Real Time PCR:
Applied Theory and Best Practices

Introduction to Real-Time PCR

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What is Real-Time PCR

Real-Time PCR, is a technique in molecular biology whereby a **target molecule** of DNA is **amplified** by repeat cycles of PCR

AND

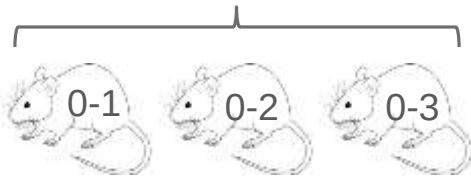
The amplification of the target molecule is **monitored** after each cycle of PCR i.e. in real time and not at its end-point, as in conventional PCR.

Scientific Question

Does the **expression level** of the Gene X change between my biological groups?

- Wild type vs Knockout
- Healthy vs Disease
- Following Treatment

Control



Knockout

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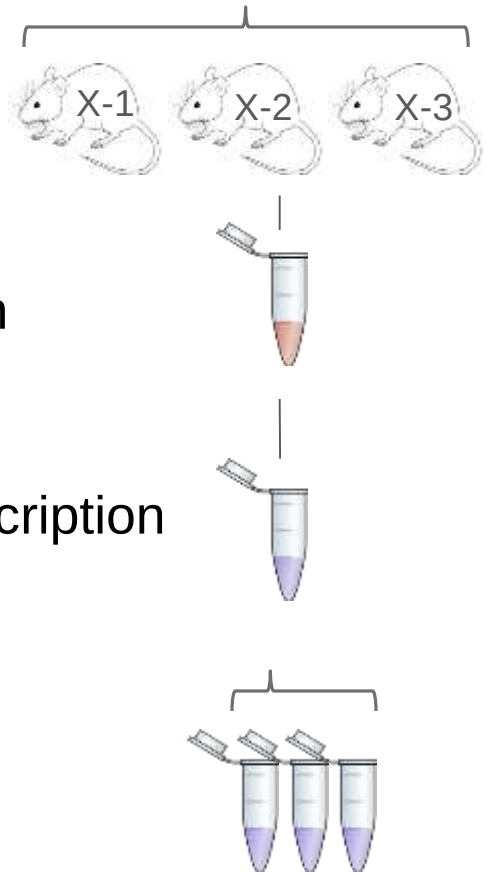
Experimental Design — Wet Lab



Considerations

- Biological Replicates
- RNA Quantity *and* Quality Assessment
- NRT Control
- Enzyme Selection
- Assay Design/Optimization
- Reference Genes
- Controls

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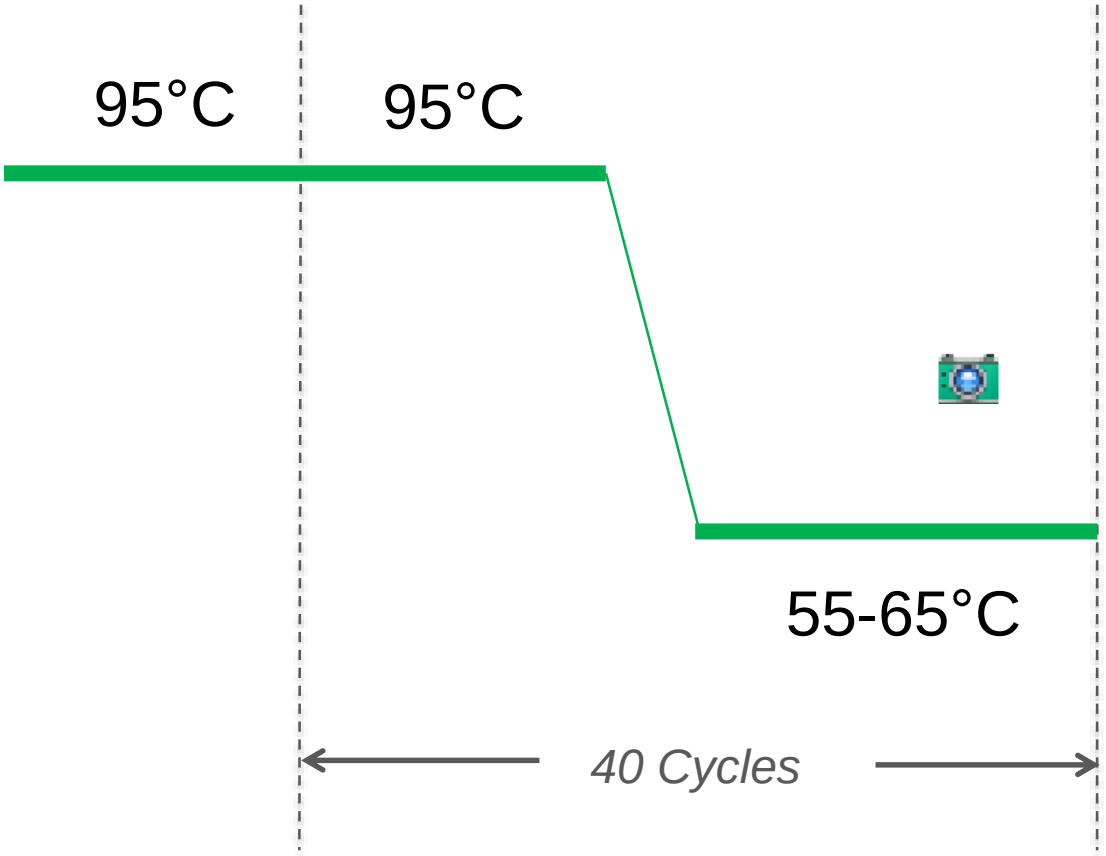
Amplification

Reagents

Supermix

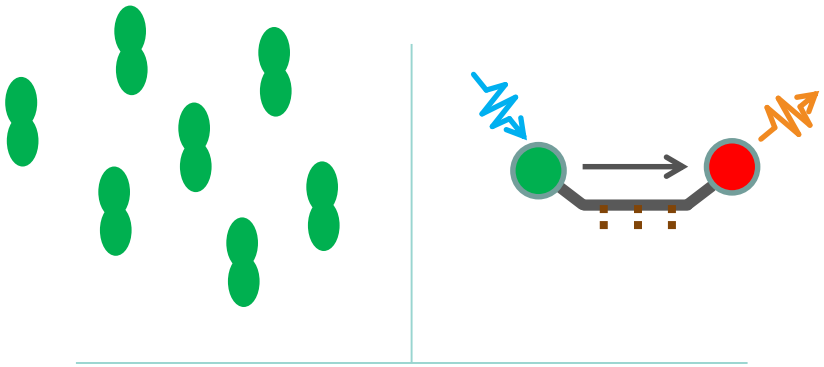
- dNTPs
- Polymerase
- Buffers
- Primers
- Template

Amplification



Hot Start Denature Anneal/Extend

Real Time Fundamentals

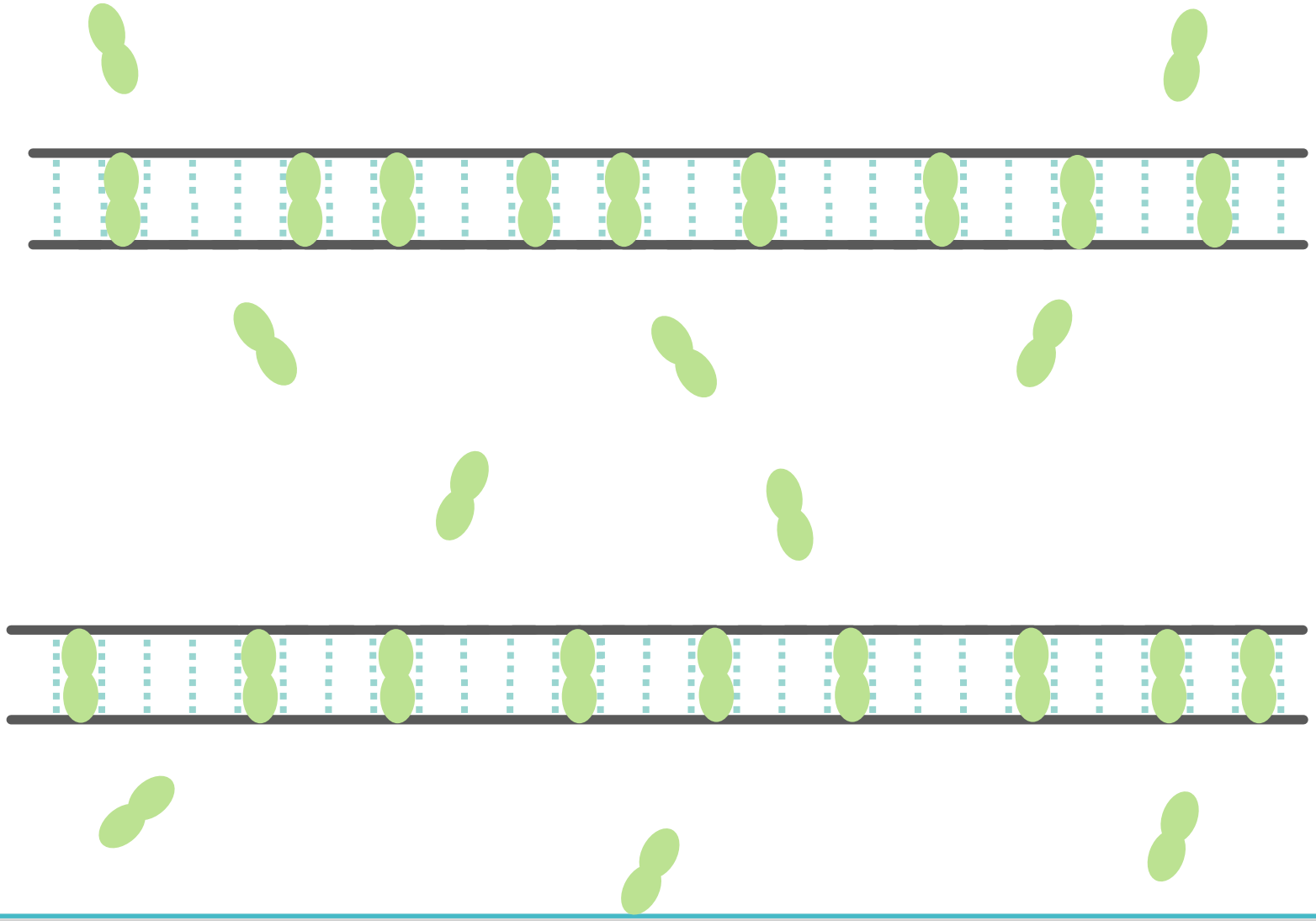


Detection

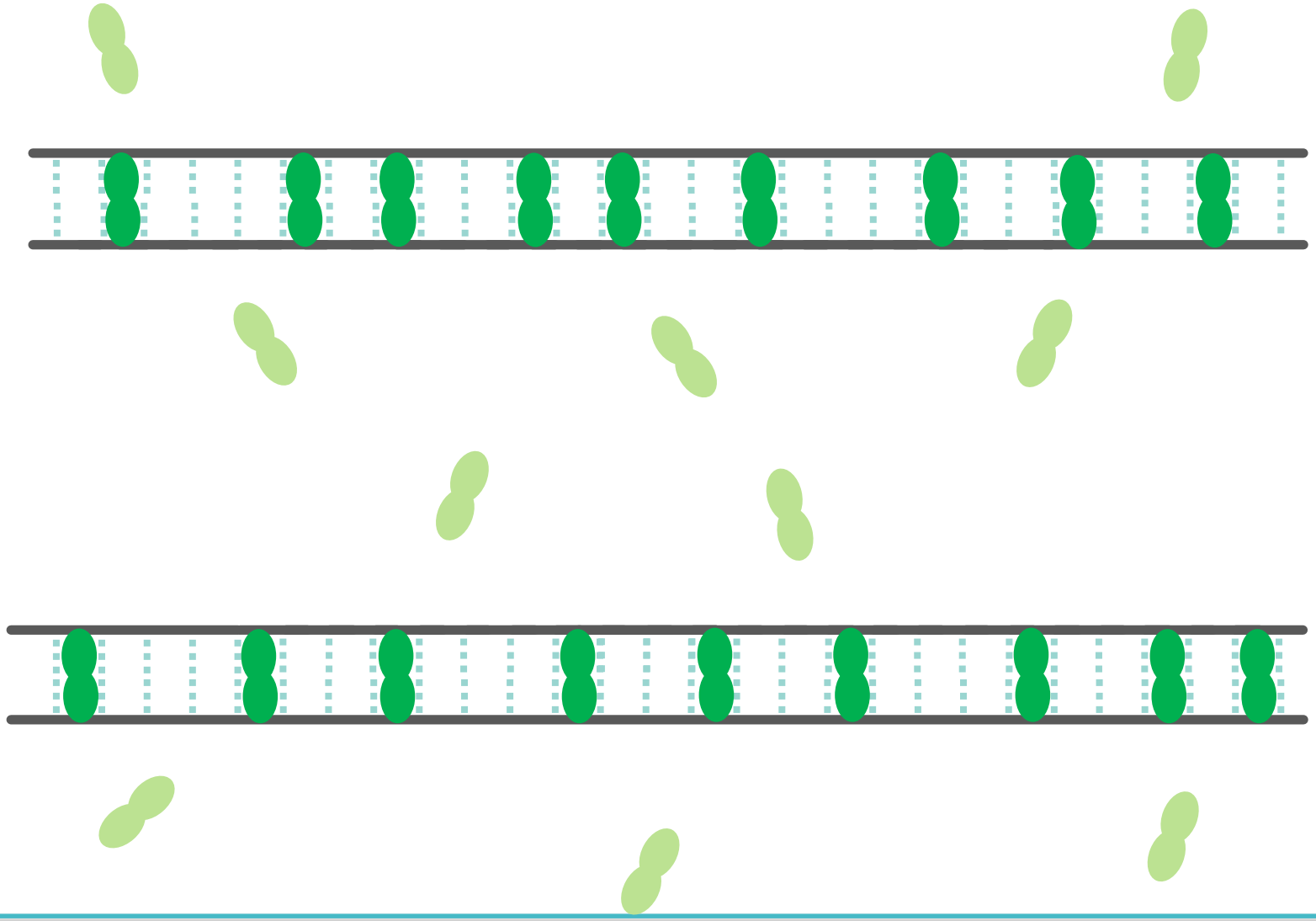
Detection

- Fluorescence
- Real-Time System

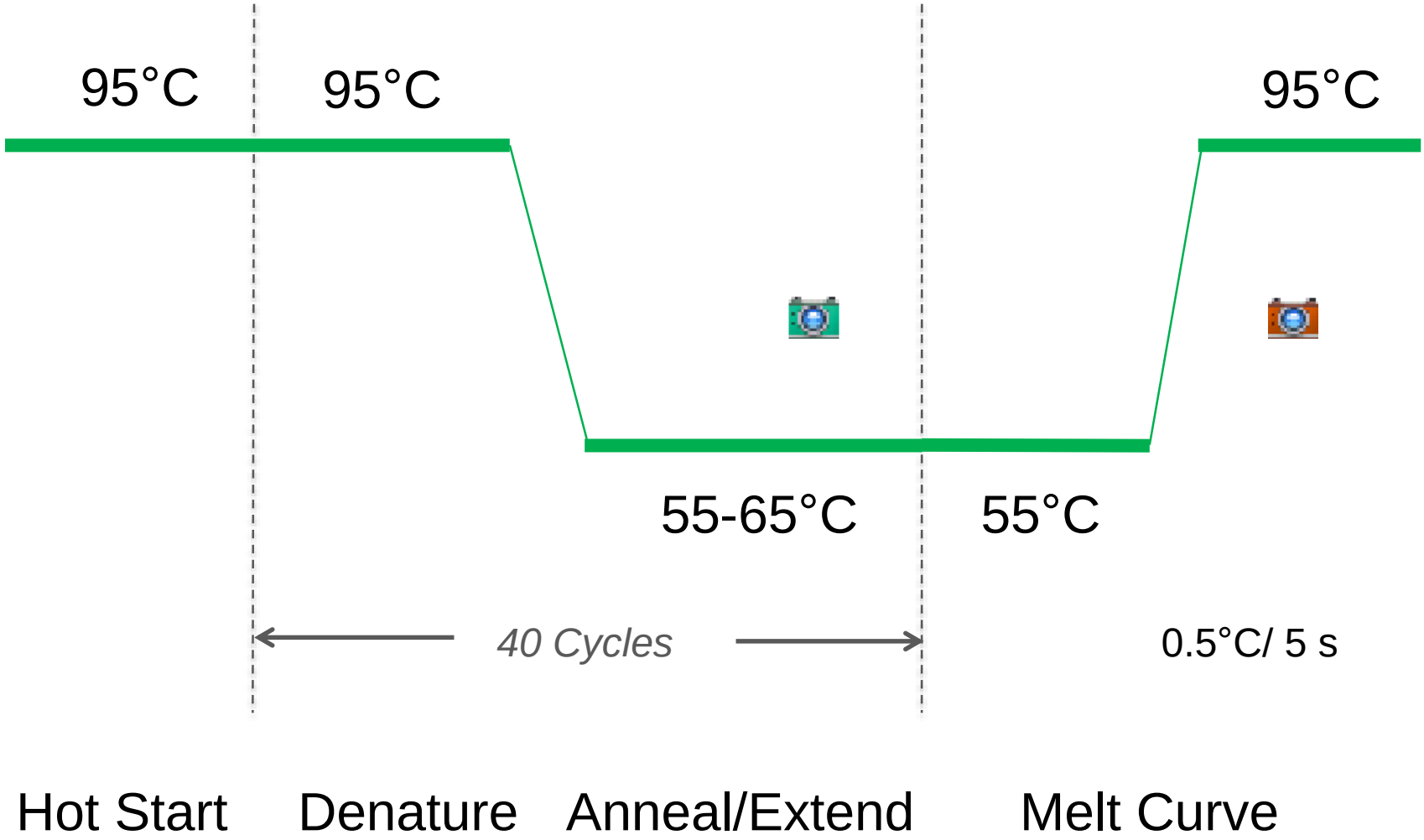
dsDNA Specific Dye



dsDNA Specific Dye



Dye Based Chemistry — Melt Curve



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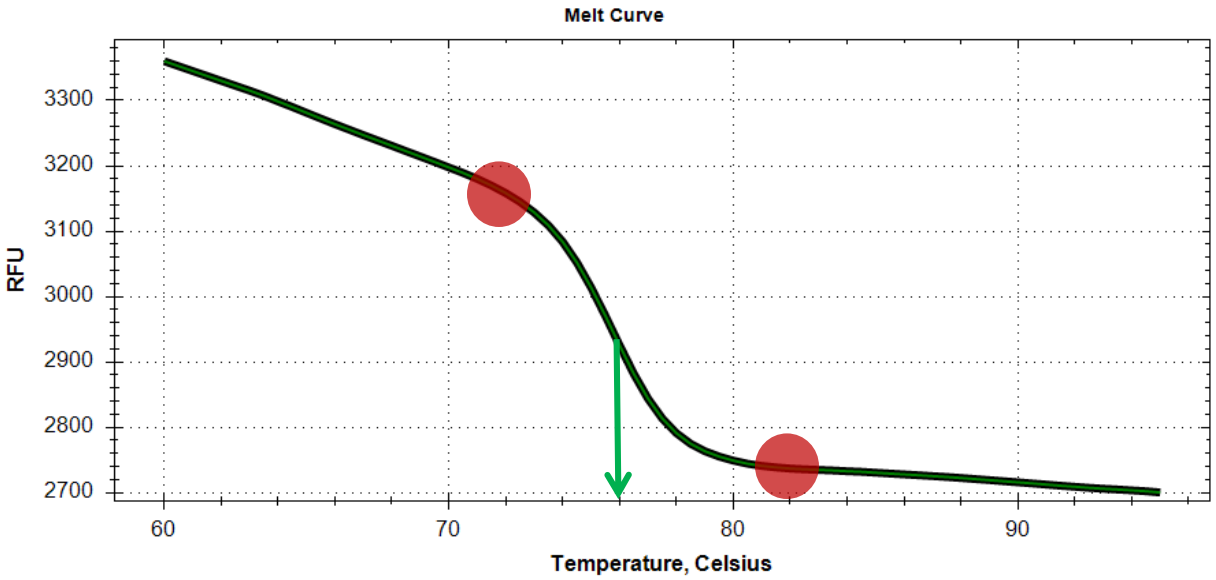

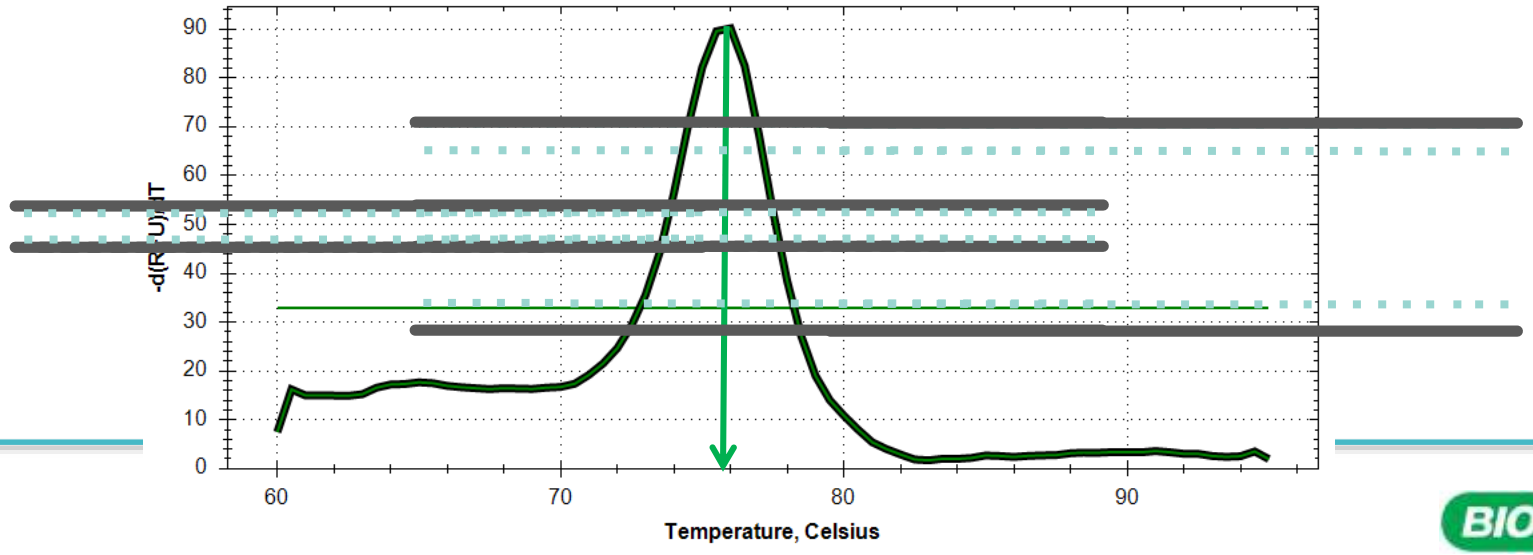

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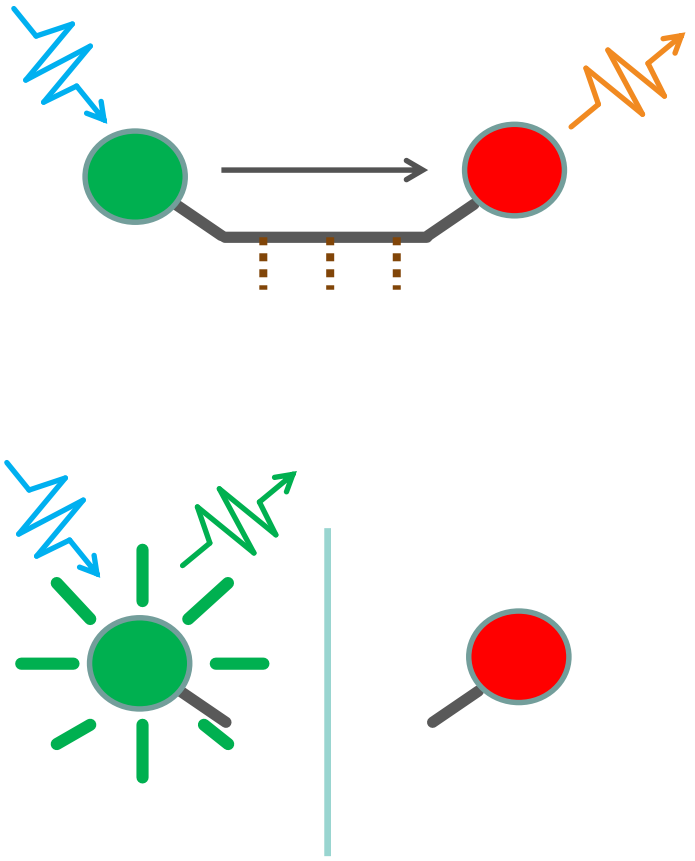
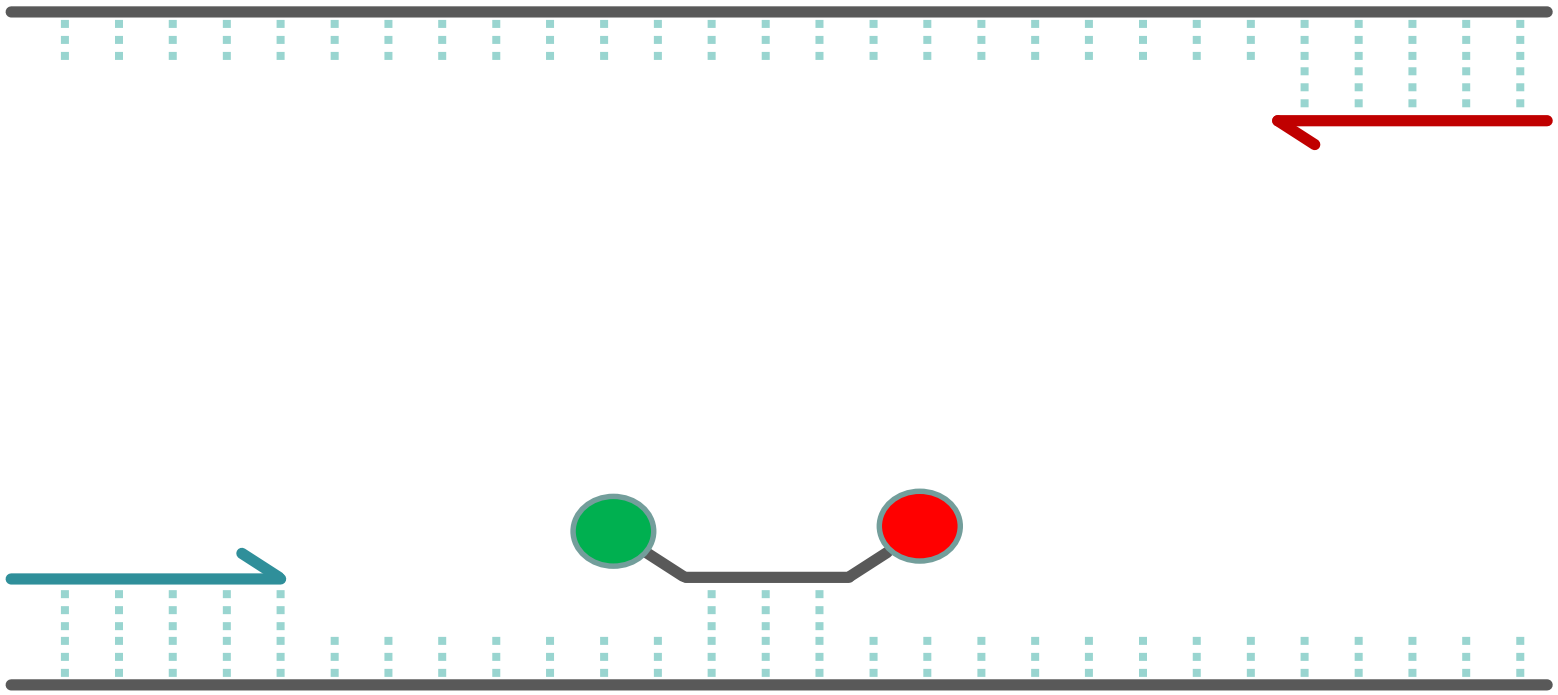
Duel Labeled Probe

- Sequence specific oligo
- 5’ Fluorescent Reporter
- 3’ Quencher
- FRET
- Fluorescent when hydrolyzed during amplification

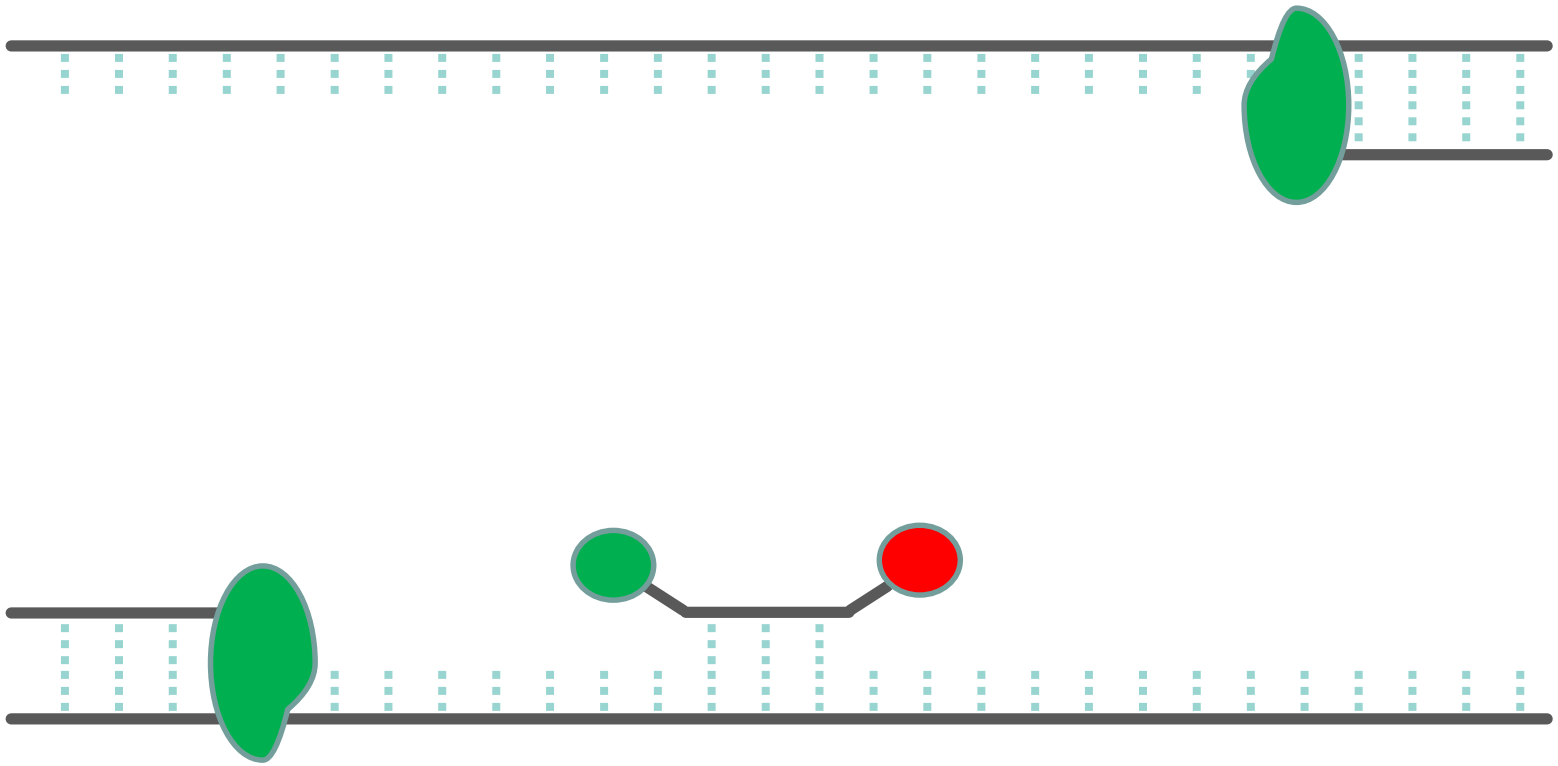


Duel Labeled Probe



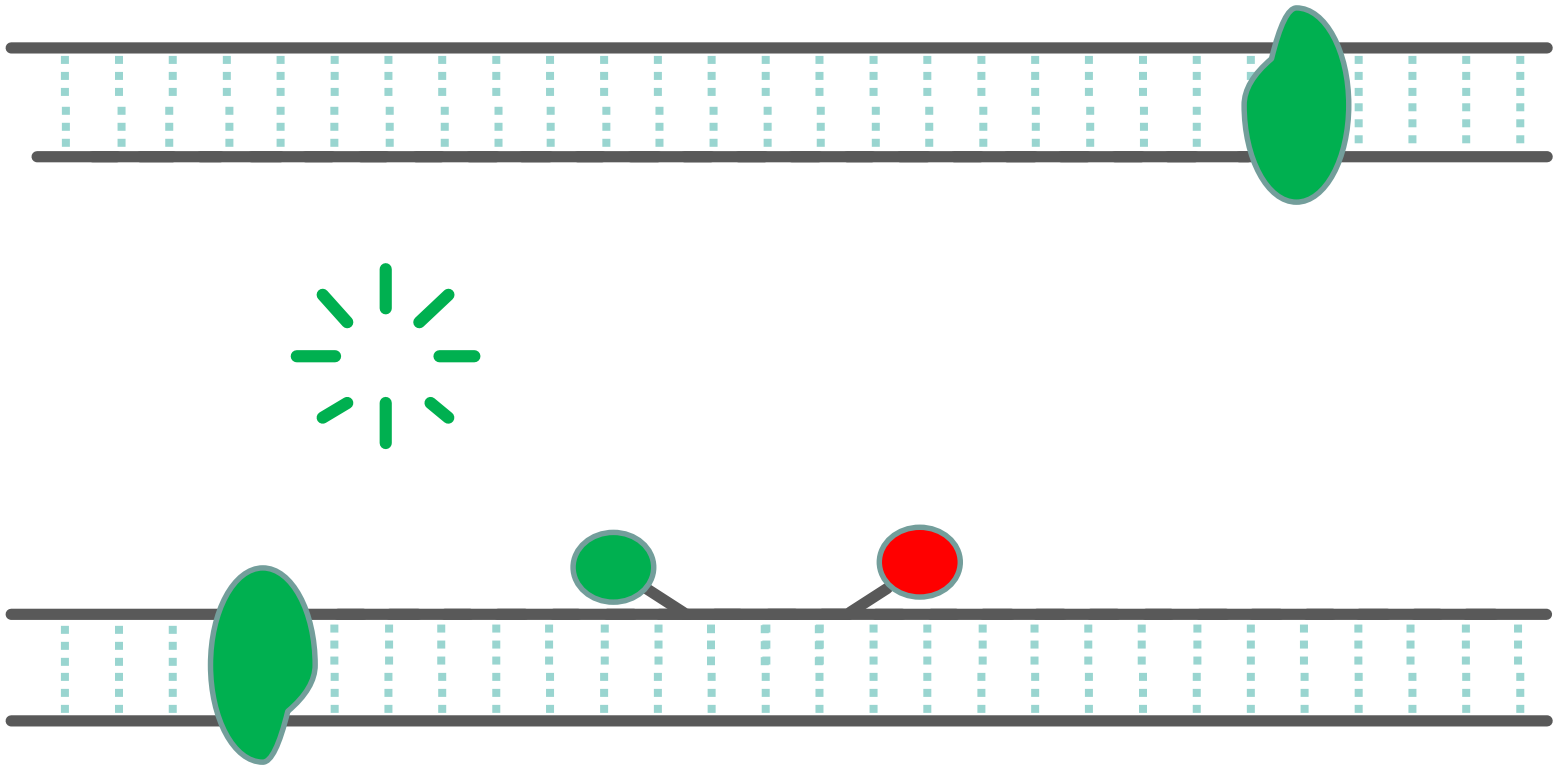
Anneal 55-65ºC

Duel Labeled Probe



Extend 55-65ºC

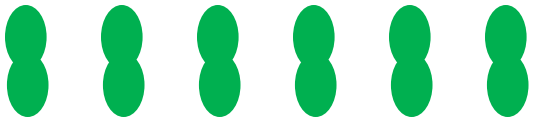
Duel Labeled Probe



Extend 55-65ºC

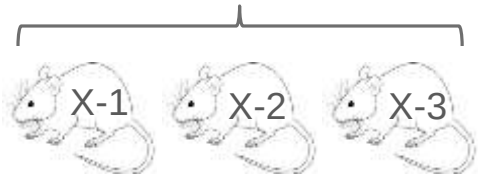
Chemistry Comparison

- **Multiplex capability**
- **Increased specificity**
- More expensive
- More complex assay design
- Need to run gels to confirm specificity



- **Inexpensive**
- **Can run Melt-Curve**
- Can’t Multiplex
- Not tolerant to non-specific amplification

Experimental Design — Wet Lab



RNA Extraction

Reverse Transcription

Amplification

Considerations

- Biological Replicates
- RNA Quantity *and* Quality Assessment
- NRT Control
- Enzyme Selection
- **Reference Genes**
- Controls

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Reference Genes

Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes

Jo Vandesompele, Katleen De Preter, Filip Pattyn, Bruce Poppe, Nadine Van Roy, Anne De Paepe and Frank Speleman

Published: 18 June 2002

Genome ***Biology*** 2002, **3(7)**:research0034.1–0034.11

- Reference gene mRNAs should be **stably expressed** across sample conditions
- **Multiple reference genes** often times are better

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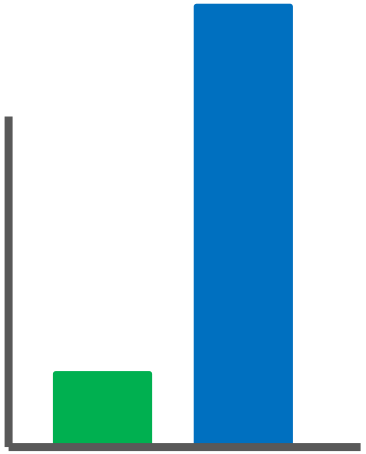
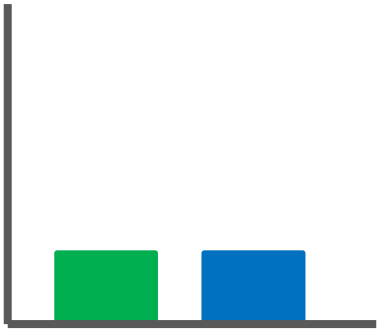
Unverified Reference Gene

- Reference gene mRNAs should be **stably expressed** across sample conditions
- **Multiple reference genes** preferred

Calculated

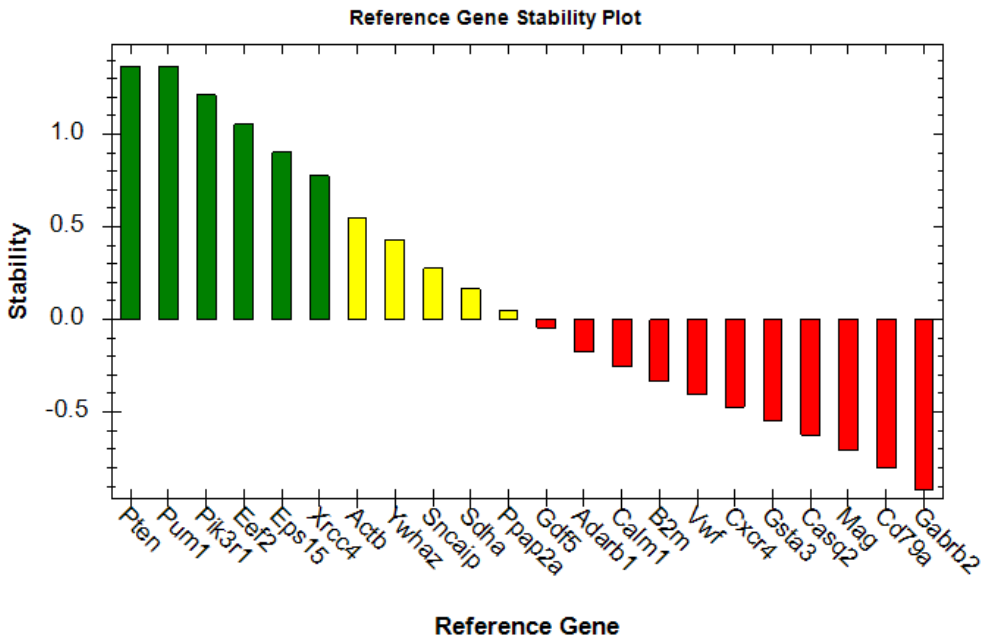


Actual



Reference Gene Selection Plate

Proper Reference Gene Selection



- Maestro provides a simple color-coded stability assessments
- Select the appropriate reference gene(s) for your experiment and begin collecting data
- Multiple reference genes can provide further stability and better normalization

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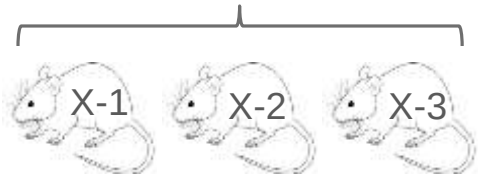
Experimental Design — Wet Lab



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- **Controls**

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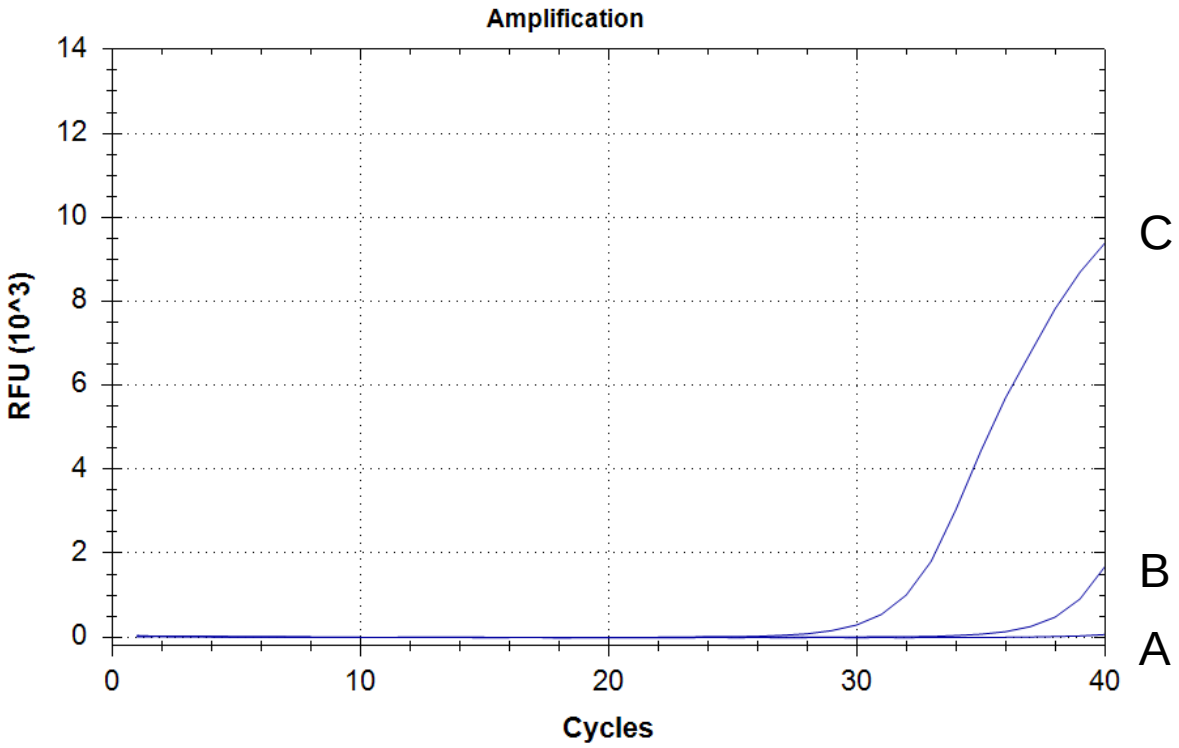
Controls — No Template Control

- dNTPs
- Polymerase
- Primers
- Buffers
- Water
- Template



Resources

- MIQE Guidelines
- Applications Guide (Fundamentals)
- Prime-PCR Guide (Protocols/Best Practices)
- Amp Reagents Guide
- Digital PCR Guide
- Reagents Comparison Guide
- Website (Tips, Tricks, More)

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