# QPCR BASICS

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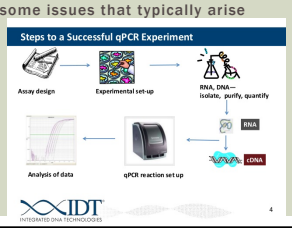
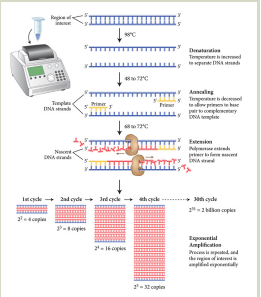
## OBJECTIVES

- To teach the theoretical concepts of how PCR operates
- To demonstrate the practical methods of how a qPCR reaction is prepared
- To provide the ability to analyze data from a qPCR run
- To highlight some issues that typically arise



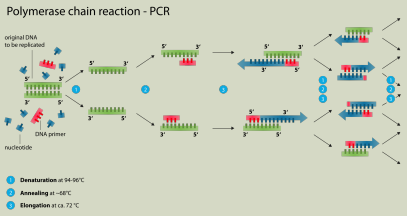

## WHAT IS PCR?

- Polymerase Chain Reaction- uses a polymerase enzyme to make copies of DNA in a chain reaction
- How does one artificially make DNA?
  - Heat it up to make the two strands come apart (Denaturation)
  - Focus on one section of DNA via primers (Annealing)
  - Each complementary strand completed (Extension)



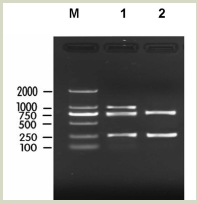
## HOW DOES IT WORK?

- PCR Reaction
  - DNA, Primers
  - Temperature
  - Taq Polymerase
  - dNTP
  - $Mg^{2+}$
- Temperature Profile
  - Stages of the reaction
  - Number of cycles



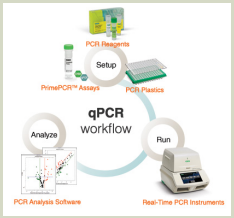

## DETECTION

- Gel Electrophoresis
  - Use charged liquid to pull DNA through a gel media
  - Include a known sample of size standards (ladder)
  - Can compare your replication with the ladder to determine if it worked!
- Limitations
  - Difficult to quantify
  - Difficult to determine presence/absence
    - Is a slight band a positive?
- Advantages
  - Cheap!!!!!!




## WHAT IS QPCR?

- Quantitative PCR (or Real time PCR)
  - It provides an estimate of your product
  - It provides this estimate after every single cycle

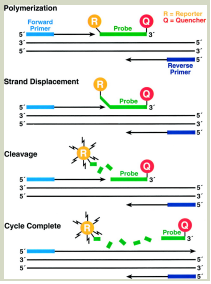


## HOW DOES IT WORK?

- The PCR part works exactly the same!
- Two methods
  - Cyber Green (Non-specific)
  - Taqman (Targeted- like primers)
- Both use a fluorescent dye method.
  - As the product increases in concentration, more dye is able to fluoresce.
  - Machine measures this fluorescence value

## TAQMAN APPROACH

- Reporter Dyes: FAM, TET, JOE, VIC, Texas Red
- Quencher: TAMRA, MGB, Black Hole
- Reference: ROX

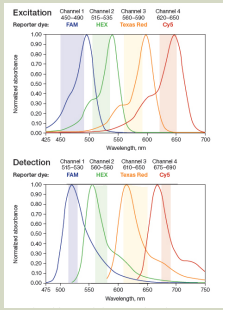



## PRIMER CHOICE

- Conserved Ranavirus region
- Other sequences more specific to type: FV3, ATV, ENHV
- FAM, VIC, NED
  - Fluoresce at different wavelengths
  - Allow multiple targets to be examined
    - Could be used to identify different strains of RV

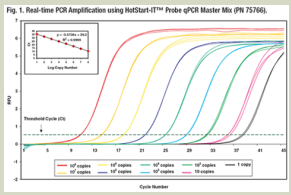
## MULTIPLEX

- Run simultaneous tests on one sample
- Use reporters that fluoresce at different wavelengths




## STANDARD CURVE

- Measurement of fluorescence is relative (Ct values)
- To create a comparison we must add in known quantities
  - Similar to the ladder in PCR.
    - Here we add in known quantities rather than known sizes.




## STANDARDS

- Plaque forming units- estimate of virus quantity
  - Requires the isolation, culturing, and counting of virus
- Gene copies
  - Order section of sequence from molecular company
  - Can verify DNA quantity
  - IDT- gBlocks

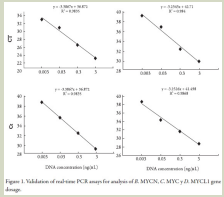



Figure 1. Validation of real-time PCR assays for analysis of *B*, MYCN; *C*, MYC; *D*, MYCL1 gene dosage.

## RANAVIRUS PROTOCOL

- TaqMan
- Primers
  - Forward: ACACCACCGCCCAAAAGTAC
  - Reverse: CCGTTCATGATGCGGATAATG
- Probe
  - CCTCATCGTTCTGGCCATCAACCAC

- Heat cycle (40 cycles)
  95°C 10 min (activate taq)
- 1) 95° 20s (denature)
- 2) 54° 20s (anneal)
- 3) 72° 30s (elongation)

## RANAVIRUS PROTOCOL

- Reaction volumes per well

4.6 ul Water
10 ul 2x TaqMan Mastermix
0.6 ul Forward Primer (300 nmol)
1.8 ul Reverse Primer (900 nmol)
1 ul Probe (250 nmol)
2 ul DNA Template (~ 100ng)
____
20 ul

## RANAVIRUS PROTOCOL

- Alternate protocol

2x TaqMan Mastermix
Forward Primer (300 nmol)
Reverse Primer (300 nmol)
Probe (200 nmol)
DNA Template (~ 100ng)

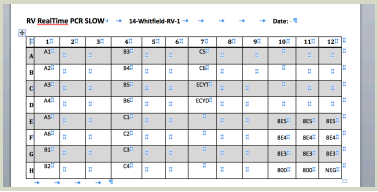
## CONTROLS

- Negative controls
  - Water
  - Sample tissue
- Positive controls
  - Infected animal
  - Virus culture
  - DNA

- Important to guard against both contamination and inhibition

## REPLICATION

- Often each sample (and standard) is run in triplicate
  - Ensures confidence in negative results and positive values
- Some laboratories use less (duplicate or singlicate)
  - Cheaper
  - Higher sample size can make up for error


RV RealTime PCR SLOW → 14-Whitfield-RV-1 → → → → Date:

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | A1 | | | B3 | | | C5 | | | | | |
| B | A2 | | | B4 | | | C6 | | | | | |
| C | A3 | | | B5 | | | ECYT | | | | | |
| D | A4 | | | B6 | | | ECYO | | | | | |
| E | A5 | | | C1 | | | | | | 8E5 | 8E5 | 8E5 |
| F | A6 | | | C2 | | | | | | 8E4 | 8E4 | 8E4 |
| G | B1 | | | C3 | | | | | | 8E3 | 8E3 | 8E3 |
| H | B2 | | | C4 | | | | | | 800 | 800 | NEG |

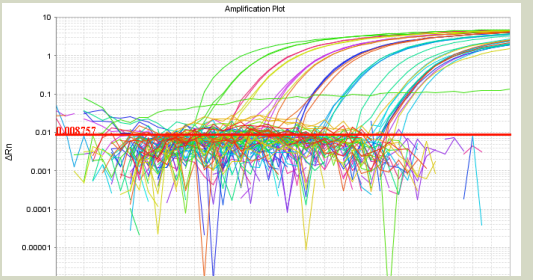
→ → → → ¶

## ANALYSIS

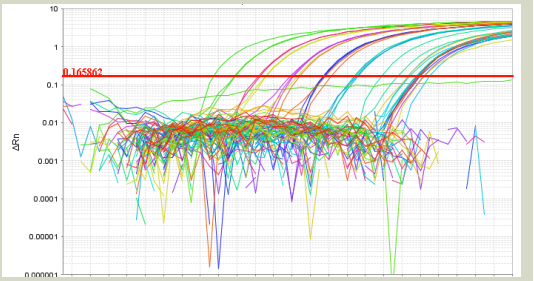
- Machine will attempt to score the samples for you based upon your standard curve.

- How do I know if it worked?
- What if some of my standards are bad?
- What if my samples have a range of values?

## OUTPUT




## CHANGING THE THRESHOLD




## MESSY DATA

## QUESTIONS?

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