm – yes, the major component will be off-scale…

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

Impact of DNA Amount into Multiplex PCR Reaction

We generally aim for 0.5-2 ng



Stochastic PCR amplification

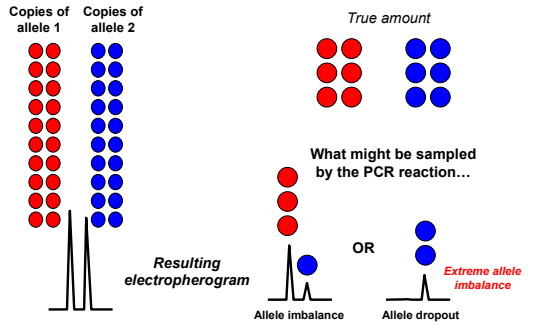
Stochastic = random selection

Stochastic Fluctuation Effects

- Unequal sampling of the two alleles present in a heterozygous individual can occur when low levels of input DNA are used (**results in allele drop-out**)
- PCR reactions **with <100 pg** (~17 diploid copies)
- Walsh *et al.* (1992) – propose avoiding stochastic effect by adjusting the number of PCR cycles in an assay so that the sensitivity limit is around 20 or more copies of target DNA (i.e., a full profile is obtained with ~125 pg)

Walsh PS, Erlich HA, Higuchi R. Preferential PCR amplification of alleles: Mechanisms and solutions. *PCR Meth Appl* 1992; 1:241-250.

Stochastic Statistical Sampling

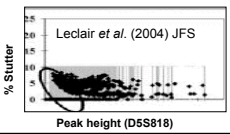


>20 copies per allele

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

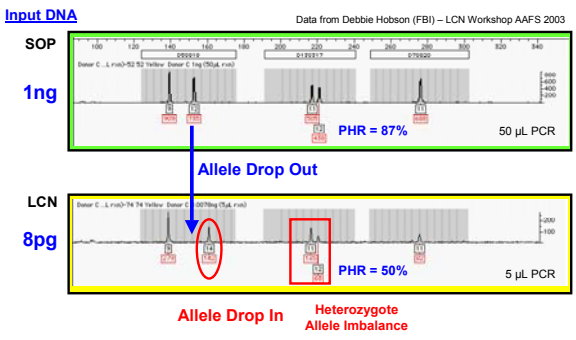
Stochastic Effect

- 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



Comparison of STR Kit Amplification SOP with LCN

Using the Same DNA Donor

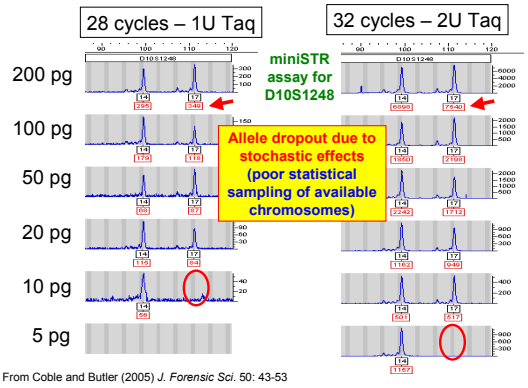


Balance of Assay Sensitivity and Potential for Stochastic Effects

- **One of the ways that assays can be made more sensitive is by increasing the number of PCR amplification cycles**
- Optimal cycle number will depend on desired assay sensitivity
- The number of PCR cycles was set to 28 for ABI STR kits to limit their sensitivity for generating full profiles to ~125 pg or 20 cells
- Sensitivity is a combination of fluorescent dye characteristics (relative to the instrument and laser excitation used) and PCR amplification conditions such as primer concentration and amount of polymerase used

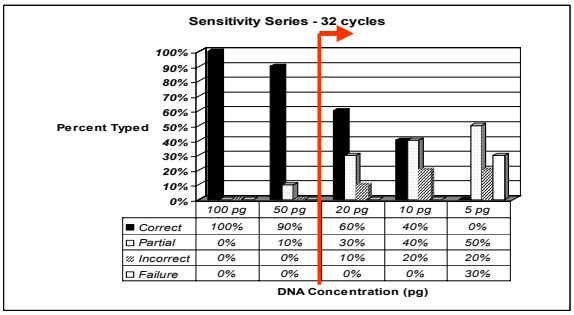
Note that Promega STR kits use higher numbers of cycles to generate roughly equivalent sensitivity to ABI kits because they have less efficient dye labels and lower primer and polymerase concentrations

Higher Sensitivity with More Polymerase and Cycle Numbers



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

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

What is a true peak (allele)?

GeneScan function — **Peak detection threshold**

Signal (S)

Noise (N)

Signal > 3x sd of noise

Genotyper function — **Peak height ratio (PHR)**

Allele 1

Allele 2

Heterozygote peak balance

PHR consistent with single source Typically above 60%

Stutter percentage

True allele

Stutter product

Stutter location above 15%

Threshold Settings for the ABI 310/3100

Detection Limit: 3x the standard deviation of the noise.
Estimated using **2x peak to peak noise**. (approximately 35 - 50 RFUs)

Limit of Quantitation: 10x the standard deviation of the noise
Estimated using **7x peak to peak noise** (150-200 RFUs)
Below this point estimates of peak area or height are unreliable.

Dynamic Range: The range of sample quantities that can be analyzed from the lowest to the highest **(linear range is also important)**

Stochastic Threshold: Level of quantifiable DNA below which peaks can show severe imbalance (peak height ratios below 60%) Approximately 150 -200 RFUs. Enhanced stutter also occurs at these signal levels.

The Scientific Reasoning behind the Concept of an Analytical Threshold (limit of detection)

- This is fundamentally an issue of reliability
- **For a peak intensity three times the standard deviation of the noise there is a limited chance that such a signal is the result of a random fluctuation**
- This is because 99.7 percent of all noise signals fall below this value (from the definition of a Gaussian curve)
- **Below this point the very real possibility exists that what you think is a peak is simply a statistical fluctuation in the baseline noise**.

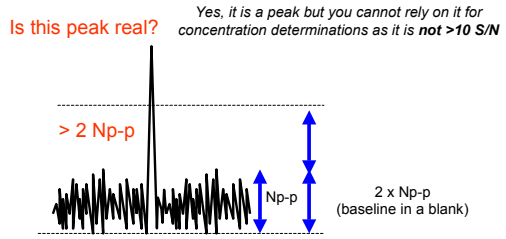
Sensitivity

- Limit of detection (**LOD**) – "the lowest content that can be measured with reasonable statistical certainty."
- Limit of quantitative measurement (**LOQ**) – "the lowest concentration of an analyte that can be determined with acceptable precision (repeatability) and accuracy under the stated conditions of the test."
- How low can you go?

EURACHEM Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*, p. 43; available at http://www.eurachem.ul.pt/guides/valid.pdf

Limit of Detection (LOD)

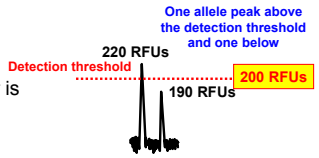
- Typically 3 times the signal-to-noise (based on standard deviation of the noise) or 2x Np-p



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

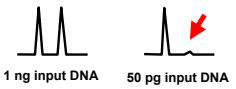
Straddle Data

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



Allelic Drop-out

- one or more sets of alleles do not amplify



TWGDAM validation of AmpFISTR Blue
Wallin *et al.* (1998) *J. Forensic Sci.* 43(4): 854-870

- Minimum cycle # (27-30 cycles examined)
- **Amplification adjusted to 28 cycles so that quantities of DNA below 35pg gave very low peaks or no peaks** (below the analytical threshold!)
- 35 pg is approx 5 cells
- (but is 35pg the analytical threshold?) Determining this value might be a useful goal of a validation study

TWGDAM validation of AmpFISTR Blue
Wallin *et al.* (1998) *J. Forensic Sci.* 43(4): 854-870

Determination of Minimum Sample

- Goal: avoid situations where peak imbalance results in only one detectable allele from a heterozygous pair.
- Perform serial dilution (1ng- 8pg) of 2 control samples which were heterozygous at all 3 loci
  - **Samples above 125pg had peak height RFUs above 150**
  - **Below 125pg peak heights were not significantly above background**
  - **At 31 pg peaks were very low or undetectable**
- **"Peaks below 150 RFU should be interpreted with caution"** **Why? Noise and stochastic fluctuation!**

Sensitivity of Detection
Moretti et al, JFS, 2001, 46(3), 661-676

- Different 310 instruments have different sensitivities; determination of stochastic threshold should be performed following in-house studies
  - Variations in quantitation systems
  - Variations in amplification systems
  - Variations in instrument sensitivity
- Peaks with heights below the threshold should be interpreted with caution
  - Caution should be used before modification of
    - Amplification cycles
    - Electrophoretic conditions

How to determine the stochastic threshold

- Examine intensity and peak height ratio of 5 samples at three different low concentrations (e.g., 60, 75, and 125 pg)
- Observe variation in peak height ratio and peak intensity
- The stochastic threshold is the point at which this variation begins a rapid increase (change in slope of line relating std dev vs concentration)
- This can also be defined as the concentration at which a set percentage of peak height ratio values fall below 60%

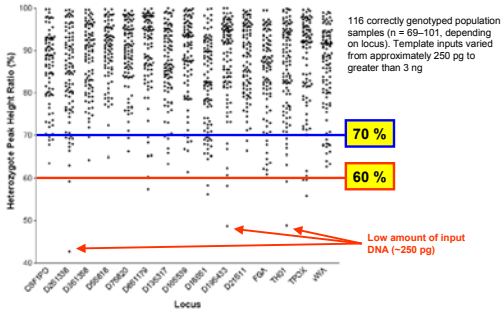
TWGDAM validation of AmpFISTR BluePCR

Wallin et al.JFS, 1998 43(4) 854-870

- In approximately 80 heterozygous loci in population samples:
  - Average peak height ratio was 92% for each locus – D3, vWA, FGA
  - Standard deviation was 7%
- Thus 99.7% of all samples should show a peak height ratio (PHR) above 71%
- Those that have a PHR of <70% may result from mixtures, low [DNA], inhibition, degradation or poor primer binding

Heterozygote Peak Height Ratios

Identifiler STR Kit Developmental Validation



116 correctly genotyped population samples (n = 69–101, depending on locus). Template inputs varied from approximately 250 pg to greater than 3 ng

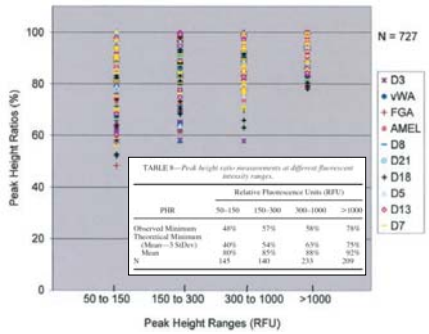
Collins PJ, Hennessy LK, Leibelt CS, Roby RK, Reeder DJ, Foxall PA. Developmental validation of a single-tube amplification of the 13 CODIS STR loci, D2S1338, D19S433, and amelogenin: the AmpFISTR Identifiler PCR amplification kit. *J. Forensic Sci.* 2004; 49(6): 1265-1277.

Peak height ratios

Moretti et al., JFS 2001, 46(3) 647-660

- PP + Cofiler gave PHR >88% n= 230+ samples with a lower range PHR (-3sd) of 59%
- Suggest using 59% as a guide
- 2% of single source samples were below this value
- Many validation studies focus on 1ng input DNA. What happens with lower amounts?

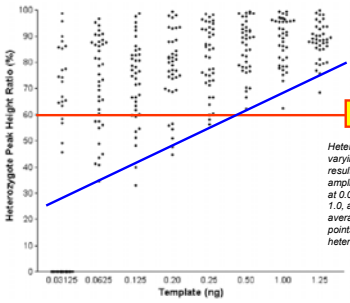
Heterozygote Peak Height Ratios



Holt CL, Buoncristiani M, Wallin JM, Nguyen T, Lazaruk KD, Walsh PS. TWGDAM validation of AmpFISTR PCR amplification kits for forensic DNA casework. *J Forensic Sci* 2002; 47(1): 66-96.

Heterozygote Peak Height Ratios

Identifiler STR Kit Developmental Validation



Heterozygote peak height ratios with varying inputs of template DNA. The results depicted are from three amplifications of a single genomic DNA at 0.03125, 0.0625, 0.125, 0.2, 0.25, 0.5, 1.0, and 1.25 ng. Multiple injections were averaged, resulting in a total of 39 data points per input amount (13 heterozygous markers × 3 repetitions).

Collins PJ, Hennessy LK, Leibelt CS, Roby RK, Reeder DJ, Foxall PA. Developmental validation of a single-tube amplification of the 13 CODIS STR loci, D2S1338, D19S433, and amelogenin: the AmpFISTR Identifiler PCR amplification kit. *J. Forensic Sci.* 2004; 49(6): 1265-1277.

Peak Height Ratio Guidelines

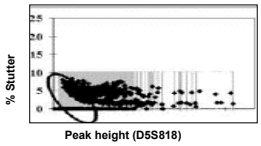
- One way to approach concentration dependence
- Profiler Plus
  - 200 to 300 RFU: 55 to 60%
  - 300 to 1000 RFU: 60 to 65%
  - above 1000 RFU: 65 to 70%
- Cofiler
  - 200 to 300 RFU: 60%
  - 300 to 1000 RFU: 60 to 65%
  - above 1000 RFU: 70% to 75%

TWGDAM validation of AmpFlSTR Blue

Wallin et al.JFS, 1998 43(4) 854-870

- Stutter increases with allele size:
- Greatest amount was 8.6% w/ sd of 0.6, thus 8.6 + 3(0.6) = 10.4 or approx. 11% is the stutter threshold
- Stutter increases at low copy #

Leclair et. al (2004) Systematic analysis of stutter percentages and allele peak height and peak area ratios at heterozygous STR loci for forensic casework and database samples. J. Forensic Sci. 49(5): 968-980



Issues with Data below the Stochastic threshold and above the analytical 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

Typically between 50 and 200 RFU – depends on validation studies

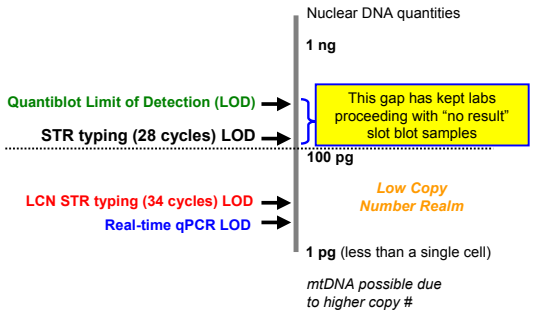
Why do you want to be in the DNA quantitation "sweet spot"?

Higher quality data which results in easier data interpretation

- Better balance across loci,
- Peaks on-scale with no pull-up from dye bleedthrough
- No split peaks from partial adenylation
- No stochastic effects on amplification

- STR kits, especially those amplifying more loci, are optimized for a narrow range of input DNA

Difference in DNA Quantitation Capability vs. STR Typing Sensitivity



Proceeding with Testing when "No DNA" Detected

If the qPCR results indicate that there is no detectable DNA, will you stop testing or will you proceed with attempting STR typing?

- The practice of proceeding even with a "no result" Quantiblot was **because the STR typing assay was more sensitive than the quantification method**.

- **What types of experiments might be done to satisfy you that "no result" from a qPCR assay is truly "no DNA"?**

qPCR for DNA Quantitation

– will it lead more labs into LCN?

DNA Quantitation with Real-Time qPCR

- RT-qPCR is a homogeneous PCR based method that enables human specific quantification
  - Is easily automated, provides electronic storage of data
  - SYBR green or targeted probes (e.g., TaqMan) can be used
- Results give quantity of amplifiable DNA – not necessarily overall quantity
  - PCR inhibition can be detected
  - Limited multiplexing can be performed
- Big advantages are speed and dynamic range
- Commercial kits are now available (e.g., **Quantifiler**)

qPCR Assays Are Also Impacted by Stochastic Sampling in the LCN Region

Quantifiler Kits User's Manual, p. 6-17

Note the larger spread in these dilution series points for the LCN samples (16 pg) because of stochastic sampling

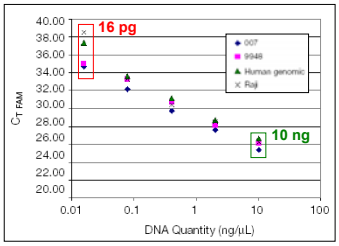


Figure 6-4 Sensitivity using the Quantifiler Y kit

Remember that DNA quantitation assays are also impacted by stochastic problems and may not be extremely reproducible on the low end, i.e., <100 pg…

Challenge with Being Able to Go Lower In DNA Quantitation Measurements

- Multi-copy marker (e.g., Alu assay) will be better than a single copy (e.g., Quantifiler) with qPCR of low quantity DNA samples
- qPCR enables measurement of lower amounts of DNA but…
- Going into the low copy number realm introduces new challenges
  - Interpretation of mixtures
  - Defining thresholds for different dyes and amplification systems
  - Defining the difference between investigative data and reliable "court-worthy" data

Caution & Challenges with LCN

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)

Reagent contamination

- Data interpretation is more complicated (due to stochastic variation during PCR amplification):
  - Allele drop-out
  - Allele drop-in
  - Increased stutter products

LCN profiles may be interpreted with careful guidelines

- May not be able to associate DNA profile with bloodstain or other visual evidence

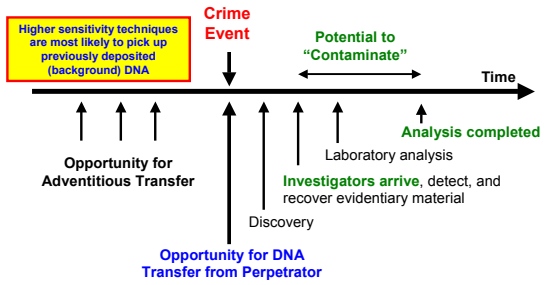
Hierarchy of Propositions

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

Premise that scientific evidence may only be interpreted if at least two competing propositions are considered

- Level III – Offense level
  - A) Suspect is the offender
  - B) Suspect is unconnected with the incident
- Level II – Activity level
  - A) Suspect broke the window at the scene
  - B) Suspect is unconnected with the incident
- Level I – Source level
  - A) Bloodstain came from the suspect
  - B) Bloodstain came from some unknown person
- Sub-level I proposition – LCN regime because there are additional uncertainties regarding source of DNA sampled … scientist cannot express a strong opinion about how DNA arrived at the site where it was recovered

Time Line Showing the Potential for DNA Deposition/Transfer



Adapted from Gill, P. (2002) *BioTechniques* 32(2): 366-385, Figure 5

Low Copy Number Limitations:

From Bruce Budowle (2005) 1st International Human Identification E-Symposium

- Allele drop out
- Allele drop in – contamination
- Increased stutter
- Heterozygote imbalance
- No thresholds
- Kits have not been subjected to QC under LCN conditions
- Reproducibility

http://www.e-symposium.com/humid/archive/drbrucebudowle.php

Low Copy Number Limitations (cont):

From Bruce Budowle (2005) 1st International Human Identification E-Symposium

- Tissue source cannot be determined
- DNA may not be relevant – casual contact/transfer
- If victim and suspect have any common access…
- Old cases may not be viable – handling
- Not for post conviction analysis
- Rarely useful for database searches
- Statistics

http://www.e-symposium.com/humid/archive/drbrucebudowle.php

Low Copy Number Philosophy

From Bruce Budowle (2005) 1st International Human Identification E-Symposium

- Cannot exclude
- Dilution for redundancy (no concentration)
- Reviewed within context of case
- Speculation
- Intelligence tool

http://www.e-symposium.com/humid/archive/drbrucebudowle.php

Low Copy Number Application

From Bruce Budowle (2005) 1st International Human Identification E-Symposium

- Investigative tool
- Missing person/remains
- Samples that can be cleansed

http://www.e-symposium.com/humid/archive/drbrucebudowle.php

Literature Summary

Early LCN Literature

- **Early work on touched objects and single cells**:
  - van Oorschot, R. A. and Jones, M. K. (1997) DNA fingerprints from fingerprints. *Nature*. 387(6635): 767
  - Findlay, I., Taylor, A., Quirke, P., Frazier, R., and Urquhart, A. (1997) DNA fingerprinting from single cells. *Nature*. 389(6651): 555-556
- **Application to routine forensic casework was pioneered by the Forensic Science Service:**
  - Gill, P., Whitaker, J., Flaxman, C., Brown, N., and Buckleton, J. (2000) 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
  - 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(2-3): 215-223
  - Gill, P. (2001) Application of low copy number DNA profiling. *Croatian Medical Journal* 42(3): 229-32

Other Useful LCN Articles (1)

- Budowle, B., Hobson, D.L., Smerick, J.B., Smith, J.A.L. (2001) Low copy number – consideration and caution. *Proceedings of the Twelfth International Symposium on Human Identification*. Available at http://www.promega.com/geneticidproc/ussymp12proc/contents/budowle.pdf.
- Buckleton, J. and Gill, P. (2005) Low copy number. Chapter 8 in *Forensic DNA Evidence Interpretation* (Eds. J. Buckleton, C.M. Triggs, S.J. Walsh) CRC Press: Boca Raton, FL, pp. 275-297.
- 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.
- 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.
- Lowe, A., Murray, C., Whitaker, J., Tully, G., and Gill, P. (2002) The propensity of individuals to deposit DNA and secondary transfer of low level DNA from individuals to inert surfaces. *Forensic Sci. Int.* 129(1): 25-34.
- Rutty, G. N., Hopwood, A., and Tucker, V. (2003) The effectiveness of protective clothing in the reduction of potential DNA contamination of the scene of crime. *Int. J. Legal Med*. 117(3): 170-174.

Other Useful LCN Articles (2)

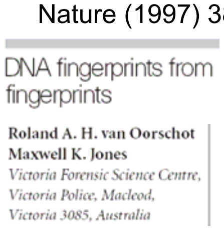
- Schneider, P.M., Balogh, K., Naveran, N., Bogus, M., Bender, K., Lareu, M., Carracedo, A. (2004) Whole genome amplification – the solution for a common problem in forensic casework? *Progress in Forensic Genetics 10 – International Congress Series* 1261: 24-26.
- Taberlet, P., Griffin, S., Goossens, B., Questiau, S., Manceau, V., Escaravage, N., Waits, L.P., and Bouvet, J. (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Res.* 24: 3189-3194.
- Van Oorschot, R.A.H., Phelan, D.G., Furlong, S., Scarfo, G.M., Holding, N.L., Cummins, M.J. Are you collecting all available DNA from touched objects? *Progress in Forensic Genetics 9 – International Congress Series* 1239: 803-807.
- Walsh, P. S., Erlich, H. A., and Higuchi, R. (1992) Preferential PCR amplification of alleles: Mechanisms and solutions. *PCR Meth. Appl.* 1: 241-250.
- Wickenheiser, R. A. (2002) Trace DNA: a review, discussion of theory, and application of the transfer of trace quantities of DNA through skin contact. *J. Forensic Sci.* 47(3): 442-450.

AAFS 2003 (Chicago) Workshop on LCN

http://www.metrokc.gov/sheriff/_img/partners/photo_afis_technology2.jpg

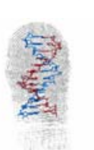
DNA from fingerprints

Nature (1997) 387: 767 Article Summary



DNA fingerprints from fingerprints

Roland A. H. van Oorschot
Maxwell K. Jones
Victoria Forensic Science Centre, Victoria Police, Macleod, Victoria 3085, Australia



http://darwin.nmsu.edu/~molbio/diabetes/images/fprint_5.gif

- Initial tests showed that they could readily obtain correct genetic profiles from swabs taken directly from the palm of a hand (13 of 13). DNA yields varied from 2 to 150 ng (average 48.6 ng). Dry hands and those that had been washed recently tended to provide the least DNA.

More Recent References on DNA from Fingerprints

- Alessandrini, F., Cecati, M., Pesaresi, M., Turchi, C., Carle, F., and Tagliabracci, A. (2003) Fingerprints as evidence for a genetic profile: morphological study on fingerprints and analysis of exogenous and individual factors affecting DNA typing. *J Forensic Sci.* 48(3): 586-592.
- Balogh, M. K., Burger, J., Bender, K., Schneider, P. M., and Alt, K. W. (2003) STR genotyping and mtDNA sequencing of latent fingerprint on paper. *Forensic Sci. Int.* 137(2-3): 188-195.
- van Oorschot, R. A., Treadwell, S., Beaurepaire, J., Holding, N. L., and Mitchell, R. J. (2005) Beware of the possibility of fingerprinting techniques transferring DNA. *J Forensic Sci.* 50(6): 1417-1422.

DNA fingerprinting from single cells

I. Findlay, A. Taylor, P. Quirke
Department of Molecular Oncology, Algernon Firth Building, University of Leeds, Leeds LS2 9LN, UK
R. Frazier, A. Urquhart
Forensic Science Service, Gooch Street North, Birmingham B5 6QQ, UK

Nature (1997) 389:555-556 Article Summary

- First time that single cells were typed using modern forensic techniques
- Used SGM assay (6 STR loci + amelogenin) with TaqGold and 34 cycles
- Analyzed 226 buccal cells from four different individuals, isolating each cell using micromanipulation procedures
- Amplified DNA in 91% (206/226) cells, obtaining a full DNA profile in 50% (114/226) and an acceptable profile (four or more STRs) in 64% of these cells (Table 1).

Findley *et al.* (1997) *Nature* article

Table 1 Details of analysis

Number of single cells analysed	226
Results obtained	206 (91%)
Amplification failure	20 (9%)
Full STR profile	114 (50%)
Acceptable profile (amelogenin,>4 STRs)	144 (64%)
Partial profile (1-4 STRs)	62 (27%)
Surplus alleles*	28 (12%)
False alleles**	11 (5%)
Allele dropout	88 (39%)

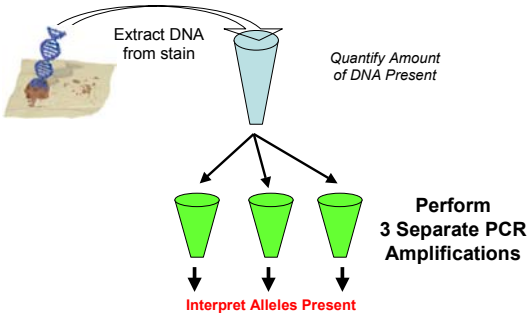
*Additional allele present in conjunction with true alleles.

**Additional allele in place of true allele. Extra-allelic peaks could be caused by contamination, somatic mutation or PCR-generated non-allelic peaks. We never saw more than two additional peaks in a profile or in 18 negatives, minimizing the possibility of cellular contamination. When surplus alleles were observed we considered the locus, but not the profile, uninformative. We observed allele dropout in 39% of cells at a rate of ~10% in each allele. If two cells are analysed then the risk of allelic dropout and misinterpretation in cells is reduced to 1%, if three cells 0.1%, and so on. Wild-card designations and conservative statistical criteria are needed to ensure that evidential value can be properly assessed.

Possible Reasons for Allele Dropout

- Failure to transfer the cell when a portion of the extract is analyzed
- Target sequence is degraded or not present in DNA template
- PCR amplification problems

Typical LCN Procedure



Develop a Consensus Profile
(based on replicate consistent results)

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")

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

Peter Gill[a,*], Jonathan Whitaker[a], Christine Flaxman[a], Nick Brown[a], John Buckleton[b]
[a]Forensic Science Service, Priory House, Gooch Street North, Birmingham B56QQ, UK
[b]ESR, Private Bag 92021, Auckland, New Zealand

Received 9 December 1999; received in revised form 12 February 2000; accepted 13 February 2000

Suggestions to 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 investigative

Contamination and efforts to avoid it

Increased Possibility of Contamination

- While LCN methods increase the sensitivity, they also increase the background DNA…
- There is a greater need for vigilance to reduce potential contamination at the crime scene, in the consumables (e.g., pipet tips, PCR tubes, etc.), and in the lab.
- Protective clothing can be worn (Rutty et al. 2003)

Contamination

- **Systematic**
  - e.g., Contaminated water or PCR buffer
- **Sporadic**
  - e.g., individual PCR tube contamination
- **To reduce risks of contamination**:
  - Careful lab cleanliness
  - Constant monitoring of reagents and consumables
- Contaminants are more likely to show up in the low molecular weight STR loci because they amplify more efficiently (miniSTRs will have a greater chance of detecting contaminating DNA)
- **A negative control can detect systematic contamination but may not detect sporadic contamination**, such as could be found in a single PCR tube

Although Rare, Some PCR Tubes Have Been Observed to Possess Contaminant DNA

- FSS observation
  - Howitt *et al.* (2003) *Proc. 14th Int. Sym. Hum. Ident.*
  - 11 casework-contaminating profiles in testing >1M samples
  - Use of negative control log and staff elimination databases
- "Contaminant" database …

Impact of Contamination on Casework

J Forensic Sci, May 2004, Vol. 49, No. 3
Paper ID JFS2003366
Available online at: www.astm.org

Peter Gill,[1] Ph.D. and Amanda Kirkham,[1] B.Sc.

Development of a Simulation Model to Assess the Impact of Contamination in Casework Using STRs

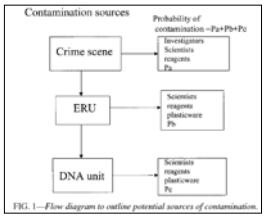


FIG. 1—*Flow diagram to outline potential sources of contamination.*

- **Use negative controls to predict the level of overall contamination in a lab**
- **Conclude that most likely outcome of a contamination event is a false exclusion**
  …if contaminating DNA is preferentially amplified over original LCN material

Secondary transfer and variable shedding

Potential DNA Transfer

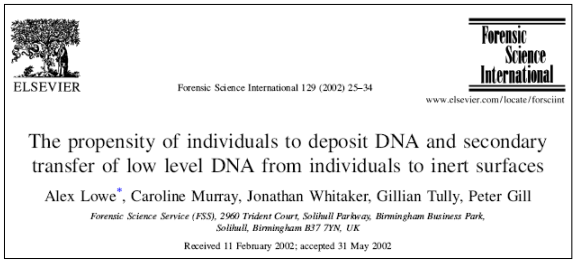
- Crime scene investigator → Scene
- Scene 1 evidence → Scene 2 evidence
- Innocent "passerby" → Scene (background DNA)

Issues of Transference and Persistence

- Transference – how easy is a DNA molecule transferred from a source to a recipient?
- Persistence – how long with a DNA molecule "stick around" after being deposited?

Lowe *et al*. (2002) *Forensic Sci. Int.* 129: 25-34

- Good shedders vs. poor shedders



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Forensic Science International 129 (2002) 25–34

Forensic Science International

www.elsevier.com/locate/forsciint

The propensity of individuals to deposit DNA and secondary transfer of low level DNA from individuals to inert surfaces

Alex Lowe*, Caroline Murray, Jonathan Whitaker, Gillian Tully, Peter Gill

Forensic Science Service (FSS), 2960 Trident Court, Solihull Parkway, Birmingham Business Park, Solihull, Birmingham B37 7YN, UK

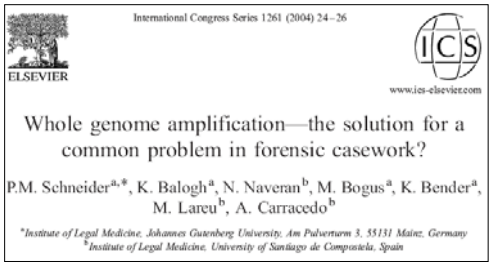
Received 11 February 2002; accepted 31 May 2002

Need for staff and police elimination databases

Whole genome amplification – is it a solution to LCN?

Whole Genome Amplification will also be subject to stochastic sampling when starting with LCN DNA templates

See Schneider *et al.* (2004) *Progress in Forensic Genetics 10*, ICS 1261: 24-26



International Congress Series 1261 (2004) 24–26

ELSEVIER

ICS

www.ics-elsevier.com

Whole genome amplification—the solution for a common problem in forensic casework?

P.M. Schneider[a,*], K. Balogh[a], N. Naveran[b], M. Bogus[a], K. Bender[a], M. Lareu[b], A. Carracedo[b]

[a]Institute of Legal Medicine, Johannes Gutenberg University, Am Pulverturm 3, 55131 Mainz, Germany
[b]Institute of Legal Medicine, University of Santiago de Compostela, Spain

Allele Dropout Seen with WGA at LCN Amounts of 50 pg and 5 pg

Schneider *et al.* (2004) *Progress in Forensic Genetics 10*, ICS 1261: 24-26

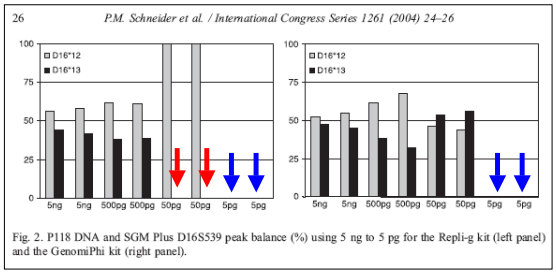


Fig. 2. P118 DNA and SGM Plus D16S539 peak balance (%) using 5 ng to 5 pg for the Repli-g kit (left panel) and the GenomiPhi kit (right panel).

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
- Use mtDNA – higher copy number per cell

Improving DNA Collection and Extraction Methods Will Help Recover More DNA

International Congress Series 1239 (2003) 803–807

Are you collecting all the available DNA from touched objects?

R.A.H. van Oorschot[a,*], D.G. Phelan[a,b], S. Furlong[a,b], G.M. Scarfo[a,b], N.L. Holding[a,b], M.J Cummins[a,b]

[a]Victoria Police, Victoria Forensic Science Centre, Forensic Drive, Macleod 3085, Victoria, Australia
[b]Department of Genetics, La Trobe University, Victoria 3086, Australia

Different Extraction Methods

Van Oorschot *et al.* (2003) *Progress in Forensic Genetics 9*, ICS 1239: 803-807

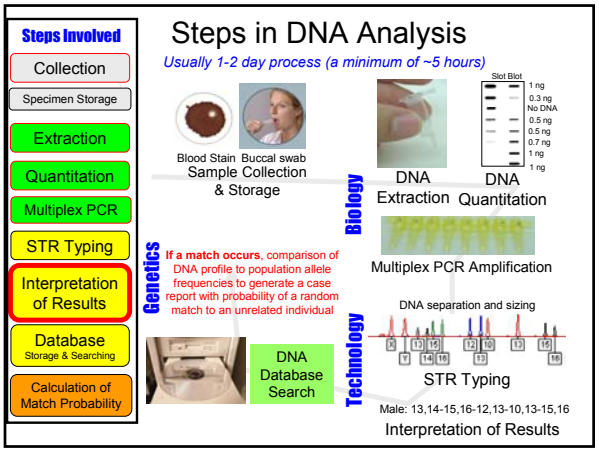
Table 1
Average[a] percentage amounts of DNA retrieved from different presentations using different extraction methods

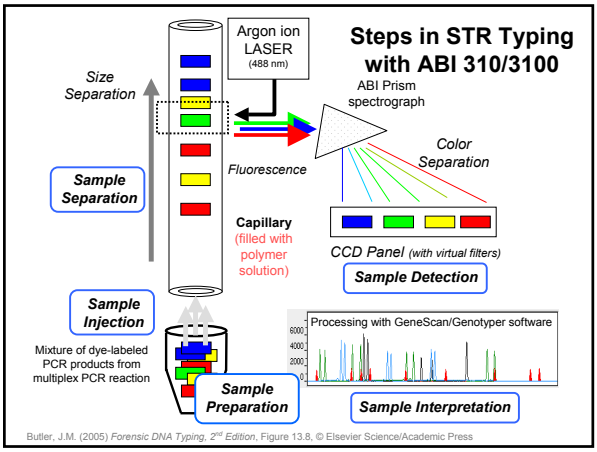
Sample extraction	100 ng in 100 µl Chelex	100 ng in 100 µl Organic	12.5 ng in 100 µl Chelex	12.5 ng in 100 µl Organic
Substrate[b]				
Dry cotton cloth	33 (19)	39 (18)	80 (36)	57 (18)
Wet cotton cloth	58 (26)	36 (10)	76 (17)	63 (18)
Dry swab stick	24 (14)	25 (13)	49 (20)	52 (17)
Wet swab stick	52 (45)	28 (15)	53 (22)	44 (13)
DNA control[2]	82 (31)	55 (30)	99 (26)	70 (28)

Note: aliquots of sample of the DNA utilised in these experiments were re-quantitated along with the samples generated from these experiments after extraction. Data were only included if these standard samples were quantitated as expected.

[a] The averages of 100 ng chelex, 100 ng organic, 12.5 ng chelex and 12.5 organic are from 9, 7, 9 and 6 repeats, respectively. The standard deviations are shown in parentheses.

[b] DNA placed on substrate and extracted from it while still wet or after drying. The DNA control represents DNA placed directly into an eppendorf tube.





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?

Signal Enhancements

- Higher PCR cycles
- Lower PCR volume (problems with inhibitors)
- Brighter fluorescent dyes
- Longer CE injection
  - 10 s @ 3 kV = 30
  - 5 s @ 2 kV = 10
- Microcon cleanup to remove salts that interfere with electrokinetic injection

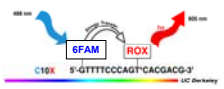
Higher Sensitivity

- Raising the number of PCR cycles creates a higher potential of allele drop-in being detected (increased noise)
- Ideally an improved fluorescent dye could be used to improve detection sensitivity and thereby permit a lower number of PCR amplification cycles to be used (peak intensity does not always correlate with stochastic effect)

High Sensitivity Energy Transfer Dye Labeling

Ju et al., Nature Medicine 2, 246 (1996)
Hung et al., Anal. Biochem, 243, 15 (1996)
Berti et al., Anal. Biochem. 292, 188 (2001)
Medintz et al., BioTechniques 32, 270 (2002)

ET dyes (example: LIZ) permit a 10-30X improvement in signal over non-ET dyes



Reduced Volume PCR

- Possibility of lower volume PCR to effectively concentrate the amount of DNA in contact with the PCR reagents
  - Gaines *et al*. (2002) *J. Forensic Sci*. 47(6):1224-1237
  - Leclair *et al*. (2003) *J. Forensic Sci*. 48: 1001-1013
- Can samples be concentrated or can extraction volume be reduced?

Leclair et al. (2003) JFS 48:1001-1013

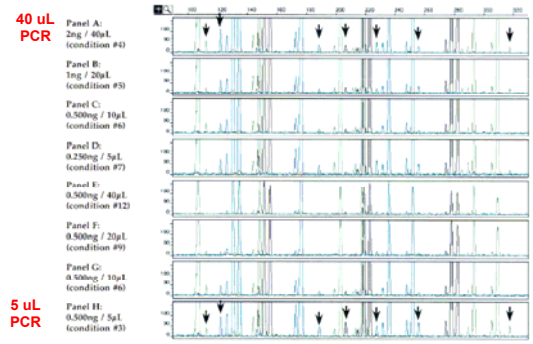


FIG. 5—*Effects of a reduction of PCR reaction volume and DNA template concentration on amplification of a casework sample with a minor profile representing 2% of the total mixture.*

Evidence Enrichment
Through Physical Capture of Sperm Cells

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Forensic Science International 137 (2003) 28–36

Forensic Science International

www.elsevier.com/locate/forsciint

Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides

K. Elliott*, D.S. Hill, C. Lambert, T.R. Burroughes, P. Gill

Research and Development, The Forensic Science Service, Trident Court, Solihull Parkway, Birmingham Business Park, Solihull B37 7YN, UK

Received 20 March 2003; received in revised form 23 June 2003; accepted 30 June 2003

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

Is LCN Effort Worthwhile?
Thoughts to Consider…

- Success rates are often low
- Requires dedicated "clean" facilities and extreme care to avoid limit contamination
- Complex interpretation procedure – requires more experienced analysts to do
- Significance of a DNA match?? – intelligence information but likely not to be probative due to unknown time when sample may have been deposited…

miniSTRs and LCN

- miniSTR assays are typically more sensitive than conventional STR kits currently in use
- Labs will start "pushing the envelope" in order to try and get a result with more sensitive assays including future miniSTR assays and kits
- Labs may move into the LCN realm without realizing it or adopting the careful LCN interpretation rules such as replicate analyses with duplicate results prior to reporting alleles

The Wisdom of Obi Wan Kenobi

http://www.starwars.com/kids/explore/lore/img/news20000902_1.jpg

Just before entering the Mos Eisley spaceport in Episode IV, Ben (Obi Wan) Kenobi warned Luke Skywalker, "You will never find a more wretched hive of scum and villainy… WE MUST BE CAUTIOUS!"

Legal Implications of Profiles

The presence of a suspect's profile does not constitute evidence that the suspect was present at the crime scene.

The absence of a suspect's profile does not constitute evidence of innocence.

Theresa Caragine (AAFS 2003 LCN Workshop)

DNA Profiles are An Investigative Tool

Finding DNA indicates contact.

Lack of a DNA profile is inconclusive.

LCN is analogous to a bigger, more powerful magnifying glass

Theresa Caragine (AAFS 2003 LCN Workshop)

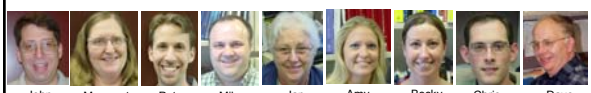
Introduction to Remaining LCN Speakers

- **Dr. Theresa Caragine**
- NYC Office of Chief Medical Examiner (OCME)
- Years of validation experience with LCN
- LCN casework since Jan 2006

- **Dr. Peter Gill**
- Forensic Science Service (FSS)
- Pioneered LCN technique
- Developed interpretation rules used in LCN
- Recent work has focused on simulation studies to understand allele dropout
- Working to develop new LCN expert system

Acknowledgments

National Institute of Justice funding from interagency agreement 2003-IJ-R-029 between NIJ and the NIST Office of Law Enforcement Standards



John Butler | Margaret Kline | Pete Vallone | Mike Coble **Now at AFDIL** | Jan Redman | Amy Decker | Becky Hill | Chris DeAngelis | Dave Duewer

Thank you for your attention…

Questions?

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

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301-975-4049
http://www.cstl.nist.gov/biotech/strbase