
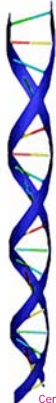


**The CFS-HumRT QPCR Assay:
Developmental Validation, Casework
Experience and Lessons Learned.**



by Melanie Richard, Forensic Biologist
Centre of Forensic Sciences

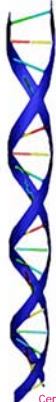


Overview

- Developmental Validation
- Quality of the DNA
- Recognizing inhibition in your QPCR
- System(s) in place to address inhibitors
- Forensic casework experience
- Direct comparison of casework experience using slot blot and QPCR

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Why Quantify?

- STR PCR works within narrow range of template DNA
- Minimum of 240pg of DNA to proceed
- STR interpretation relies on amount amplified
- Preservation of sample
- DAB standard 9.4.2.1

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Why Use QPCR?

- Automation
- High throughput
- Objective, software facilitated assessment
- Improved amplification strategies
- Potential for increased STR PCR Success
- Preservation of sample


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Why In-house kit?

- Commercial kit not available in 2001
- Benefits
 - Cost effective (~4X cheaper @ \$0.90/rxn)
 - Greater flexibility in terms of assay needs
 - Improved Quality Control re: optimization
 - Not impacted by production issues


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Assay Components

- ABI Custom Oligo Synthesis Service
 - 100uM VIC labelled TaqMan®-MGB probe
 - 130,000 pmols primers
- TaqMan® Universal PCR Master Mix
 - includes uracil-N-glycosylase
- TaqMan® Control Human Genomic DNA
 - 10ng/μL, quantity verified in house


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Assay Design (2001)

- QPCR not used in forensic science
- 3 factors considered critical
 - Specific for human DNA
 - Reliable with a variety of sample types
 - Sensitivity as good/better as slot blot method
- ~1 month to design
- ~6 months to develop
- ~1 year to validate for casework
- August 2003 on-line


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Assay Design

- TaqMan®-MGB - specificity
- Single copy, non-polymorphic target
 - Same amplicon size/copy # for all samples
 - Similar amplification performance as STRs
- Primer Express Software (ABI)used to design primers/probe
 - GenBank STR sequences as candidates
 - HUMTH01 (Accession D00269)

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Assay Design


Probe

- 31bps downstream from repeat region
- 67% GC, Tm=70°C, 15bps

Primers

- Forward GC=43%,Tm=58°C, 21bps
- Reverse GC=57%,Tm=60°C, 21bps

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Probe & Primer Sequences


forward primer → Probe ←

GTGGGCTGAA AAGCTCCCGA TTATCCAGCC TGGCCACAC AGTCCCCTGT
CACCCGACTT TTCGAGGCT AATAGGTCGG ACCGGGTGTG TCAGGGGACA

ACACAGGGCT TCCGAGTGCA GGTCACAGG AACACAGACT
← reverse primer
TGTGTCCCGA AAGCTCAGT CCAAGTGTCC TTGTGTCTGA

Amplicon Length=62bp

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Universal PCR Parameters

Stage 1 Stage 2 Stage 3 (40 cycles)

50.0°C 95.0°C 95.0°C 60.0°C

2:00 10:00 0:15 1:00


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Assay Optimization

- Primer concentrations independently varied
 - System robust, all 25 different combinations generated product
- 5 concentrations of probe tested
 - Sensitivity impacted
- The factors considered in selection
 - lowest CT
 - highest ΔR_n value
 - least variability among replicates
 - preparation simplicity


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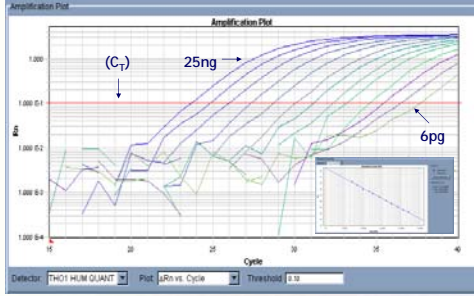
Assay Specificity

- 560 human DNA samples from 4 different databases examined demonstrates no population specific prevalence of mutations
- The sequence specific target was detected with some primate species, including chimpanzee and gorilla
- Addition of 1000 fold more interfering DNA to QPCR had no effect on accuracy of quantification of human DNA

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Standard Curve



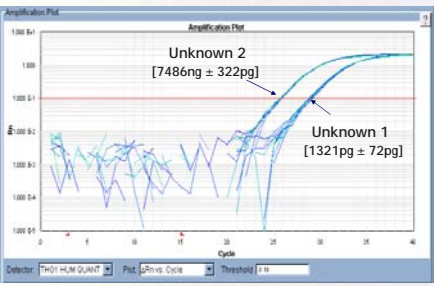
Amplification Plot

Detector: TH01 HUM QUANT Plot: Ln vs. Cycle Threshold: 1.00

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Reproducibility (N=10)



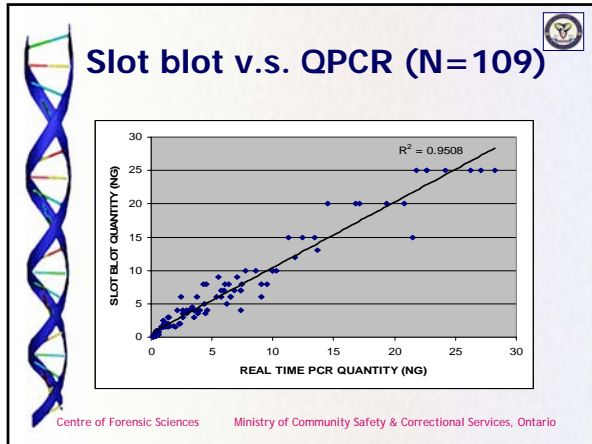
Amplification Plot

Detector: TH01 HUM QUANT Plot: Ln vs. Cycle Threshold: 1.00

Unknown 2 [7486ng ± 322pg]

Unknown 1 [1321pg ± 72pg]

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-
- Validation Summary**
- Primer/probe sequences developed
 - PCR optimization
 - Specificity
 - Human population studies
 - Sensitivity and precision with DNA standards
 - Reproducibility
 - Comparison to QuantiBlot™
- Presented at Promega 2001 & IAFS 2002
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Validation Published

Richard M, Frappier R and Newman J. Developmental Validation of a Real-Time Quantitative PCR Assay for Automated Quantification of Human DNA. J Forensic Sci 2003;48(5):1041-46.


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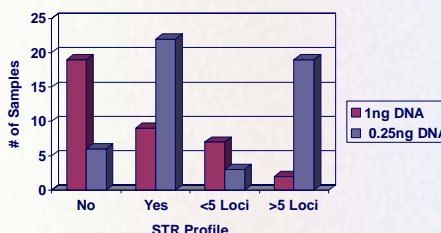
Non-Probativ Casework

- Selection Criteria
 - 1ng of DNA failed to generate complete STR profile
 - Further PCR using 250pg of DNA was attempted
 - Most re-amplification results confirmed PCR inhibition
- Sample Types
 - Cut-outs/swabs from clothing (15)
 - Cigarette butts (4)
 - Stamp (1)
 - blood swabs from scene/items (7)
 - Swab of fake beard (1)
 - Cell-component of urine sample (1)

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STR Results with Difficult Casework Samples

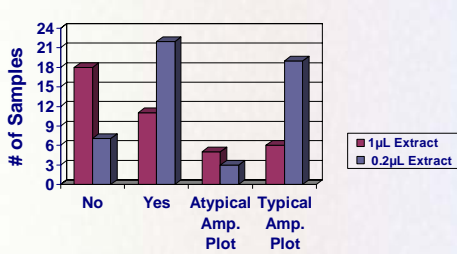


STR Profile	1ng DNA	0.25ng DNA
No	19	6
Yes	9	22
<5 Loci	7	3
>5 Loci	2	19

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QPCR Results with Difficult Casework Samples



QPCR Result	1µL Extract	0.2µL Extract
No	18	7
Yes	10	22
Atypical Amp. Plot	5	3
Typical Amp. Plot	6	18

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Factors Effecting DNA Quality

Extraction Method

- Organic extraction methods with micro-filtration yield high purity extracts as do some magnetic bead systems


Degradation

- Fragments are too short to allow for STR amplification

Inhibition

- Material has been co-extracted that blocks amplification

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Effect of Quality on Quantitation


Slot Blot

- Quantitation is not affected by inhibitors

QPCR

- Like other PCR systems it is susceptible to inhibition and as a result quantitation may be affected


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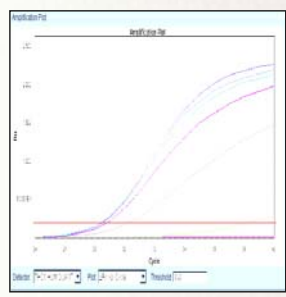
Types of PCR Inhibitors

- Heme
- Phenol
- Adhesives
- Textile Fabric Dyes
- Humic Substances
- Heavy Metals
- Polysaccharides
- Salts

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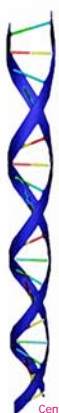


Impact on Quantitation

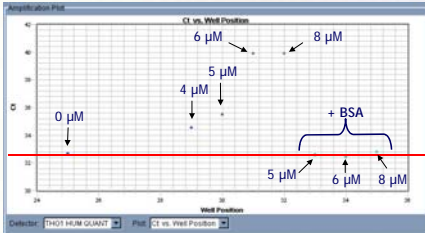


None
Inaccurate
False Negative

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Effect of BSA on Inhibition



0 μ M
4 μ M
5 μ M
6 μ M
8 μ M
+ BSA
5 μ M
6 μ M
8 μ M

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Casework Protocol


Duplicate Analysis

- Test 1 = 1 μ l neat extract with no BSA added
- Test 2 = 0.2 μ l of extract with 0.16 μ g/ μ l BSA (Sigma Fraction V)

Interpretation

- Utilize result from Test 2 for quantitation
- Utilize results from Test 1 & 2 to determine STR amplification strategy


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QPCR Data Evaluation

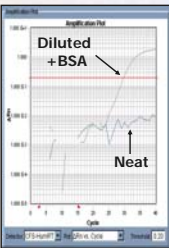
- Concordance of duplicate analysis
 - Evaluated based on ratio calculation
- Morphology of amplification plot
- Purity of DNA
 - Colour of extract (dyes)
 - Extraction method utilized
- Sample type

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Non-concordant Result

- Neat sample fails however diluted test sample does amplify
- Amplification plot morphology "normal"
- Rely on quantity determined by diluted test sample

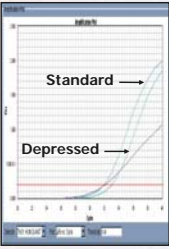


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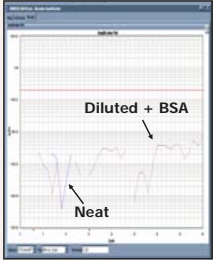

"Abnormal" QPCR Plot

- Test sample crosses ct threshold but plot morphology is "Abnormal"
- Cannot rely on quantity determination
- QPCR test of further dilution(s) is required



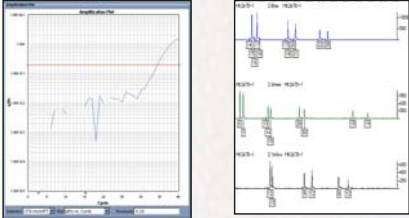
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Bloodstain on Jeans



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Further Dilution Identified Inhibition

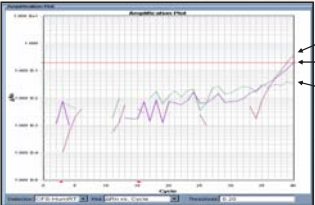


* 1/10 dilution+BSA for coloured extracts


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Extract from Cigarette Butt

- 1/10 dilution was the only sample which exhibited quantifiable DNA (~0.1ng/μl)
- Sample type is known to be problematic

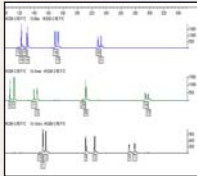


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Inhibition & Limited DNA

- Extract re-purified to further remove PCR inhibitors associated with the cigarette butt filter paper
- Sample re-quantified & 1ng amp in STR-PCR system




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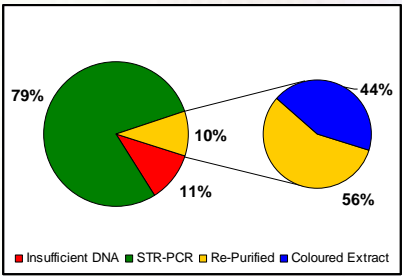
Re-Purification Strategy

- Re-suspend in 400µL TNE
- 2 Phenol/Chloroform extractions
- 1 Chloroform extraction
- Volatilization (45min.)
- Micro-filtration to 15µl
- QPCR of 1µl neat extract, 1/5 & 1/10 dilutions with BSA

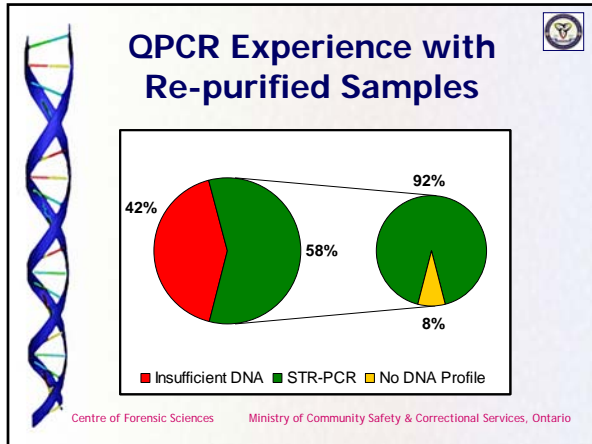
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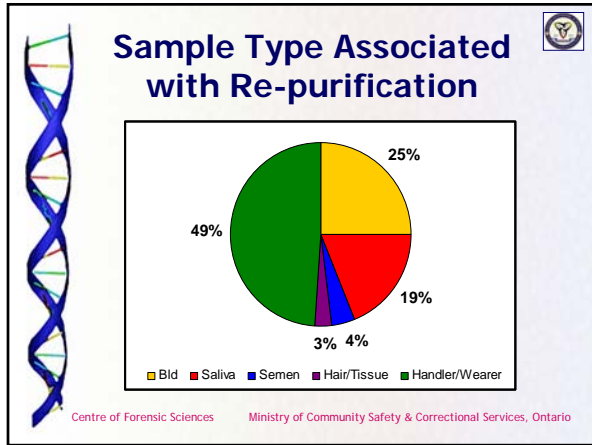


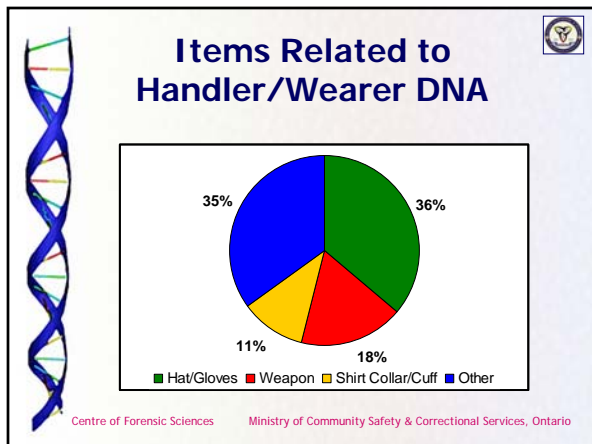
QPCR Experience with Casework Samples

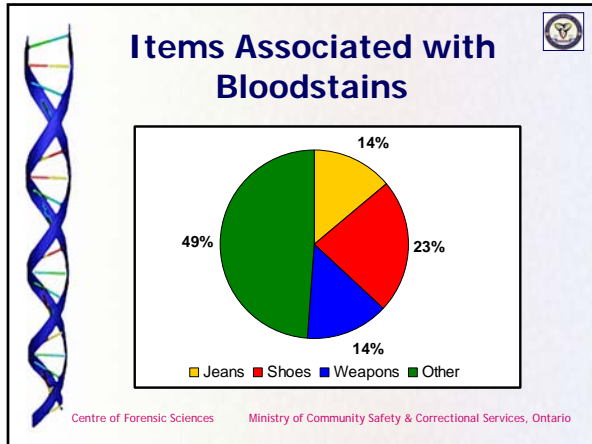


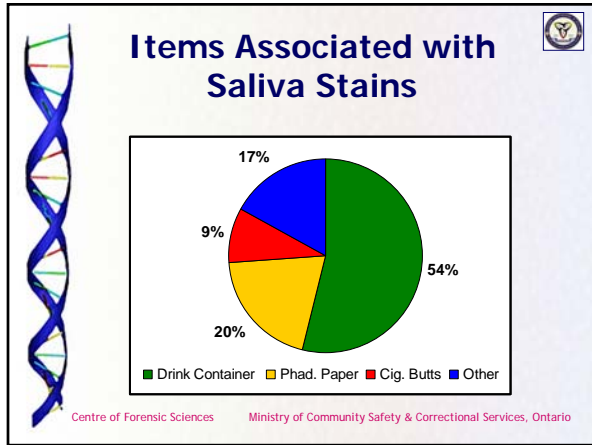
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QPCR Take Home Message

Benefit

- Wide dynamic range, greater sensitivity, automated process, more information

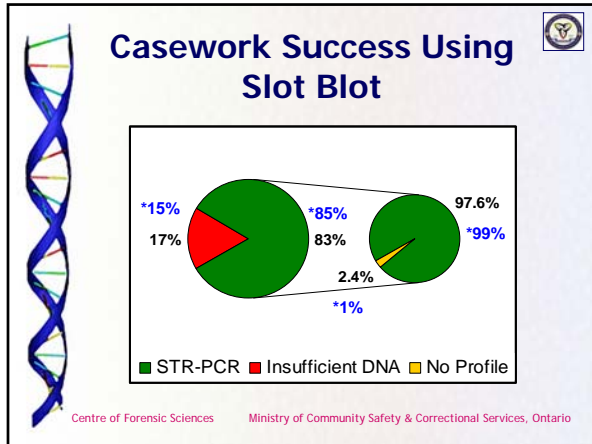
Drawback

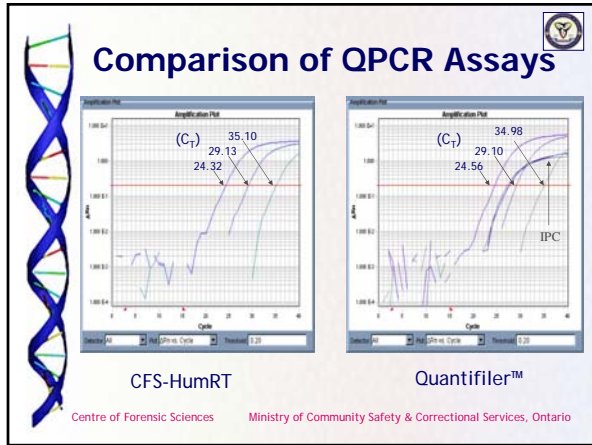
- Susceptible to PCR inhibitors, accurate quantity determination may require further work

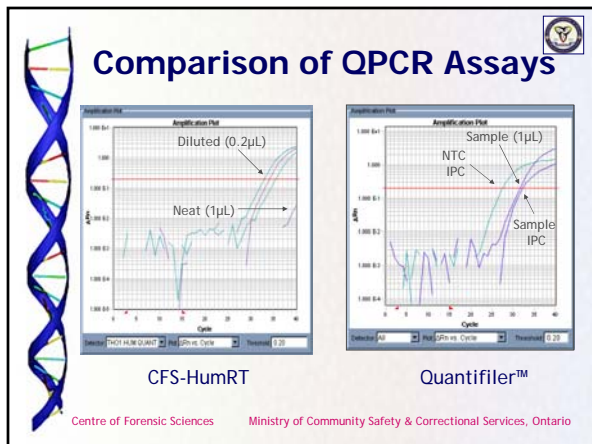
Limitation

- Regardless of the mechanism used to detect inhibition, need to be aware of false negatives and have a system in place to address them

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