Advanced Topics in STR DNA Analysis

DNA Quantitation with Real-time qPCR and Low Copy Number Issues

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NIST

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Outline for This Section

- Why Quantify DNA?
- Slot blot vs. real-time qPCR
- qPCR theory
- qPCR assays available
- Low-copy number challenges
- Stochastic threshold

Purpose of Human-Specific DNA Quantitation

- All sources of DNA are extracted when biological evidence from a crime scene is processed to isolate the DNA present.
- Thus, non-human DNA such as bacterial, fungal, plant, or animal material may also be present in the total DNA recovered from the sample along with the relevant human DNA of interest.
- For this reason, the DNA Advisory Board **(DAB) Standard 9.3 requires human-specific DNA quantitation** so that appropriate levels of human DNA can be included in the subsequent PCR amplification.
- **Multiplex STR typing works best with a fairly narrow range of human DNA** – typically 0.5 to 2.0 ng of input DNA works best with commercial STR kits.

Calculation of the Quantity of DNA in a Cell

1. **Molecular Weight of a DNA Basepair = 618g/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 = 1.85 x10^{12} g/mol**
   There are 3 billion base pairs in a haploid cell ~3 x 10^9 bp
   (~3 x 10^9 bp) x (618 g/mol/bp) = 1.85 x 10^{12} g/mol
3. **Quantity of DNA in a Haploid Cell = 3 picograms**
   1 mole = 6.02 x 10^{23} molecules
   (1.85 x 10^{12} g/mol) x (1 mole/6.02 x 10^{23} molecules)
   = 3.08 x 10^{-12} g = 3.08 picograms (pg)
   A diploid human cell contains ~6 pg genomic DNA
4. **One ng of DNA contains the DNA from 167 diploid cells**
   1 ng genomic DNA (1000 pg)/6pg/cell = **~333 copies of each locus**
   (2 per 167 diploid genomes)

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

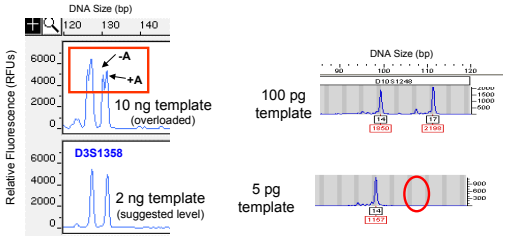
Higher quality data which results in easier data interpretation

- 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

Impact of DNA Amount into PCR

We generally shoot for 0.5-2 ng



Current Quantification Methods

- UV 280/254 – not sensitive not human or DNA specific
- Yield gel – not human specific, tells sample quality, not sensitive
- Fluorescence – not human specific, sensitive
- Slot blot – Human specific, sensitive, poor dynamic range
- RtPCR- human specific, very sensitive, good dynamic range

Slot Blot vs Real-time qPCR

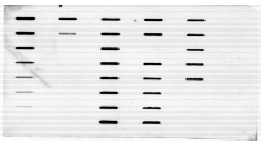
Slot Blot (Quantiblot)

- 2-3 hours of rinses, incubations, pipettings, washes, exposures, and developments
- Involves comparison to standards run simultaneously
- Semiquantitative by manual comparison or through scanner
- Quantity obtained will not reflect PCR inhibitors (amount of "amplifiable" DNA)

Real-time qPCR (Quantifiler or other assay)

- 1 hour setup time and 2 hour run time
- Involves comparison to standards run simultaneously
- Automated quantitation
- Quantity obtained reflects amount of "amplifiable" DNA

Sample Setup and Data Analysis with Slot Blot versus Real-time qPCR



Assay Sensitivity and Dynamic Range

Quantiblot-ECL 40 pg - 2.0 ng

ACES 2.0 40 pg - 4.0 ng
No longer available
(ACES tended to work better on degraded DNA)

Real Time qPCR 1.0 pg - 16 ng

qPCR has lower detection limit and larger dynamic range

Quantitative PCR

- What is rtPCR or qPCR?
- How does it work?
- How does it compare to traditional methods of Human DNA quantitation?
- What techniques are available?
- What systems are available?

History

- RtPCR is a very recently developed technique
  - Developed by Higuchi in 1993
  - Used a modified thermal cycler with a UV detector and a CCD camera
  - Ethidium bromide was used as intercalating reporter As [dsDNA] increased fluorescence increased
- First paper on qPCR:
  - Higuchi, R.; Fockler, C.; Dollinger, G.; Watson, R. "Kinetic PCR analysis: real-time monitoring of DNA amplification reactions" Biotechnology (N Y). 1993 Sep;11(9):1026-30
- Warning: RT-PCR also means reverse transcriptase PCR which is used when working with RNA

PCR amplification

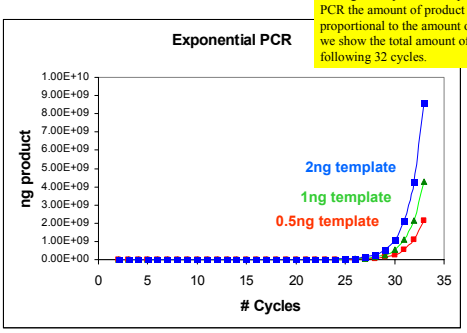
- Theoretically the quantity of PCR template T doubles with each cycle.
- After 2 cycles the quantity of product is 2T
- After N cycles the quantity of product is

$$P = (2)^n T$$

  - Thus there is a exponential relationship between the original quantity of product and the amount of template

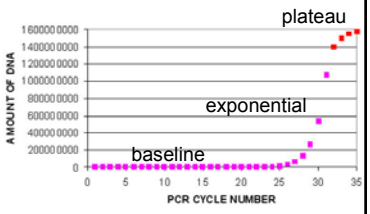
PCR Product Amount is Proportional to the Amount of Input DNA Template

During the exponential expansion of the PCR the amount of product produced is proportional to the amount of template. Here we show the total amount of product following 32 cycles.



What is qPCR?

- To use PCR as a quantitative technique, the reaction must be clearly defined
- In fact there are several stages to a PCR reaction
  - Baseline stage
  - Exponential stage
  - Plateau stage

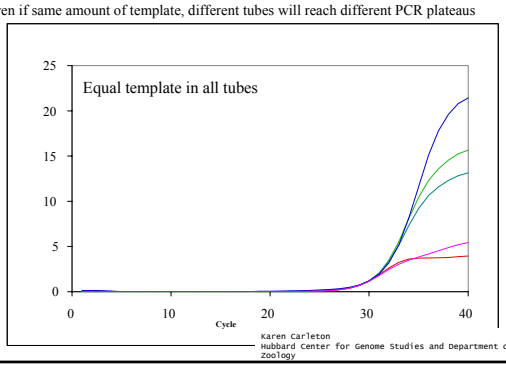


PCR plateaus

- PCR product can not double forever
  - Limited by
  - Amount of primer
  - Taq polymerase activity
  - Reannealing of product strands
- Reach plateau
  - No more increase in product
- End point detection
  - Run for fixed # cycles and then quantify on agarose gels

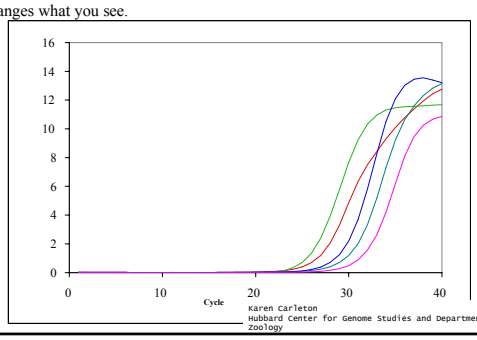
Problem #1: End point plateau does not depend on T

Even if same amount of template, different tubes will reach different PCR plateaus



Problem #2: For endpoint detection, how many cycles should you do?

Different wells reach plateau at different cycle numbers. When you look changes what you see.



Issues for quantitation by non RT-PCR methods

- In spite of its use in mixture resolution, PCR is not technically a quantitative technique
- The time and rate at which plateau appears varies with temperature, tube position, inhibitors, matrix
- Once plateau appears, increase in product concentration is non linear
- Standards can be added but they must have the same primer binding sites and similar sequence to target

Solution

- Use data when still in exponential phase
  - PCR product proportional to initial template
- Need to look at PCR product each cycle
  - Use fluorescent detection, where fluorescence is proportional to PCR product
- Use real time PCR machine which records fluorescence for each well at each cycle

Karen Carleton
Hubbard Center for Genome Studies and Department of Zoology

Quantitation using the PCR Reaction

- PCR proceeds exponentially doubling each cycle:

$$Y_n = Y_{n+1}(1+E_c)$$

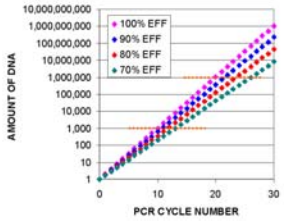
Where E_c is the efficiency (E_c = 1 for a perfect amplification) and Y_n is the yield of product for a particular cycle

- During the exponential stage of the reaction E_c is relatively constant and the reaction yield Y is a function of the quantity of input DNA, X

$$Y = X\,(1+E_c)^n$$

Effect of efficiency on [DNA]

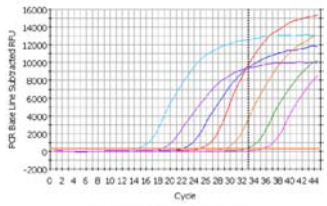
- E_c is a function of:
  - Hybridization efficiency
  - Quantity of reactants/target DNA
  - Temperature



http://www.med.sc.edu:85/pcr/realtime-home.htm

Real Time PCR

- Quantitation of DNA is a based on the number of cycles required to reach a threshold intensity, C_t.
- The greater the amount of starting DNA, the sooner this threshold value is reached.



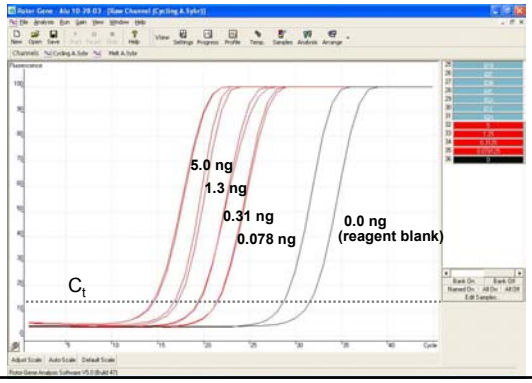
C_t

http://www.med.sc.edu:85/pcr/realtime-home.htm

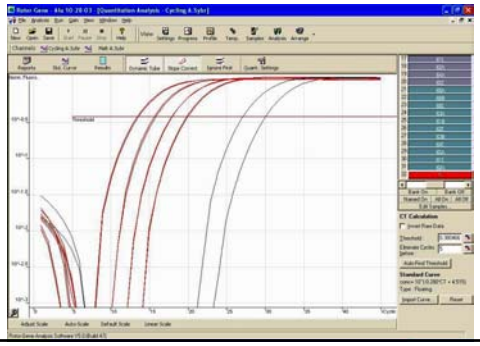
Quantitation using C_t

- The log of DNA template concentration vs C_t is plotted using a series of stds yielding a calibration curve
- The unknown is then run and the number of cycles required to reach threshold, C_t is compared to the calibration curve.

Development of a standard curve

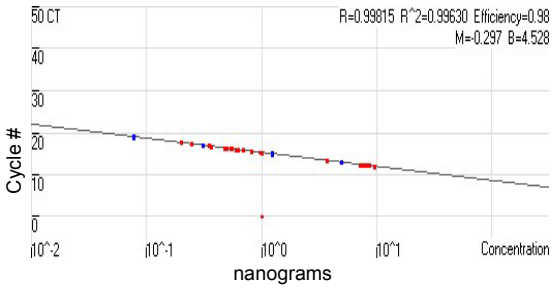


The output data is plotted on a log scale and the fractional # cycles required to reach C_t is measured



Standard curve

Plot the cycle # at threshold CT vs concentration



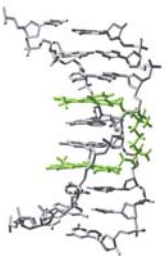
Concentration = 10^(-0.297*CT+ 4.528)

Detection Methods

- Fluorescent intercalating dye - SYBR Green
  - Fluorescence increases with concentration of dsDNA
- Taqman probes
  - Fluorescence increases as quenched probe is digested
- Molecular beacons
  - Fluorescence increases as quenched probe hybridizes to template

SYBR green product detection

- Easy
  - Fluorescence only with dsDNA
  - Use with existing PCR primers
- Generic,
  - Detects all double stranded products, including primer dimers
  - However, can be very specific with proper primer design
- Singleplexed
  - Multiple probes cannot be used

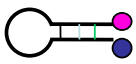


dsDNA Intercalation

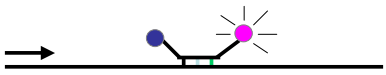
http://www.probes.com/handbook/figures/1557.html

Molecular beacons

- Consist of ssDNA with an internal complementary sequence that keeps reporter and quencher dyes close → No fluorescence

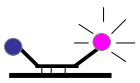
Molecular beacon  Reporter Quencher

- Following denaturation, beacon anneals to template, separating both dyes and yielding fluorescence proportional to PCR product concentration



Molecular Beacons

- Improved specificity and multiplexing
  - Non-specific amplification will not produce a signal
  - Can multiplex several probes (quantify nuclear, Y, int std.)
- Can be tricky to design
  - Loop portion – binds to DNA template
  - Stem portion – must be complementary to other stem
  - Probe must denature from template below 72° so Taq polymerase does not chew it up during extension step



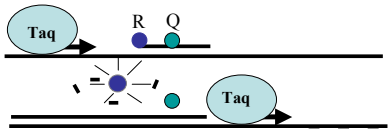
$T_{anneal} < T_m < T_{ext}$

Above T_m loop structure reforms and probe leaves template

Taqman

Probe also binds to PCR product during extension but is always quenched

- 5'-3' exonuclease activity of Taq polymerase digests probe and frees reporter dye from quencher
- Free dye accumulates with PCR product



Probes vs SYBR Green

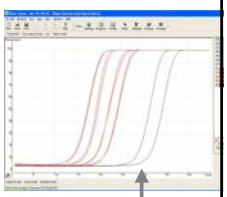
- SYBR Green
  - Singleplex probes (Alu)
  - If no sample, amplification of contaminants occurs at high cycle #
  - If inhibition, no result or poor efficiency curve
- Probes (Taqman, Mol. beacons)
  - Multiplex targeted probes – Quant Y, nuclear DNA, int. std
  - Inhibition and no sample can yield no result (if single locus probe)
  - to check for inhibition, an internal std. is used
- Choice: Simplicity (SYBR green) vs Multiplexing (probes)

Single vs Multilocus Targets

- SYBR Green – Multilocus Probe
  - Alu inserts occur at multiple locations throughout the genome - sensitive
  - If no sample, amplification of contaminants occurs at high cycle #
  - Syber green requires no special kit –Inexpensive
- Probes (Taqman, Mol. beacons)
  - Single location in genome
  - an internal std. is used to check for amplification and correct for changes in efficiency
  - Lower sensitivity due to noise at low copy number
- Choice: Sensitivity (SYBR green) vs Internal Standard Precision (probes)

Effects of Inhibitors on Alu Assay

- Use Alu sequence, present at 1,000's of copies/cell
  - Assay is sensitive to ambient human DNA in air and water
  - Normal Reagent blanks have a Ct at about 27-29 cycles
- If inhibitors are present – no amplification occurs or efficiency is altered
  - Thus low level ambient DNA serves as an internal control for inhibitors
- For non Alu based RtPCR, an internal standard is required to detect inhibition



Reagent Blank

Comparison Studies Slot Blot vs RT qPCR

	Reference	RTi-PCR	Quantiblot
1	5	5.38	6.25
2	1.25	1.14	0.56
3	0.3125	0.29	0.56
4	0.078125	0.08	0.12
5	5	4.92	8.75
6	1.25	1.32	0.63
7	0.3125	0.30	0.81
8	0.078125	0.09	0.23

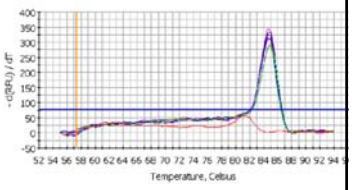
Calibration studies in McCord lab with experimental primers

sample	rtPCR	slot blot	Tho1 Allele
blood on stick	0.32	0.50	1880
blood on metal	0.40	0.50	1890
blood on concrete	0.40	0.50	1860
blood on leaves	0.08	0.20	1540
blood on cardboard	0.27	0.24	1450
blood on cloth	0.04	0.05	577
blood on denim	0.25	1.00	1240

From validation work of Jan Nicklas and Eric Buel
Nicklas, J.; Buel, E. (2003) *J. Forensic Sci.* 48(5): 936-944

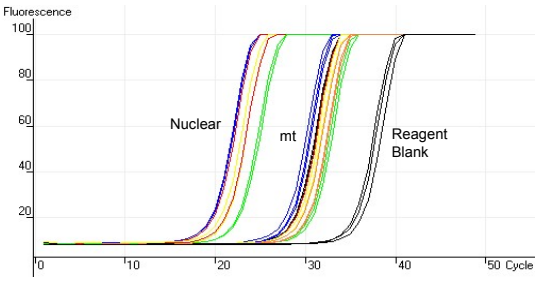
Future Applications of qPCR

- Determination of Mt vs Nuclear DNA
- Determination of Y vs Nuclear DNA
- Determination of sample degradation
- Sample screening by melt curves



http://pathmicro.med.sc.edu/pcr/realtime-home.htm

Sequential detection of mtDNA and nuclear DNA (Alu)



Quality of data depends on technique!

Quantitation Information

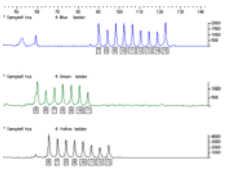
Threshold	0.0175
Left Threshold	10.000
Standard Curve Imported	No
Standard Curve (1)	conc= 10^(-0.198*CT + 2.796)
Standard Curve (2)	CT = -5.057*log(conc) + 14.140
Reaction efficiency (*)	0.57672 (* = 10^(-1/m) - 1)
M	-5.0568
B	14.14006
R Value	1.000
R^2 Value	1.000
Start normalising from cycle	1
Noise Slope Correction	Yes
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
No Template Control Threshold	10%
Sample Page	Page 1

R-Value: Perfect 1.000 !!

Rayna and Sarah

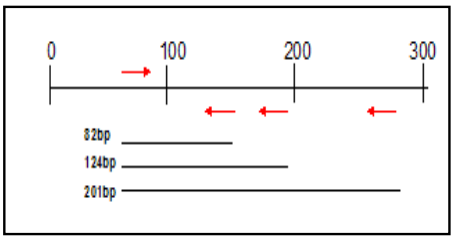
Work in FIU Laboratory- with assistance of Vermont Crime Lab

- Development of miniplex STRs for degraded DNA typical sizes 60-120 bp.
- Slot blot works poorly on these samples
- So develop a series of different primers to selectively amplify degraded dna



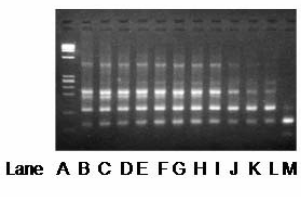
Determination of DNA Quality by qPCR

*Alu*Ya5 Primers – Nicklas and Buel



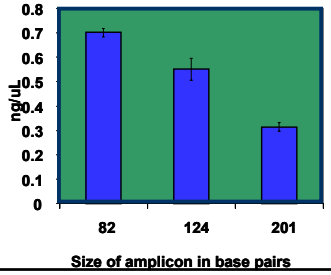
Primer design

Results using primers from Nicklas & Buel Vermont Crime Lab

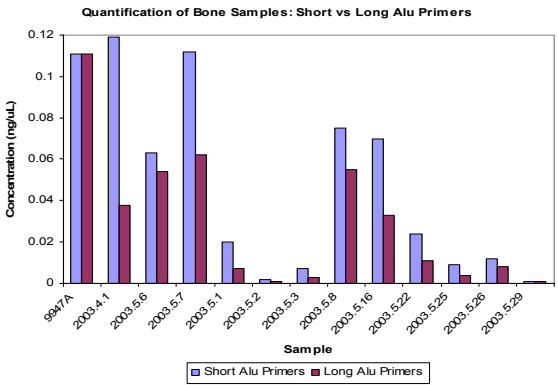


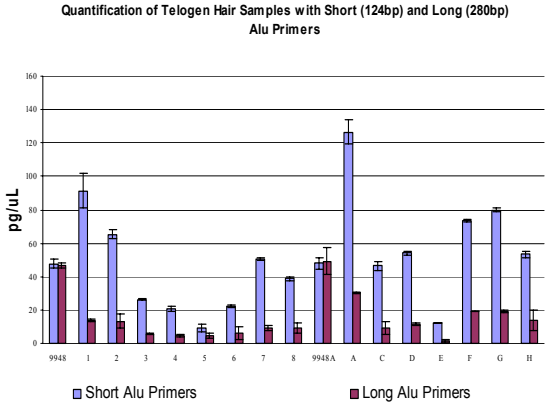
Lane A –ΦX HaeIII
Lane B – No digestion
Lane C – 30 sec digestion
Lane D – 1 min digestion
Lane E – 2 min digestion
Lane F – 3 min digestion
Lane G – 4 min digestion
Lane H – 8 min digestion
Lane I – 12 min digestion
Lane J – 16 min digestion
Lane K – 24 min digestion
Lane L – 32 min digestion
Lane M – 48 min digestion

Quantitation of DNase I degraded DNA using 3 primer sets



An example of the quantitation results obtained with a degraded DNA sample. Error bars represent 95% confidence interval.





RT-qPCR Instruments Cited

- Corbett Research Rotorgene
  - Phenix Research, Hayward, CA
- ABI 7000 or 7500 Sequence Detection System
- ABI 7700 (discontinued)
- ABI 7900HT Sequence Detection System
  - Applied Biosystems Foster City, CA

ABI 7000

Corbett Rotorgene

Real-Time qPCR Efforts

- Marie Allen – nuclear and mtDNA assay (BioTechniques 2002, 33(2): 402-411)
- Eric Buel – Alu system (JFS 2003, 48(5):936-944)
- Centre for Forensic Sciences – nuclear; TH01 flanking region (JFS 2003, 48(5):1041-1046)
- John Hartmann – Alu system (SWGDAM Jan 2003)
- CA-DOJ – TH01 assay (NIJ DNA Grantees June 2003)

- SYBR Green assay – human-specific with right PCR
- Quantifiler kit (ABI) – separate nuclear and Y assays

NIST Lessons Learned from Real Time-qPCR Assays

Using ABI 7500 (early work with ABI 7000 and some Roche LightCycler)

- Results are RELATIVE to standards used
- Single source and mixed source samples with same UV concentrations differ with RT-qPCR assays
- Need to keep instrument clean to avoid background fluorescence problems
- Assay reagent costs:
  - Quantifiler: $2.46/sample (only permits 2 µL/sample)
  - SYBR Green: $0.80/sample (up to 10 µL/sample)
  - QuantiBlot: $0.54/sample (5 µL/sample)

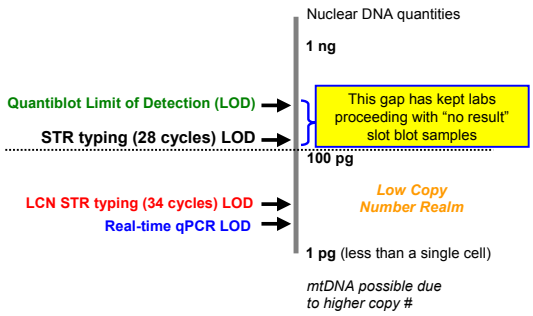
http://www.cstl.nist.gov/biotech/strbase/DNAquant.htm

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"?

Difference in DNA Quantitation Capability vs. STR Typing Sensitivity



DNA Quantitation Summary

- 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 can be used
- Results give quantity of amplifiable DNA – not necessarily overall quantity
  - PCR inhibition can be detected
  - Multiplexing can be used
- Big advantages are speed and dynamic range
- Commercial kits are now available

Acknowledgements

- Jan Nicklas and Eric Buel - Vermont Crime Laboratory
- Jiri Drabek
- Denise Chung, Kerry Opel
- Nancy Tatarek
- John Butler, Yin Shen

- Major support provided by
- The National Institute of Justice
- The OU Provost's Undergraduate Research Fund
- Ohio University Research Incentive Fund

Chemistry & Biochemistry @ OHIO UNIVERSITY

References

On-line

http://www.med.sc.edu:85/pcr/realtime-home.htm
http://www.realtimeprimers.org/
http://dna-9.int-med.uiowa.edu/realtime.htm
http://dorakmt.tripod.com/genetics/realtime.htm

In Print

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Low-Copy Number (LCN) Work

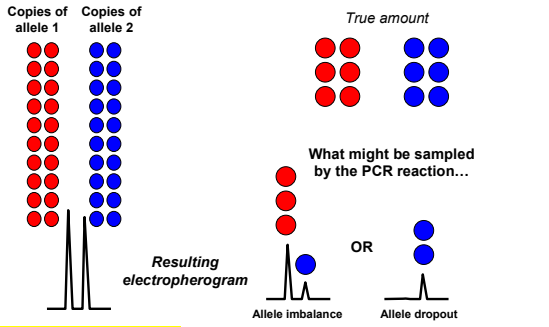
- **Early work on touched objects and single cells**:
  - van Oorschot, R. A. and Jones, M. K. (1997) DNA fingerprints from fingerprints. *Nature*. 387(6635): 767
  - Findlay, I., Taylor, A., Quirke, P., Frazier, R., and Urquhart, A. (1997) DNA fingerprinting from single cells. *Nature*. 389(6651): 555-556
- **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

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

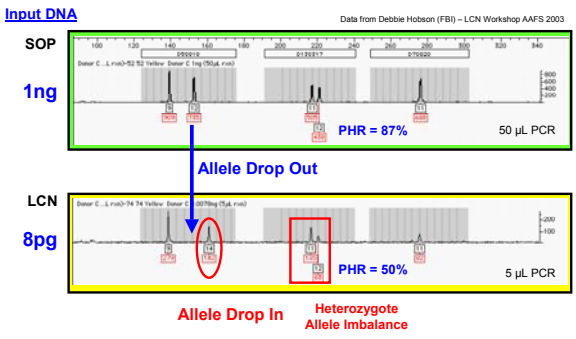


>20 copies per allele

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

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

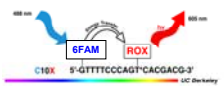
Higher Sensitivity

- 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)

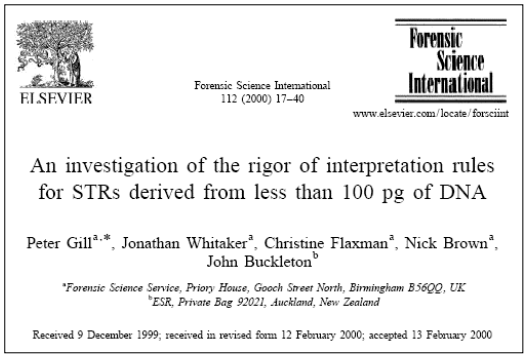
ET dyes (example: LIZ) permit a 10-30X improvement in signal over non-ET dyes



Challenge with Being Able to Go Lower In DNA Quantitation Measurements

- 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

New Interpretation Rules Required for LCN



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

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Suggestions to Optimal Results with LCN

- At least two* PCR amplifications from the same DNA extract
- 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

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