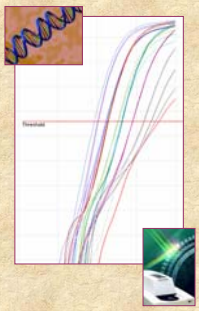
qPCR at AFDIL: Our Experiences Quantitating mtDNA & More

Toni M. Diegoli

Part of the American Academy of Forensic Sciences 2008 Annual Meeting
Workshop: "Human DNA Quantification Using Real-Time PCR Assays"

Overview

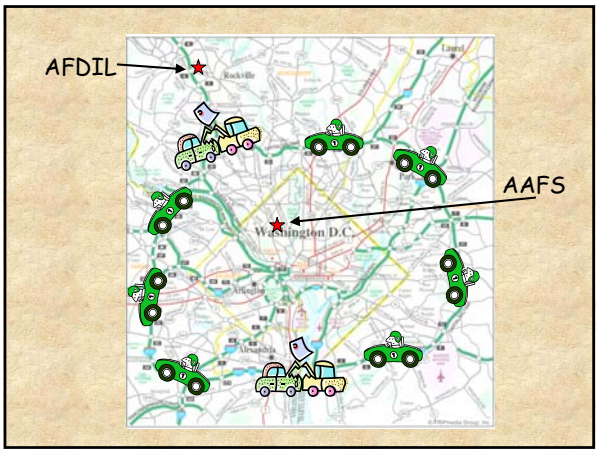


- Why qPCR at AFDIL?
- How qPCR used at AFDIL?
- Details of the assay
- "Validation"
- Examples of assay use at AFDIL
- A Bit About Plexor® HY

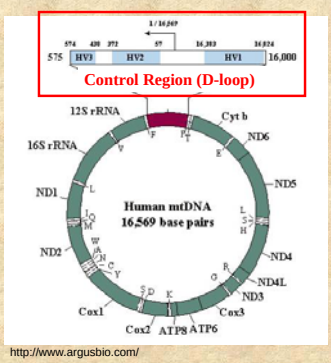
AFDIL
Armed Forces DNA Identification Laboratory

Armed Forces DNA Identification Laboratory = AFDIL!

- mitochondrial DNA sequencing laboratory
  - casework and research sections
  - nuclear section



Mitochondrial DNA Testing



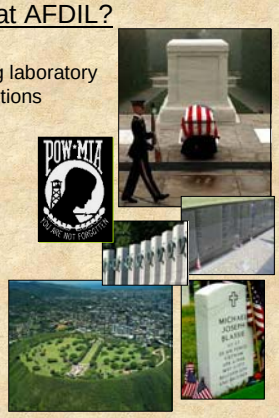
http://www.argusbio.com/

CR → HV1, HV2, (HV3)

- high copy number
- lack of recombination
- maternal inheritance
- high mutation rate

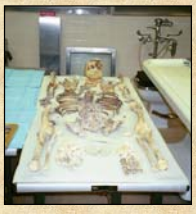
Why qPCR at AFDIL?

- mitochondrial DNA sequencing laboratory
  - casework and research sections
  - nuclear section
- common sample types:
  - bone/tooth
  - old (degraded)
  - fragmented/small
  - dirty
- common extract problems:
  - low copy number
  - degraded
  - inhibited



Why qPCR at AFDIL?

- Quantifiler → nuclear quantitation
- mtDNA Databasing
- Bone/tooth extraction protocols
  - development
  - evaluation
  - validation
- difficult samples
  - presence of inhibitors?
  - alternate amplification strategy?

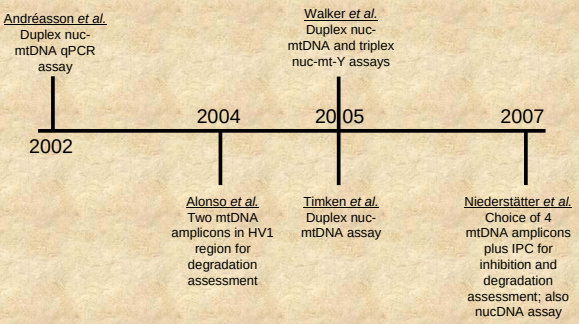


Considerations When Choosing a qPCR assay

- published or manufactured?
  - desired target
  - in-house capabilities
  - cost
- additional capabilities?
  - inhibition detection?
  - degradation assessment?
- chemistry?
  - ability to multiplex?
  - specificity? sensitivity?
  - cost?
- instrumentation?
  - mechanism of dye excitation, heating/cooling, etc.
  - number of dyes that can be detected
- ease of set-up?
  - who will be routinely performing quantitation?

Know types of samples to be analyzed in your lab

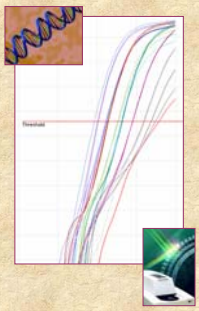
Published Forensic mt-qPCR Assays



One of the first forensic qPCR publications

Forensics-specific modifications to Andréasson, et al paper

Overview



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- **Details of the assay**
- "Validation"
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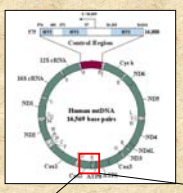
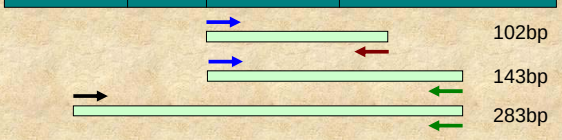
Details of the Assay



- Cytochrome C Oxidase II
- non-coding
- tRNA lysine
- ATPase 8

one amplicon per run



Details of the Assay

Internal PCR Control – IPC

- plasmid amplified simultaneously with mtDNA fragment
- used to detect inhibition
- detected using a FAM-labeled probe
- results in 156bp IPC amplicon

Details of the Assay

Primer	Sequence (5' → 3')
mt8294F[1]	CCACTGTAAAGCTAACTTAGCATTAACC
mt8154F	GGGTATACTACGGTCAATGCTCTGA
mt8395R	GGGCCATACGGTAGTATTTAGTTGG
mt8436R[1]	GTGATGAGGAATAGTGTAAGGAGTATGG
IPC R	TCGTTTCGGAGCGTTGGTTAG
IPC F	AGGTTGCTAACTATGAAACACTGGC

1. As described in Andreasson, et al. (2002).

Probe	Sequence (5' → 3')
mtDNA	VIC-CCAACACCTCTTTACAGTGAA-MGB/NFQ
IPC	6FAM-CAGCACTTCTTTTGAGCAC-MGB/NFQ

Details of the Assay

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Probe	Sequence (5' → 3')
mtDNA	VIC-CCAACACCTCTTTACAGTGAA-MGB/NFQ
IPC	6FAM-CAGCACTTCTTTTGAGCAC-MGB/NFQ

- minor groove binder
- purchased from Applied Biosystems
  - HPLC-purified
  - formulation: liquid
  - diluted in 1xTE
  - synthesis scale: 100 pmol/µl
- 5' dye label and 3' MGB/NFQ

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Probe	Sequence (5' → 3')
mtDNA	VIC-CCAACACCTCTTTACAGTGAA-MGB/NFQ
IPC	6FAM-CAGCACTTCTTTTGAGCAC-MGB/NFQ

- can be prepared in-house, or purchased from Sigma-Aldrich
- when purchased:
  - purification: desalt or HPLC
  - formulation: dry (resuspend overnight in uv'd H_2O)
  - synthesis scale: 1.0 µmol

Details of the Assay

Primer Storage
- 200µl aliquots of each 10µM primer
- polyallomer tubes
- -20 degrees
- stock concentration: 100µM

Probe Storage
- 40µl aliquots of each 10µM probe (single-use)
- protected from light
- -20 degrees
- stock concentration: 100µM

DNA Storage
- standards, IPC
- 4 degrees
- polyallomer tubes

Master Mix Storage
- working stock: 4 degrees
- stock bottles: -20 degrees
- original container

Details of the Assay

Volume (µl)	Stock Concentration	Reagent	Final Concentration
2	2.5mg/ml	BSA	**0.25mg/ml**
0.3	10µM	mtDNA Forward Primer	**0.15µM**
0.3	10µM	mtDNA Reverse Primer	**0.15µM**
0.4	10µM	mtDNA Probe (VIC)	**0.2µM**
0.4	10µM	IPC Probe (FAM)	**0.2µM**
0.6	10µM	IPC Forward Primer	**0.3µM**
0.6	10µM	IPC Reverse Primer	**0.3µM**
0.4	5000/µl	IPC	**100/µl**
10	2x	Taqman® Universal PCR Master Mix (Applied Biosystems)	**1x**
Optional			
0.4	5 units/µl	TaqGold *(2 additional units)*	**0.1 units/µl**
0.6	--	water	--
20	**Final Reaction Volume** (*after sample is added*)		

Reaction Set-Up Considerations

Questions to ask:

- What did bone look like prior to extraction?
- What are the circumstances of recovery (background)?
- Was the extract brownish or clear?
- Would one expect there to be large or small amounts of DNA in the extract?
- What is the volume of extract that will be added to the qPCR reaction?



Reaction Set-Up Considerations

Adjustments to make:

- Run samples in duplicate or triplicate
- Dilute samples
- Prepare in plastic tubes with low DNA-binding capacity
- Be certain to saturate pipette tip by pipetting up and down before distributing to tube/well
- Alter the sample input volume
- Add additional Taq

Documentation is key!

Reaction Set-Up Considerations

Eliminating DNA loss and denaturation during storage in plastic microtubes

BY CLAIRE GAILLARD AND FRANÇOIS STRAUSS

http://www.axygen.com/Products/maxymumRecovery.jsp

Reaction Set-Up Considerations: Using Additional TaqGold

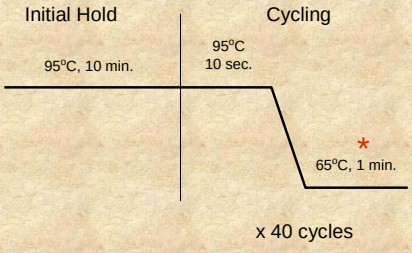
Standard	No Extra Taq Gold				Avg: No	1.25U Extra Taq Gold			Avg: 1.25U
1000pg/ul	32.73	32.25	31.74	30.70	31.86	28.45	28.29	28.33	28.36
100pg/ul	29.35	29.25	29.43	29.14	29.29	28.72	28.86	27.91	28.50
10pg/ul	28.80	28.67	28.93	28.90	28.82	29.06	29.05	29.04	29.05
1pg/ul	28.93	28.88	28.79	29.01	28.90	29.03	28.97	29.12	29.04
0.1pg/ul	28.68	28.88	28.91	28.85	28.83	29.23	29.14	29.26	29.21
0.01pg/ul	27.99	28.94	28.68	28.65	28.65	29.26	29.29	28.47	29.01
Negative	28.91	28.66	28.68	28.82	28.77	29.20	29.18	n/a	29.19

Average Ct: 29.30
Standard Deviation: 1.15

Average Ct: 28.91
Standard Deviation: 0.34

Reaction Set-Up:

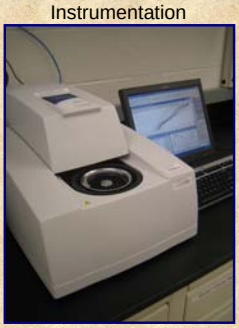
Thermal Cycling Parameters



x 40 cycles

Reaction Set-Up

Instrumentation

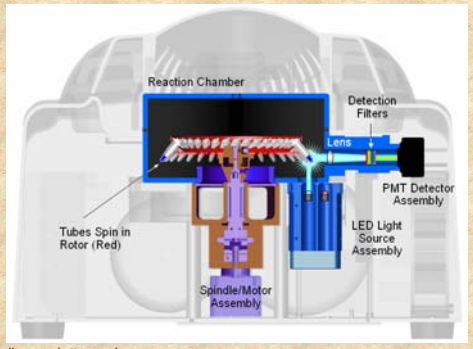


Rotor-Gene™ 3000
Corbett Research

Machine Specifications

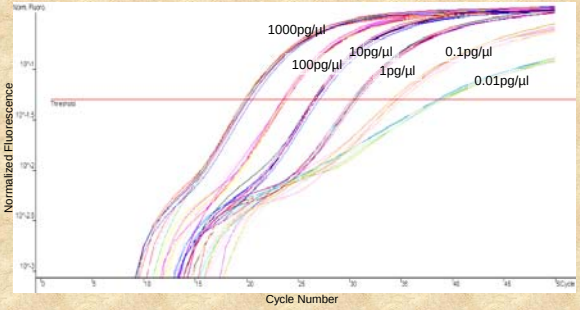
- 72 tubes per run
- approximately 90 minutes per run
- no passive reference (Rox) required
- Software Version 6.0

qPCR Platform: Rotor-Gene™ 3000



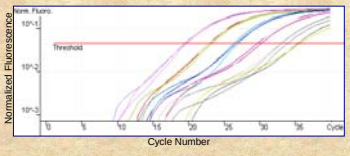
http://www.corbettresearch.com

The Details: Typical Amplification Plot

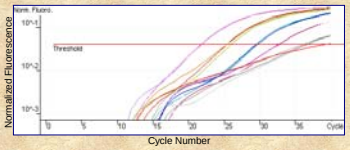


143bp amplicon

The Details: Typical Amplification Plots

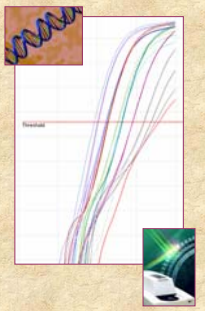


102bp amplicon



283bp amplicon

Overview



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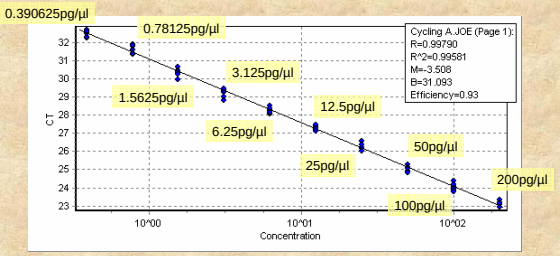
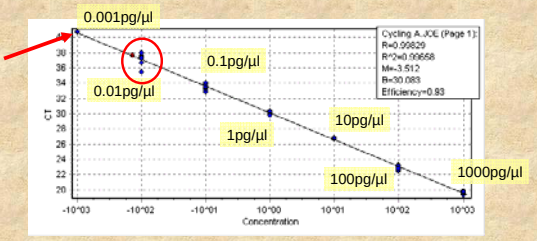
Validation of Standards



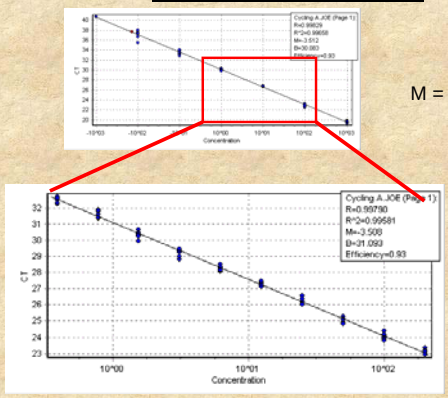
200pg/µl → 0.390625pg/µl

Validation of Standards



1000pg/µl → 0.001pg/µl

Validation of Standards



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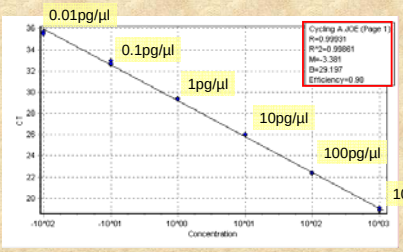

1000pg/µl → 0.01pg/µl

TLE + glycogen + polyallomer tubes

Validation of Standards

Dynamic Range Comparison of Some qPCR Assays

qPCR Assay	Dynamic Range
Mito Assay – ADFIL	1ng/µl – 0.00001ng/µl
Mito Assay – Published	10ng/µl – 0.000075ng/µl
Quantifiler	50ng/µl – 0.023ng/µl
Plexor HY – Standard	50ng/µl – 0.0032ng/µl
Plexor HY – Alternative	20ng/µl – 0.00128ng/µl

Validation of Standards

Standard Ct Variation Between Runs

Standard Concentration (pg/µl)	Average Ct	Standard Deviation	Percent Variance
1000	19.67	0.53	2.69%
100	22.85	0.49	2.15%
10	26.22	0.58	2.21%
1	29.62	0.72	2.42%
0.1	33.11	0.90	2.73%
0.01	36.60	1.42	3.88%

qPCR Standards: Challenges

- mt-Genomes per cell vary from 100s to 1000s
- concentrations listed on tubes useless for mtDNA
- quant in relation to total genomic DNA

?

Converting pg/µl to mtGE/µl for the 9947a Standards

plasmid DNA

- contains target sequences
- known concentration (mtGE/µl)

Step 1. Run 9947a Standards **as Unknowns** against the mtGE/µl Standards

genomic DNA

- known concentration (pg/µl)
- learn mtGE/µl

9947a standards

- known concentration (pg/µl)
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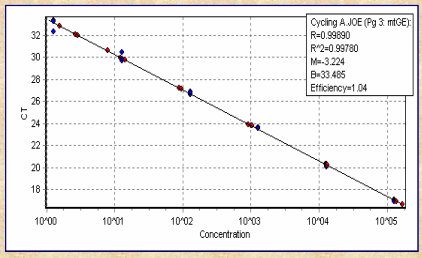
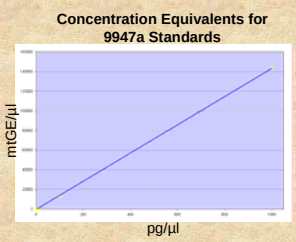
Converting pg/µl to mtGE/µl for the 9947a Standards



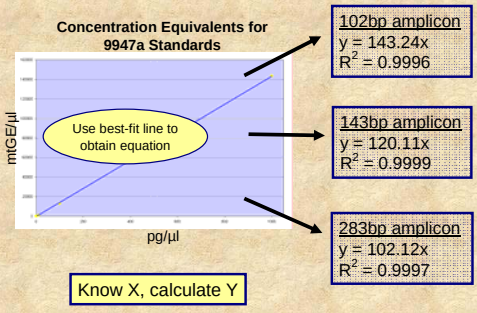
Step 1. Run 9947a Standards Against the mtGE/µl Standards

Converting pg/µl to mtGE/µl for the 9947a Standards



Step 2. Plot Concentration Equivalents for the 9947a Standards

Converting pg/µl to mtGE/µl for the 9947a Standards

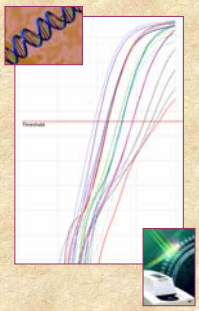


Step 3. Use Best-Fit Line to Obtain Equation for Each Amplicon

Converting pg/µl to mtGE/µl for the 9947a Standards

- For the AFDIL Research Section's purposes, quantitating a sample **relative** to the amount of mtDNA present in a specific concentration (in pg/µl) of total genomic 9947a DNA is sufficient.
  - "this sample contains the same amount of mtDNA as 50pg/µl 9947a"

Overview



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Examples of Assay Use at AFDIL

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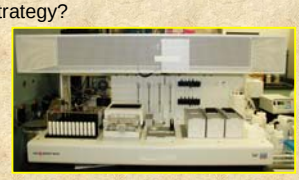
- Bone/tooth extraction protocols
  - development
  - modification
  - validation
- difficult samples
  - presence of inhibitors?
  - alternate amplification strategy?



mt-qPCR Assay Use for mtDNA CR Databasing

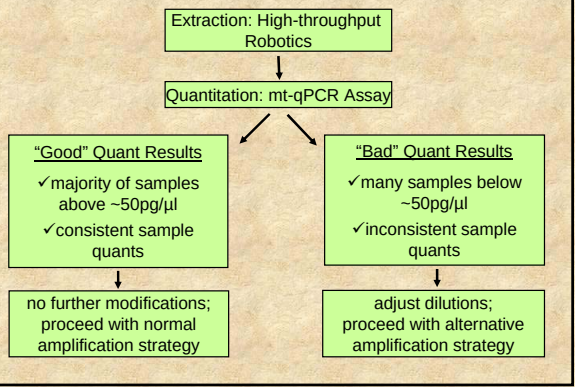
Why do we need to know the quantity of mtDNA databasing samples?

- as a quality control measure when receiving samples from collaborators
  - extracts that arrive with quantitation information
  - extracts that arrive without quantitation information
  - samples extracted in-house
  - storage conditions?
- allow uniform, high-throughput treatment of samples

mt-qPCR Assay Use for mtDNA CR Databasing

Quant Results	Sample Treatment
>50pg/µl	Aggressive amplification strategy
<50pg/µl >1pg/µl	Consider modifications in amplification strategy and/or concentration
<1pg/µl	Unlikely to yield useful results

mt-qPCR Assay Use for mtDNA CR Databasing



Examples of Assay Use at AFDIL

- mtDNA Databasing
- **Bone/tooth extraction protocols**
  - **development**
  - **modification**
  - **validation**
- difficult samples
  - presence of inhibitors?
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mt-qPCR Assay Use for Extraction Protocol Evaluation

Bone Powdering Techniques:
- liquid nitrogen (experimental)
- blender (casework SOP)

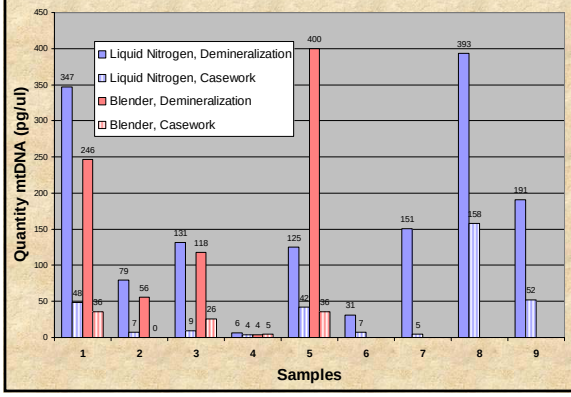

Experimental vs. Standard Practice

Bone Extraction Techniques:
- demineralization (experimental)
- casework (SOP)



Odile M. Loreille, Toni M. Diegoli, Jodi A. Irwin, Michael D. Coble, Thomas J. Parsons. High efficiency DNA extraction from bone by total demineralization. FSI: Genetics 2007; 1(2): 191-195.

mt-qPCR Assay Use for Extraction Protocol Evaluation



Examples of Assay Use at AFDIL

- mtDNA Databasing
- Bone/tooth extraction protocols
  - development
  - modification
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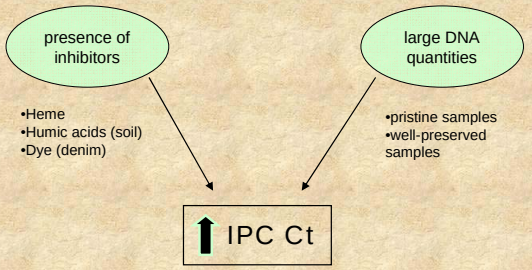
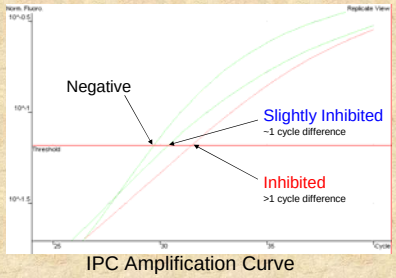
- **difficult samples**
  - **presence of inhibitors?**
  - **alternate amplification strategy?**

mt-qPCR Assay Use: Inhibition Detection

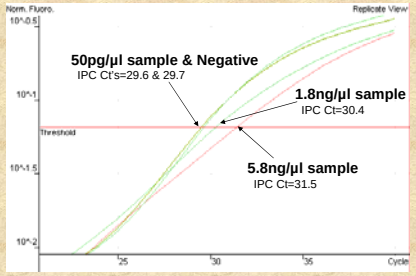


mt-qPCR Assay Use: Inhibition Detection



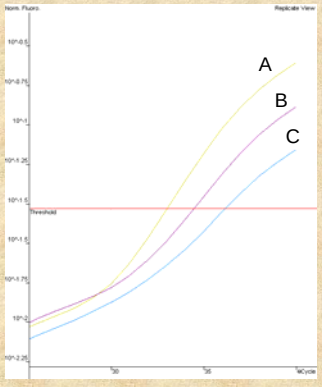
IPC Amplification Curve

mt-qPCR Assay Use: Inhibition Detection



Effect of DNA Quantity on the IPC Amplification Curve

mt-qPCR Assay Use: Inhibition Detection



Using Inhibition Detection to Evaluate Novel Extraction Protocols A, B, & C

mt-qPCR Assay Use: Degradation Assessment

Size Ranges of Validated Primer Pairs for mtDNA Amplification at AFDIL

Amplification Strategy	Size Range (bp)
Entire Control Region	1197
Hypervariable Regions	366-469
Primer Sets	210-287
Mini-Primer Sets	125-178

mt-qPCR Assay Use for Difficult Samples

Quantitation: mt-qPCR Assay

✓283bp amplicon → ✓143bp amplicon → ✓102bp amplicon

proceed with aggressive amplification strategy		proceed with modified amplification strategy		proceed with conservative amplification strategy	
Entire CR	1197	~~Entire CR~~	~~1197~~	~~Entire CR~~	~~1197~~
HVI/HVII	366-469	~~HVI/HVII~~	~~366-469~~	~~HVI/HVII~~	~~366-469~~
Primer Sets	210-287	Primer Sets	210-287	~~Primer Sets~~	~~210-287~~
MPS	125-178	MPS	125-178	MPS	125-178

mt-qPCR Assay Use: Degradation Assessment

mt-qPCR Assay Results Using the Three Amplicons on Four Degraded Samples

	mt-qPCR Assay Results (pg/ul)		
Sample	283bp amplicon	143bp amplicon	102bp amplicon
A	NR	0.059*	0.11
B	NR	0.22	1.12
C	NR	0.15	0.89
D	NR	0.079*	0.21

*Less than 3 sample replicates gave results.

Lessons Learned

- Take steps to ensure regularity of runs with low copy number samples:
  - polyallomer tubes
  - pipette sample up and down
  - run multiple replicates of both samples and standards
  - dilute samples and standards in TLE/TE
  - consider using additional Taq (up to 2 units)
- Use knowledge of the sample to conserve extract and avoid repeat quantitation runs.

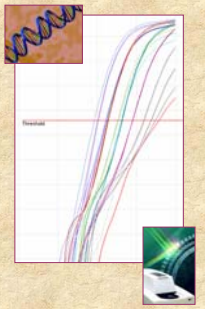
CONSISTENCY IS KEY!!

It's all **relative**!

Summary

- The AFDIL Research Section was interested in quantitating mtDNA in order to aid:
  - mtDNA databasing efforts
  - development and validation of novel extraction protocols
  - understanding of the best approach to deal with difficult samples
- The chosen assay answered these needs in different ways:
  - a means to quantitate mtDNA in an extract
  - an indicator of inhibition in an extract
  - an assessment of the level of degradation of an extract

Overview



- Why qPCR at AFDIL?
- How qPCR used at AFDIL?
- Details of the assay
- "Validation"
- Examples of assay use at AFDIL
- **A Bit About Plexor® HY**

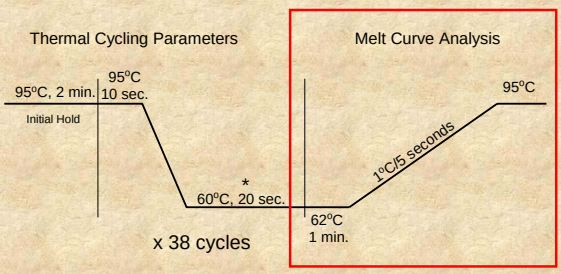
Ways in which Plexor HY may be helpful for LCN

Assay Component	Target Location	Amplicon Length	
Autosomal	Chromosome 17	99bp	multicopy
Y	Short arm	133bp	multicopy
IPC	Novel sequence	150bp	

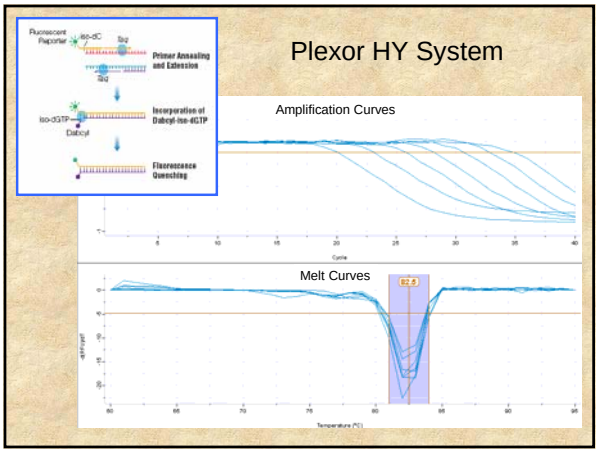
- lower limit of standard curve is 3.2pg/µl (6.4pg input)
- variable input volume possible (2-9µl)
- alternate standard curve (0.00128ng/µl – 20ng/µl)
- multiplex design conserves extract
- melt curve analysis confirms specificity of reaction

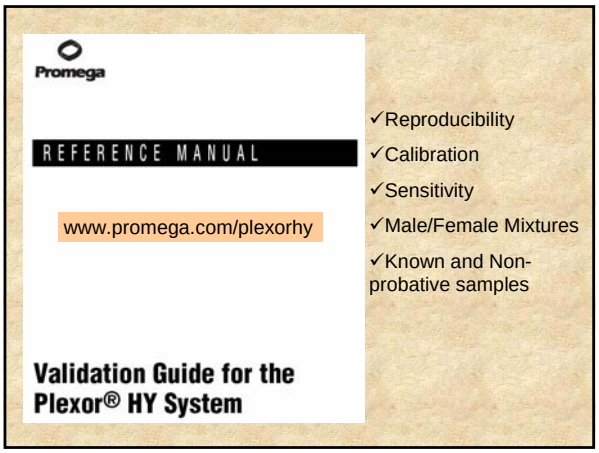
Plexor HY Thermal Cycling Profile

for Rotor-Gene 3000



Plexor HY System





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AFDIL
Armed Forces DNA Identification Laboratory

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