

**Development and Optimization of a Multiplexed  
Quantitative Real Time PCR Assay:  
A “Mini” Roundtable Discussion”**

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# Multiplexed Quantitative Real Time PCR Definition:

- Simultaneous amplification and measurement of Multiple DNA species in the same sample within the same well (tube)
  - i.e., two or more primer sets with two or more probes
- Will not include biallelic discrimination assays under this definition since they have a common primer set and two probes.

# The Pro's and Con's:

## Pro's:

- Uses less sample
- Less cost/ sample(?)
- Generate data rapidly, HT
- Many New Fluorophores
- Canned assays
- Can perform one or two step PCR

## Con's:

- Matched amplicon sizes(?)
- Similar Primer and Probe Melting Temps.
- Requires more time, optimization

# Considerations:

- Development time
- Choose Dyes that are spectrally separated and high raw signal.
- Limiting primer for higher abundant species.
- Check to ensure limiting primer does not impact accuracy of data. Perform primer limited vs. non-primer limited.
- Discern optimal probe concentrations.
- Determine the linear dynamic range of MPX system.
- If using Comparative Ct Method for quantification, ensure assay is robust with matched PCR efficiencies between target and housekeeping gene.

# Example of Development and Optimization of a Multiplexed Real Time qPCR Assay:

## Development of a Quantitative PCR (TaqMan) Assay for Relative Mitochondrial DNA Copy Number and the Common Mitochondrial DNA Deletion in the Rat

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Changes in mitochondrial DNA copy number and increases in mitochondrial DNA mutations, especially deletions, have been associated with exposure to mutagens and with aging. Common deletions that are the result of recombination between direct repeats in human and rat (4,977 and 4,834, bp, respectively) are known to increase in tissues of aged individuals. Previous studies have used long-distance PCR and Southern blot or quantitative PCR to determine the frequency of deleted mitochondrial DNA. A quantitative PCR (TaqMan) assay was developed to detect both mitochondrial DNA copy number and deletion frequency in the

rat. This methodology allows not only the determination of changes in the amount of mitochondrial DNA deletion relative to total mitochondrial DNA but also to determine changes in total mitochondrial DNA relative to genomic DNA. As a validation of the assay in rat liver, the frequency of the common 4,834 bp deletion is shown to increase with age, while the relative mitochondrial DNA copy number rises at a young age (3–60 days), then decreases and holds fairly steady to 2 years of age. *Environ. Mol. Mutagen.* 44:313–320, 2004. © 2004 Wiley-Liss, Inc.

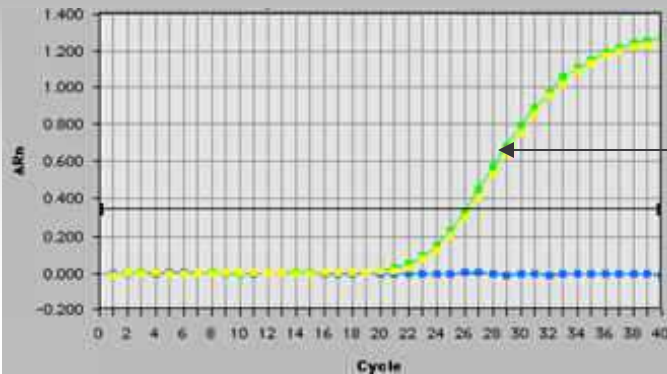
# Primer and Probe Design:

- Designed using Primer Express and Following General Guidelines
- Choose Fluorophore labels that are Spectrally Separated

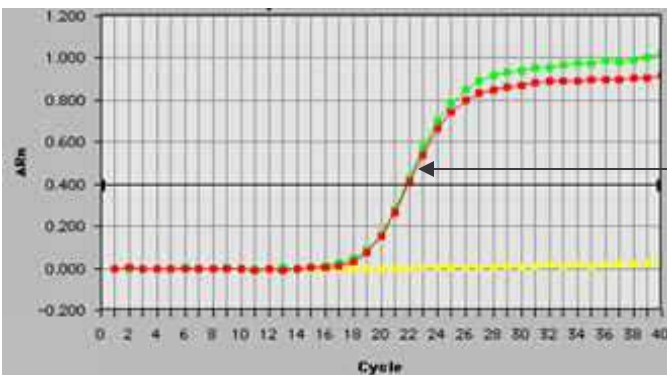
Table I. Sequences of the TaqMan Primers and Probe

Primer/probe	Sequence (5' - 3')
<b>Mitochondrial D-Loop</b>	
Forward	GGTTCCTTACTTCAGGGCCATCA
Reverse	GATTAGACCCGTTACCATCGAGAT
Probe	6-FAM-TTGGTTCATCGTCCATACGTTCCCCTTA-TAMRA
<b>Mitochondrial deletion</b>	
Forward	AAGGACGAACCTGAGCCCTAATA
Reverse	CGAAGTAGATGATCCGTATGCTGTA
Probe	VIC-TCACTTTAATCGCCACATCCATAACTGCTGT-TAMRA
<b><math>\beta</math>-actin</b>	
Forward	GGGATGTTTGCTCCAACCAA
Reverse	GCGCTTTTGACTCAAGGATTTAA
Probe	VIC-CGGTCGCCTTCACCGTTCCAGTT-TAMRA

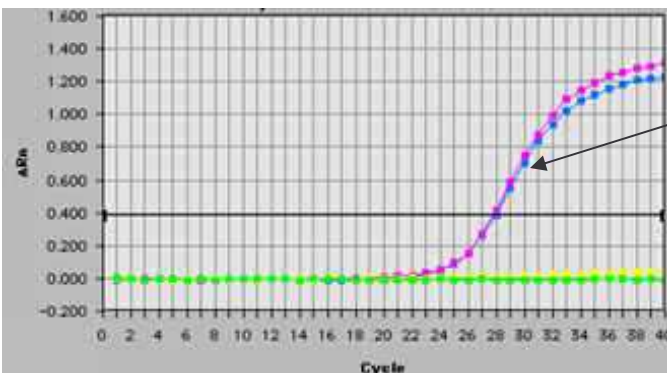
# Establish Species Relative Abundance:



**β-Actin: To be Multiplexed with D-Loop (Less Abundant)**



**D-Loop: To be Multiplexed with β-Actin and Mitochondrial Deletion (more abundant for both)**



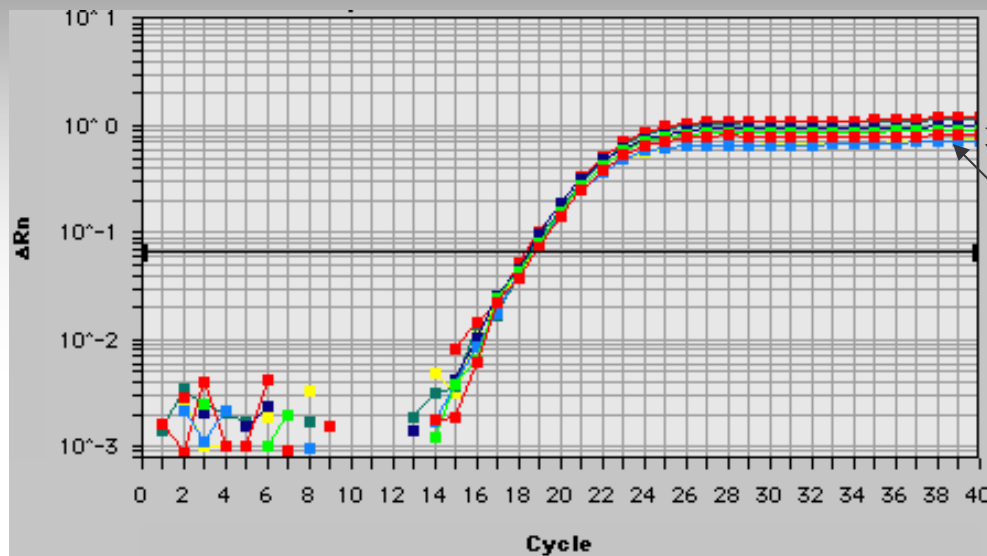
**Mito. Del.: To be Multiplexed with D-Loop (Less Abundant)**

**Probes and Primers used at Default Values of 250 nM probe and 900 nM primers**

# Limiting Primer Experiment I:

FOR 300 REV 300	FOR 300 REV 200	FOR 300 REV 100	FOR 300 REV 50
FOR 200 REV 300	FOR 200 REV 200	FOR 200 REV 100	FOR 200 REV 50
FOR 100 REV 300	FOR 100 REV 200	FOR 100 REV 100	FOR 100 REV 50
FOR 50 REV 300	FOR 50 REV 200	FOR 50 REV 100	FOR 50 REV 50

# Probe Titrations Experiment:



**D-Loop:**

**Probe:**

200 nM

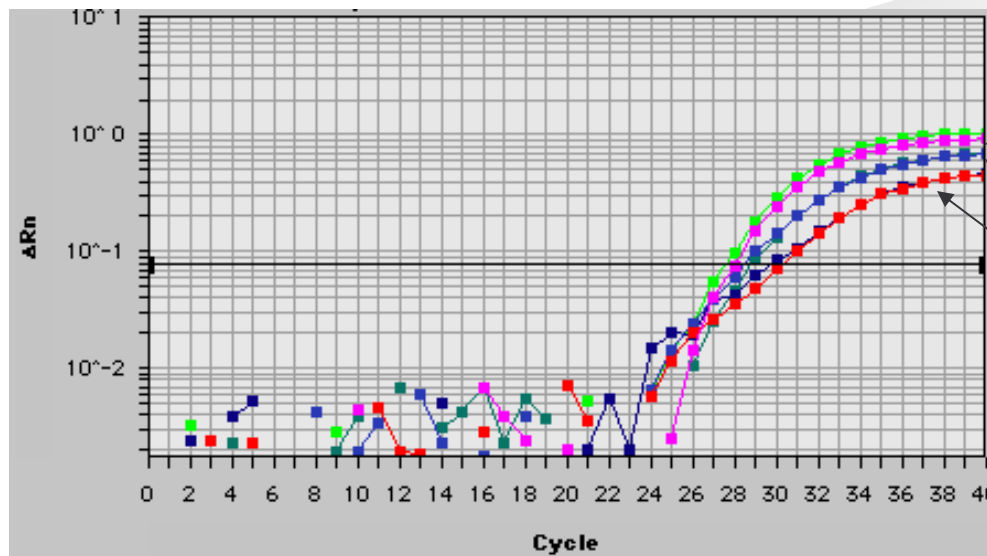
100 nM

50 nM

**Primers:**

100 nM

R and F



**Mito. Deletion:**

**Probe:**

200 nM

100 nM

50 nM

**Primers:**

200 nM

R and F

# Limiting Primer Experiment II:

**TABLE II. Effects of Altering Primer Concentrations on  $C_T$  and  $\Delta C_T$**

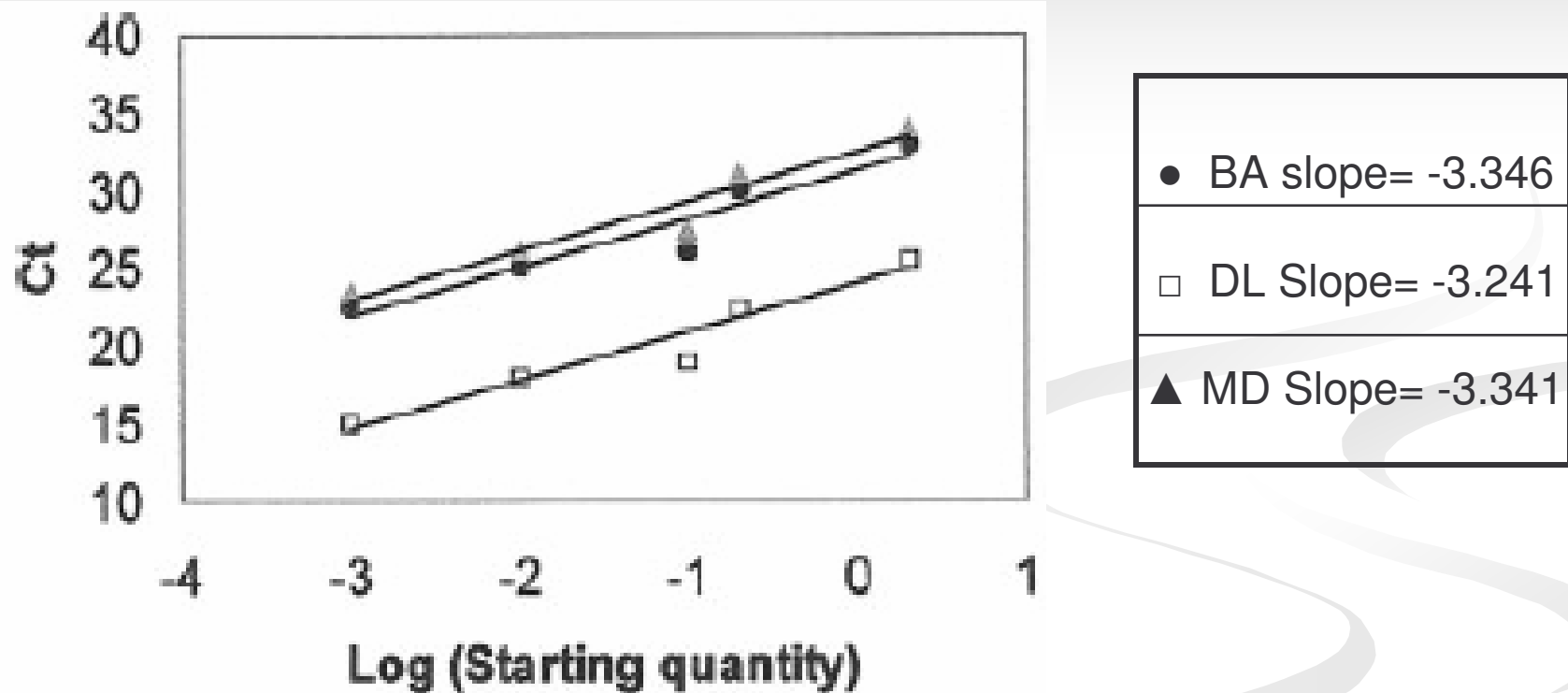
<b>B-actin primer</b>	<b>D-Loop primer</b>	<b><math>\beta</math>-actin <math>C_T</math></b>	<b>D-Loop <math>C_T</math></b>	<b>D-Loop <math>C_T</math> - <math>\beta</math>-actin <math>C_T</math> (<math>\Delta C_T</math>)</b>
200 nM	100 nM	27.55	18.54	-9.01
200 nM	50 nM	27.09	19.03	-8.06
200 nM		27.45		
100 nM	200 nM	28.48	18.38	-10.1
50 nM	200 nM	29.95	18.45	-11.5
	200 nM		18.42	

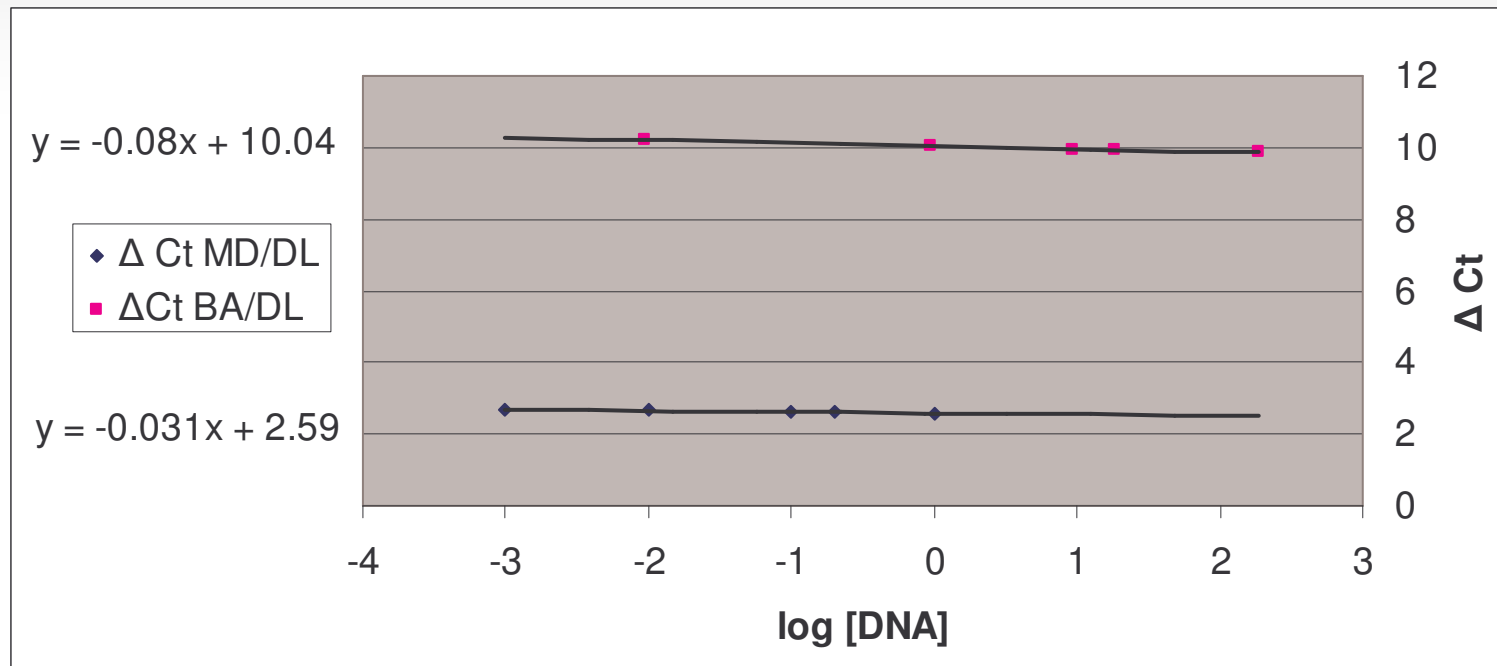
<b>Mito. Deletion primer</b>	<b>D-Loop primer</b>	<b>Mito. deletion primer <math>C_T</math></b>	<b>D-Loop <math>C_T</math></b>	<b>Mito. Deletion <math>C_T</math> - D-Loop <math>C_T</math> (<math>\Delta C_T</math>)</b>
200 nM	200 nM	23.54	18.43	5.11
200 nM	100 nM	22.48	18.31	4.17
200 nM	50 nM	22.82	18.74	4.08
200 nM		23.32		
100 nM	200 nM	25.36	18.36	7.00
50 nM	200 nM	28.41	18.22	10.10

All probes were used at 100nM

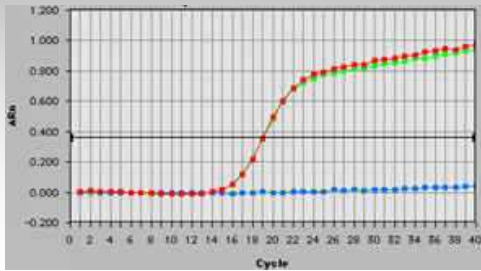
# Establish Linear Dynamic Range and PCR Efficiencies:



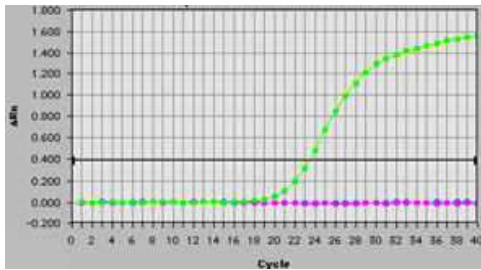
# Validation Experiment: Allows Usage of the Comparative Ct Method



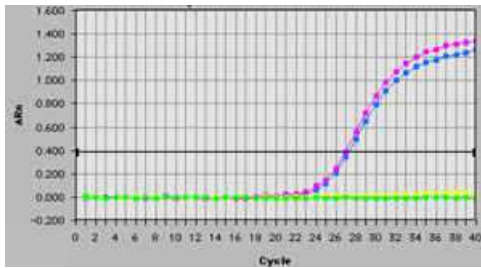
# Establish that Optimized Assay Does Not Affect Ct Values:



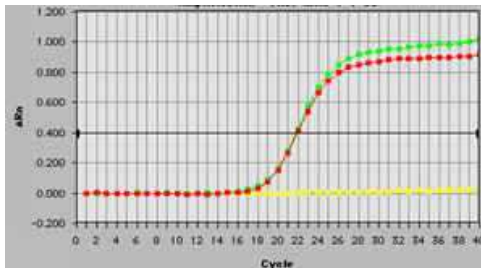
D-Loop with Mito. Del.



Mito Del. with D-Loop



$\beta$ -Actin with D-Loop



D-Loop with  $\beta$ -Actin

Each assay is run with two control samples at the same concentration

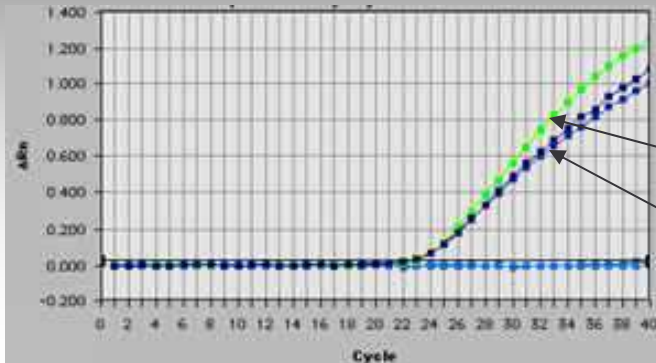
# Reproducibility Over Time

- One control sample of same DNA concentration
- ran in all 23 different runs
- 23 different runs occurred over a 12.5 month period
- delta Ct values were evaluated over the assay period
- Standard deviations did increase over the 12.5 month period for both multiplexed Assays

# **Triplex: Three Probes, Three Primer Sets**

- Choose three fluorophores that are spectrally separated**
- Employ use of non-fluorogenic quenchers**
- Similar development as duplex**

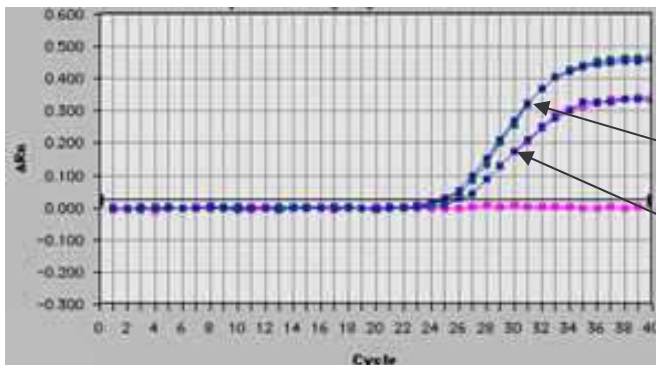
# Some Failures: Attempt at Triplex



FAM labeled endogenous control :

Endogenous control alone

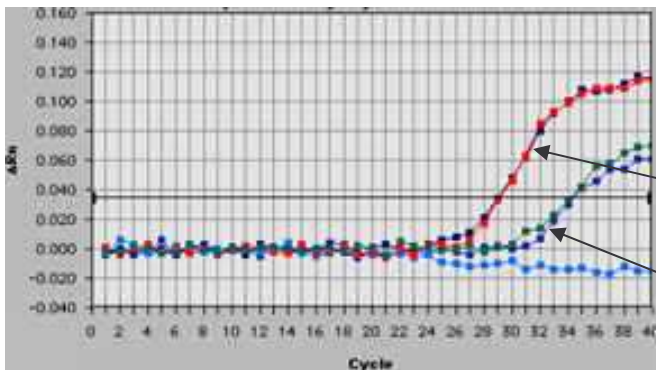
Run in a triplex



Joe labeled Target:

Target alone

Target in a triplex



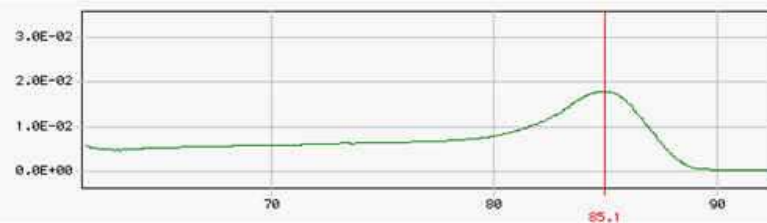
Tamra labeled Target:

Target alone

Target in a triplex

# SYBR Green I Multiplex:

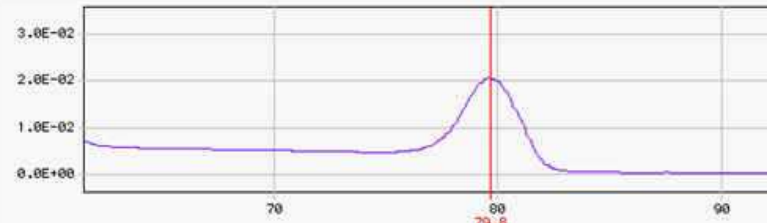
Human Primer set



Melting Temperature

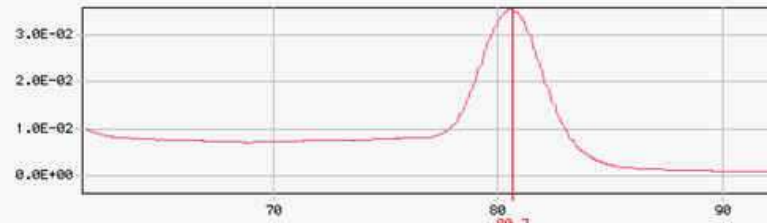
85.1

Cow primer set



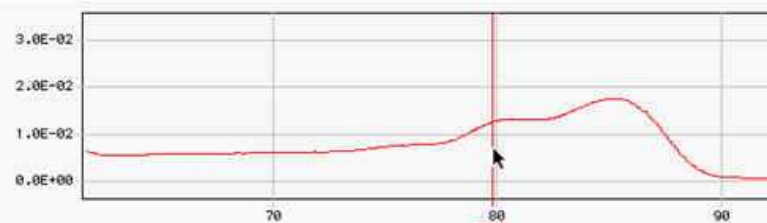
79.8

Chicken Primer Set



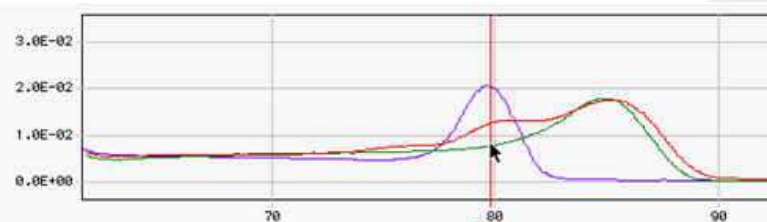
80.7

Mealworm sample



79.8/85.1

Alignment:  
Human, cow, and  
sample



**What Works Best For You?**

The background of the slide features a light gray gradient. In the lower right quadrant, there are several thick, overlapping, wavy lines in shades of gray, creating a sense of movement and depth.