Multiplex qPCR Assays at the California DOJ: Diagnosing DNA in Challenging Samples

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Outline

I. "Diagnosing" DNA

II. California DOJ Multiplex qPCR Assays

III. Triplex qPCR Assay: DNA Quantity and Quality
   1. assessing DNA degradation
   2. assessing PCR inhibition
   3. a casework example

IV. Quadruplex qPCR Assay: Triplex qPCR + Y

V. Implementing a Validated Non-Commercial Assay

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"Diagnosing" DNA

biological evidence

DNA extract

- How much human DNA is present ?
- Is PCR inhibitor present ?
- Is the DNA degraded ?
- What sex was the source of DNA ?
- Is the sample a mixture of male and female DNA ?
- Is human mitochondrial DNA present ?

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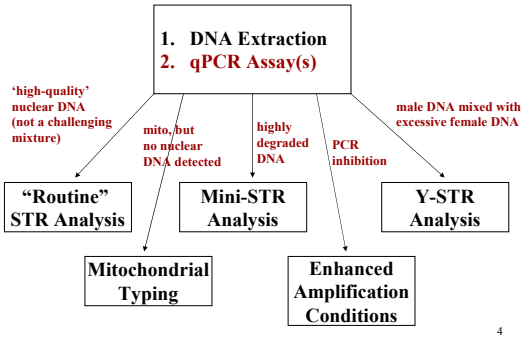
Goal: Develop qPCR Tools to Diagnose DNA → Increase Efficiency of Analysis



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CA DOJ Multiplex qPCR Assays

- 1st Generation: Duplex qPCR Assay (on-line 2005)
  - human nuclear DNA quantity
  - human mitochondrial DNA quantity
    - mainly in Missing Persons DNA Program
- 2nd Generation: Triplex qPCR Assay (2006)
  - human nuclear DNA quantity
  - DNA quality (inhibition and degradation)
  - ~1000 reactions per month throughout CA DOJ
- 3rd Generation: Quadruplex qPCR Assay (2007)
  - Triplex assay + Y-chromosome-specific quantity

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CA DOJ qPCR Publications

- Duplex qPCR (nuclear-mitochondrial)
  - Timken, et al., *JFS*, v. 50, pp. 1044-1060 (***2005***)
- Triplex qPCR (nuclear-degradation-IPC)
  - Swango, et al., *FSI*, v. 158, pp. 14-26 (***2006***)
  - Swango, et al., *FSI*, v. 170, pp. 35-45 (***2007***)
- Quadruplex qPCR (Triplex + Y)
  - Hudlow, et al., *FSI-Genetics*, article in press on-line (***2007***)

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Why Use a Non-Commercial qPCR Assay?

- potential for greater sensitivity relative to commercial assays (e.g., VT Alu assay(s))

- potential for cost savings relative to commercial assays (also potential time savings)

- potential to use an assay more suitable for forensic extracts (e.g., using a longer qPCR target sequence to obtain a better estimate of STR-amplifiable DNA for degraded samples)

- potential to use an assay designed to address sample *quantity* and *quality* issues relevant to your specific downstream analyses

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Developing, Validating, and Implementing a Non-Commercial qPCR Assay

1. Assay Research and Development (3-11 months)
   - selection of target sequences for testing
     - specificity, avoid known SNPs (primer/probe binding site mutations), good sequence for probe design (not too AT-rich)
   - primer/probe design, testing, and optimization for singleplex
     - optimize for signal strength, sensitivity, precision, specificity
   - primer/probe testing and optimization for multiplex
     - look for cross reactivity that can lower PCR efficiencies
     - adjust primer concentrations to limit PCR for high copy number targets
     - assess robustness and reliability for challenging samples

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Developing, Validating, and Implementing a Non-Commercial qPCR Assay

2. Developmental Validation Studies (SWGDAM Guidelines) (3-8 months)

Plate Precision	"Accuracy"*	Sensitivity
Reproducibility	Species Specificity	Mixtures
Stability: Inhibited Samples	Stability: Degraded Samples	Case-type Samples (& Population)

* comparison to quants obtained with previously validated system(s); take results through STR to determine suitability (and limitations) of new system for typing

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Developing, Validating, and Implementing a Non-Commercial qPCR Assay

3. Documentation and Protocols (Sign-Off); Training and Competencies (3-6 months, and on-going)
   - technical review
   - summaries for validation binder
   - write-up publication/presentation
   - protocol, checksheets, analysis and interpretation guidelines, supplemental analysis software (Excel)
   - modifications to LIMS
   - modifications to QA/QC procedures
     - reagent prep sheets
     - QC procedures
     - equipment-related procedures (e.g., new dye channel)
   - analyst training and competency runs

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Developing, Validating, and Implementing a Non-Commercial qPCR Assay

4. Implementation at DOJ-BFS Regional Labs (≥ 2 months)
   - up-date protocols, QC/QA procedures
   - SWGDAM "Internal" validation ("site" validation) – (next slide)
   - analyst training and competency runs (first competency run is the Lab's Qualifying Test)

- required for commercial or non-commercial assay -

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Developing, Validating, and Implementing a Non-Commercial qPCR Assay

4. Implementation at DOJ-BFS Regional Labs (≥ 2 months)
   - SWGDAM "Internal" validation

Plate Precision	"Accuracy"	Sensitivity
Reproducibility	Species Specificity	**Mixtures**
Stability: Inhibited Samples	Stability: Degraded Samples	Case-type Samples (& Population)

- required for commercial or non-commercial assay -

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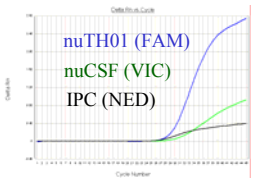
Triplex qPCR Assay: DNA Quantity & Quality

- DNA Degradation
  - the qPCR Degradation Ratio

- PCR Inhibition
  - the ΔIPC
  - the Normalized Inhibition Factor (NIF)



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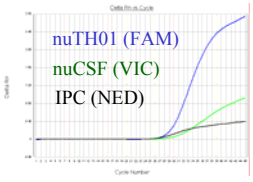
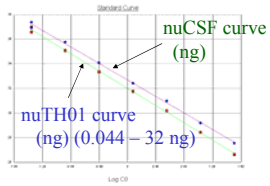
Triplex qPCR Assay: DNA Quantity & Quality

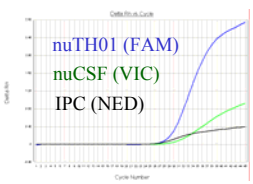
- PCR co-amplification and detection of **three different targets**
- nuTH01 assay – "long" nuclear target (~180 basepairs in length)
  - spans the TH01 CODIS STR locus
  - primary quantification assay
  - detected with FAM-labeled TaqMan™ probe
- nuCSF assay – "short" nuclear target (67 bp)
  - flanks the CSF1PO CODIS STR locus
  - for assessing DNA degradation
  - detected with VIC-labeled TaqManMGB™ probe
- IPC assay – internal PCR control
  - synthetic single-stranded oligonucleotide (77 bp)
  - detected with NED-labeled TaqManMGB™ probe



Triplex qPCR Assay: DNA Quantity & Quality

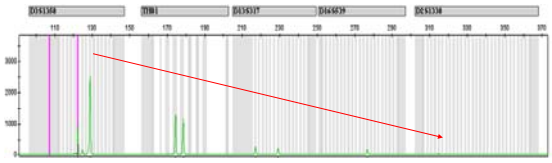
- validated for ABI 7500 real-time instrument
- uses 2-4 uL sample volume (20 uL PCR)
- reaction mix composition (~$2/sample):
  - custom primer-probe mix (+ BSA 0.16 ug/uL)
  - ABI TaqMan Universal Master Mix
  - 2.5 U additional AmpliTaq Gold polymerase
- DNA standard = Promega Human Male DNA
  (***single*** DNA dilution series → two standard curves)





The qPCR Degradation Ratio: Using Short (nuCSF) and Long (nuTH01) qPCR Targets to Assess DNA Degradation

Identifiler STRs (VIC (Green)) for a Degraded DNA Sample



- "wedge" or "ski slope" effect
- more small DNA fragments than large DNA fragments
- should be able to assess DNA degradation by using qPCR to quantify the ratio of small-to-large DNA target sequences

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The qPCR Degradation Ratio: Using Short (nuCSF) and Long (nuTH01) qPCR Targets to Assess DNA Degradation

nuCSF assay – detects and quantifies DNA fragments larger than ~67 bp.

nuTH01 assay – detects and quantifies DNA fragments larger than ~180 bp.

In degraded DNA, there will be more fragments >67 bp than >180 bp …

qPCR Degradation Ratio

$$= \frac{\text{nuCSF Quantity (ng)}}{\text{nuTH01 Quantity (ng)}}$$

For high-molecular weight DNA, Degradation Ratio is ~ 1.

For highly-degraded DNA, Degradation Ratio is > 1.

The bigger the Degradation Ratio, the more fragmented the DNA.

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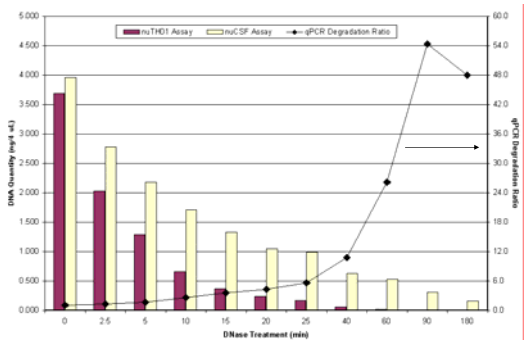
Triplex Quantifications and Degradation Ratios for DNaseI-Degraded Samples

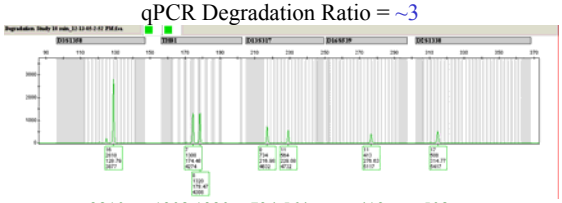


Degradation Ratio and Identifiler STR Results

("1 ng" template, nuTH01 component of the triplex qPCR assay)

10 Minute DNase Time Point

qPCR Degradation Ratio = ~3



2810 RFU | 1308,1320 | 734,564 | 413 | 508

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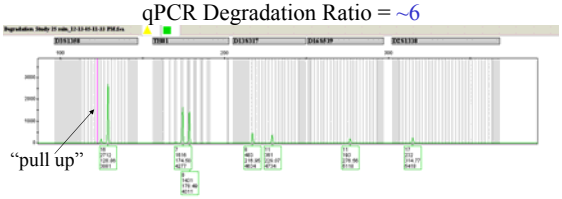
Degradation Ratio and Identifiler STR Results

("1 ng" template, nuTH01 component of the triplex qPCR assay)

25 Minute DNase Time Point

qPCR Degradation Ratio = ~6



2712 RFU | 1616,1431 | 483,361 | 193 | 232

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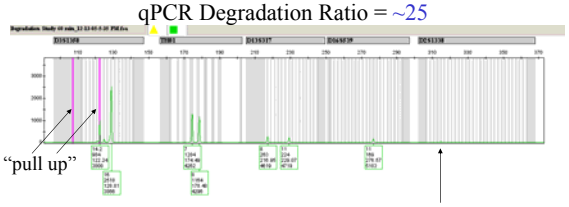
Degradation Ratio and Identifiler STR Results

("1 ng" template, nuTH01 component of the triplex qPCR assay)

60 Minute DNase Time Point

qPCR Degradation Ratio = ~25



2518 RFU | 1284,1164 | 263,224 | 169 | "0" (below threshold)

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Interpreting the qPCR Degradation Ratio

Degradation Ratio	STR Implications
1 – 3	none
3 – 5	"wedge" effect, possible cross-dye pull-up
>5 (>10 ⇒ artifacts expected to be significant)	increasing "wedge" effect, pull-up, dropped-out alleles at larger loci, off-scale peaks, called stutter peaks, -A shouldering

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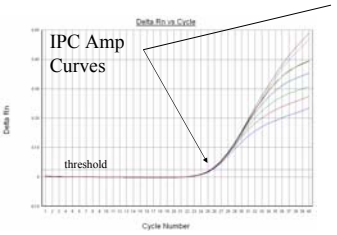
Using the IPC qPCR Target to Detect PCR Inhibition

- template is a 77 nucleotide synthetic DNA sequence ('alien sequence' - designed to be non-homologous to GenBank sequences)
- spiked into primer-probe mix (~90,000 copies/sample)



- for non-inhibited samples, IPC assays should amplify to give (nearly) identical Ct values

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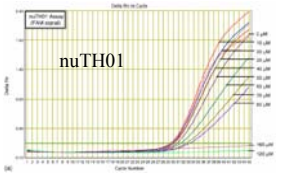
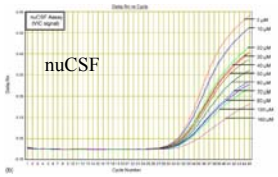
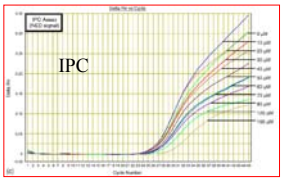
Increasing PCR Inhibition (hematin 0-160µM) Leads to Delayed Amplifications







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What we'd like the IPC
to tell us about PCR inhibition:

1) Is qPCR giving a reliable estimate of DNA quantity? (Is PCR inhibition delaying the qPCR amplification to the extent that we are significantly underestimating the DNA quantity?)

2) Will there be PCR inhibition in our downstream STR amplifications?

These are two different questions !

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What we'd like the IPC
to tell us about PCR inhibition:

1) Is qPCR giving a reliable estimate of DNA quantity? (Is PCR inhibition delaying the qPCR amplification to t underestimating t quantity?)

delay in amplification of IPC = "**ΔIPC**"

2) Will there be PCR inhibition in our downstream STR amplifications?

These are two different questions !

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What we'd like the IPC
to tell us about PCR inhibition:

1) Is qPCR giving a reliable estimate of DNA quantity? (Is PCR inhibition delaying the qPCR amplification to the extent that we are significantly underestimating the nuTH01 (or nuCSF) DNA quantity?)

2) Will there be PCR inhibition in our downstream STR amplificat

normalized inhibition factor = "**NIF**"

These are

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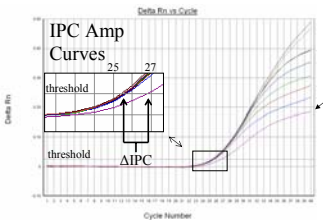
Using the IPC qPCR Target to Detect PCR Inhibition

Delay in Ct of IPC = **ΔIPC**

(semi-automatically calculated and reported using Excel worksheet (S. Myers))

= (IPC Ct of sample) – (average IPC Ct of non-inhibited controls)

= 26.8 – 25.6 = 1.2 cycles



- PCR inhibition detected by delay in Ct of IPC, relative to non-inhibited samples

So, what information does the ΔIPC provide?

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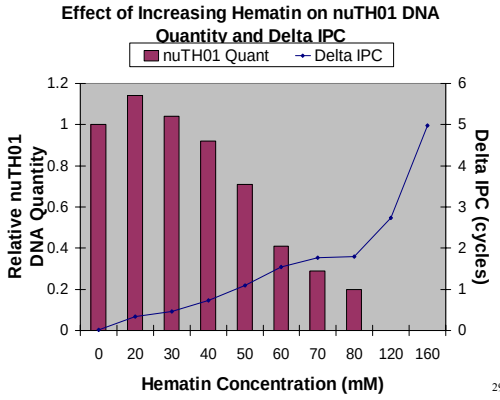

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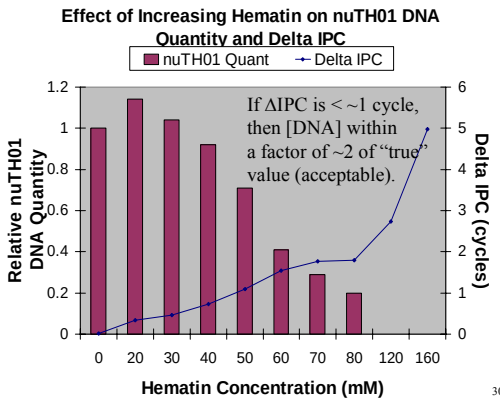

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What we'd like the IPC to tell us about PCR inhibition:

1) Is the qPCR quant a reliable estimate? (Is PCR inhibition delaying the qPCR amplification to the extent that we are significantly underestimating the nuTH01 (or nuCSF) DNA quantity?) Calculate & interpret the ΔIPC.

2) Will there be PCR inhibition in our downstream STR amplifications?

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What we'd like the IPC to tell us about PCR inhibition:

1) Is the qPCR quant a reliable estimate? (Is PCR inhibition delaying the qPCR amplification to the extent that we are significantly underestimating the nuTH01 (or nuCSF) DNA quantity?) Calculate & interpret the ΔIPC.

2) Will there be PCR inhibition in our downstream STR amplifications?

These are two different questions !

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Will there be PCR inhibition in our downstream STR amplifications?

Won't the ΔIPC by itself answer this question?

Not necessarily …

The qPCR amplification and the downstream STR amplification might be run under very different conditions with respect to PCR inhibition.

Look at two examples.

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Will there be PCR inhibition in our downstream STR amplifications?

Example #1:

qPCR amp: 1 uL of neat extract in 20 uL volume
- nuTH01 DNA = 0.1 ng/uL
- ΔIPC = 0.2 cycles ("mild" inhibition)

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Will there be PCR inhibition in our downstream STR amplifications?

Example #1:

qPCR amp: 1 uL of neat extract in 20 uL volume
- nuTH01 DNA = 0.1 ng/uL
- ΔIPC = 0.2 cycles ("mild" inhibition)

⇩

STR amp: 10 uL of neat extract in 25 uL volume

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Will there be PCR inhibition in our downstream STR amplifications?

Example #1:

qPCR amp: 1 uL of neat extract in 20 uL volume
- nuTH01 DNA = 0.1 ng/uL
- ΔIPC = 0.2 cycles ("mild" inhibition)

⇩

STR amp: 10 uL of neat extract in 25 uL volume
- major inhibition seen in STR alleles !!

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Will there be PCR inhibition in our downstream STR amplifications?

Example #1:

qPCR amp: 1 uL of neat extract in 20 uL volume
- nuTH01 DNA = 0.1 ng/uL
- ΔIPC = 0.2 cycles ("mild" inhibition)
- extract is 5% of PCR volume

STR amp: 10 uL of neat extract in 25 uL volume
- major inhibition seen in STR alleles !!
- extract is 40% of PCR volume
- **⇒ inhibitors <u>concentrated</u> in the STR amp !**
- **⇒ inhibitors <u>diluted out</u> in qPCR amp !**

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Will there be PCR inhibition in our downstream STR amplifications?

Example #2:

qPCR amp: 1 uL of neat extract in 20 uL volume
- nuTH01 DNA = 5 ng/uL
- ΔIPC = 1 cycle (significant inhibition)

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Will there be PCR inhibition in our downstream STR amplifications?

Example #2:

qPCR amp: 1 uL of neat extract in 20 uL volume
- nuTH01 DNA = 5 ng/uL
- ΔIPC = 1 cycle (significant inhibition)

⇩

STR amp: 0.2 uL of neat extract in 25 uL volume

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Will there be PCR inhibition in our downstream STR amplifications?

Example #2:

qPCR amp: 1 uL of neat extract in 20 uL volume
- nuTH01 DNA = 5 ng/uL
- ΔIPC = 1 cycle (significant inhibition)

⇩

STR amp: 0.2 uL of neat extract in 25 uL volume
- no PCR inhibition seen; all STR loci amp fine

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Will there be PCR inhibition in our downstream STR amplifications?

Example #2:

qPCR amp: 1 uL of neat extract in 20 uL volume
- nuTH01 DNA = 5 ng/uL
- ΔIPC = 0.5 cycles (significant inhibition)
- extract is 5% of PCR volume

STR amp: 0.2 uL of neat extract in 25 uL volume
- no PCR inhibition seen; all STR loci amp fine
- extract is 0.8% of PCR volume

⇒ **inhibitors <u>diluted out</u> in STR amp !**

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Will there be PCR inhibition in our downstream STR amplifications?

Potential for PCR inhibition in downstream STR amps depends on **relative amounts of PCR inhibitor and DNA** in sample.

To achieve the desired DNA template quantity in the downstream amp, will the inhibitor be concentrated or diluted out?

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Will there be PCR inhibition in our downstream STR amplifications?

Potential for PCR inhibition in downstream STR amps depends on **relative amounts of PCR inhibitor and DNA** in sample.

To achieve the desired DNA template quantity in the downstream amp, will the inhibitor be concentrated or diluted out?

the Normalized Inhibition Factor (or "NIF")

$$= \frac{\Delta IPC \text{ (cycles)}}{nuTH01 \text{ (ng DNA)}}$$

(W. Hudlow, CA DOJ)

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Will there be PCR inhibition in our downstream STR amplifications?

the Normalized Inhibition Factor (or "NIF")

$$= \frac{\Delta IPC \text{ (cycles)}}{nuTH01 \text{ (ng DNA)}}$$

- small NIF ⇒ downstream inhibition unlikely
- large NIF ⇒ downstream inhibition more likely

Example #1: NIF = (0.2 cycles)/(0.1 ng) = **2**

Example #2: NIF = (1 cycle)/(5 ng) = **0.2**

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Will there be PCR inhibition in our downstream STR amplifications?

the Normalized Inhibition Factor (or "NIF")

$$= \frac{\Delta IPC \text{ (cycles)}}{nuTH01 \text{ (ng DNA)}}$$

Current Lab *Guidelines* for nuTH01 NIF used with Identifiler™ STR amp:

NIF < 1	then inhibition in STR amp is not expected
NIF > 1	then inhibition in STR amp is more likely

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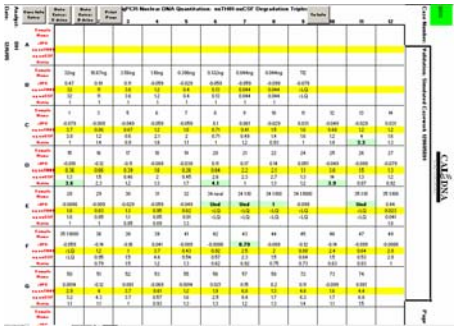
Spreadsheet for Semi-Automatic Calculation of Degradation Ratio, ΔIPC, and NIF



developed by Steve Myers (CA DOJ)

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The Triplex Assay: A Casework Example
(Colleen Spurgeon, DOJ Richmond DNA Lab)

Missing Remains Evidence
- partial cranium
- partially submerged in water/mud
- exposed for years/decades (?)

Expectation (based on experience)
- exposure => DNA degraded ?
- soil, bone => PCR inhibition ?
- low yield of DNA in extract ?
- a challenging sample, could require multiple analysis attempts to achieve useful typing info

NOT the actual sample

Picture from http://www.orkneyjar.com/archaeology/minehowieburial.htm

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The Triplex Assay: A Casework Example

DNA Extraction
- portion of skull excised
- extensively cleaned
- powderized in freezer mill (LN_2) to give 2 g bone dust
- "burgundy" liquid after Phenol/Chloroform extraction
- 20 uL of colorless extract after Centricon100 filtrations

Triplex qPCR Results
- 2 uL of extract used for qPCR (in 20 uL qPCR volume)
  - nuTH01: 0.072 ng/2uL
    - nuTH01 $[DNA]_{neat}$ = 0.036 ng/uL
  - nuCSF: 2.1 ng/2uL
    - nuCSF $[DNA]_{neat}$ = 1.1 ng/uL
  - Degradation Ratio = 29 !!

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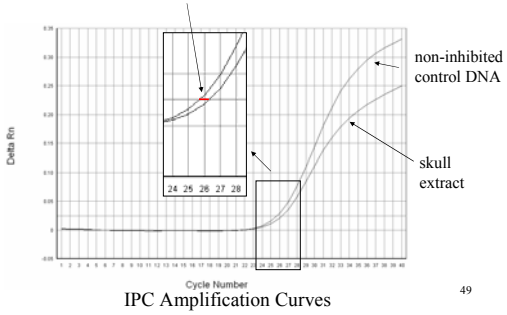
The Triplex Assay: A Casework Example

Triplex qPCR Results (continued)

ΔIPC = 0.5 cycles



IPC Amplification Curves

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The Triplex Assay: A Casework Example

Triplex qPCR <u>Diagnosis</u>:

Degradation Ratio = 29	=>	HIGHLY Degraded DNA. MiniSTRs not on line, only alternative was Identifiler STR analysis.
nuTH01 [DNA] = 0.036 ng/uL	=>	Lower than target 0.075 ng/uL for optimal Identifiler STR amplification; may need to concentrate the sample?
ΔIPC = 0.5 cycles	=>	< 1 cycles, so qPCR results are expected to be "accurate."
NIF = 0.5 cycles/0.072 ng = 6.9	=>	>> 1, so anticipate possible inhibition problems in downstream STR amp.

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The Triplex Assay: A Casework Example

DNA Analysis <u>Strategy</u>:

- concentrate the sample from 18 to 10 uL (vacuum evaporation) to increase the DNA concentration from 0.036 to 0.064 ng/uL (close to target of 0.075 ng/uL)

- compensate for PCR inhibitors by running the STR amplification under enhanced conditions ("Superjuice" - 5U additional Taq polymerase per reaction and double the concentration of BSA)

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The Triplex Assay: A Casework Example

ABI Identifiler STR Analysis Results:

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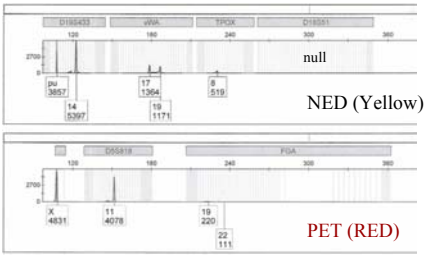
The Triplex Assay: A Casework Example

ABI Identifiler STR Analysis Results:



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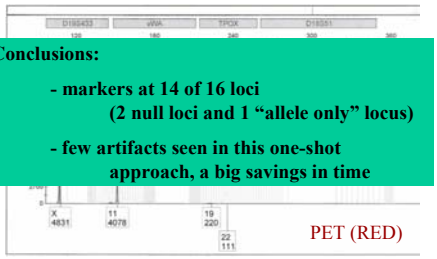
The Triplex Assay: A Casework Example

ABI Identifiler STR Analysis Results:



Conclusions:

- **markers at 14 of 16 loci**
  (2 null loci and 1 "allele only" locus)
- **few artifacts seen in this one-shot**
  approach, a big savings in time

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nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR Assay

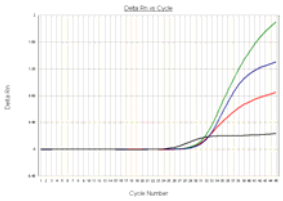
- multiplex PCR amplification and real-time detection of **four different DNA targets**
- triplex qPCR (dyes changed) PLUS Y-chromosome target

✓ nuTH01 - nuclear target: ~180 basepairs (bp) in length

\+ nuSRY - Y-specific target: 137 bp (adjacent to single-copy *SRY* gene)
- detected with VIC-labeled TaqManMGB™ probe

✓ nuCSF - short nuclear target: 67 bp – for assessing degradation

✓ IPC (internal PCR control) – for assessing PCR inhibition



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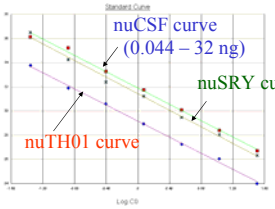
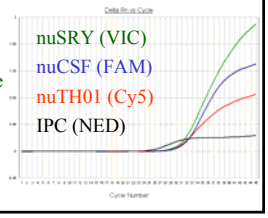
nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR Assay

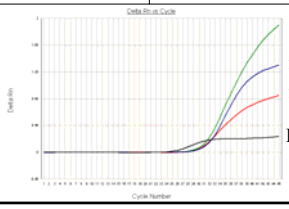
- validated for ABI 7500 real-time instrument
- uses 2-4 uL sample volume (20 uL PCR)
- reaction mix composition (~$2.25/sample):
  - custom primer-probe mix (+ BSA 0.16 ug/uL)
  - ABI TaqMan Universal Master Mix
  - 2.5 U additional AmpliTaq Gold polymerase
- DNA standard = Promega Human Male DNA
  (***single*** DNA dilution series → three standard curves)





Using & Interpreting the Quadruplex qPCR Results

For:	Result(s) to Use:
Routine STR Analysis	nuTH01
STR Analysis of Male:Female Mixtures	ratio of nuSRY:nuTH01 (nuSRY for Y-STR amp, if ratio <~0.1)
Mini-STR Analysis	nuTH01 or nuCSF
Detection of PCR Inhibition	IPC (to determine "ΔIPC"), NIF
DNA Degradation Analysis	Ratio of nuCSF:nuTH01 (to determine "Degradation Ratio")



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Implementing a Validated Non-Commercial qPCR Assay

- talk to developers & users of the non-commercial assay
  - get up-to-date information on the assay
  - get as much validation information and documentation as possible from the lab
  - understand interpretation and assay limitations before starting; get tips for avoiding & solving problems
  - if possible, obtain reagent(s) for testing
  - obtain data sets from originating lab for comparing to your results; send example data back to originating lab
- assess your QC/QA and workflow issues
- discuss any proposed changes in procedure with originating lab
- many internal validation and implementation issues are the same for custom and commercial assays

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Thank you.

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