

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

Steps to a Successful qPCR Experiment

Analysis of data

qPCR reaction set-up

RNA, DNA - isolate, purify, quantify

Assay design

Experimental set-up

IDT
HYBRIDIZATION TECHNOLOGIES

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)

Denaturation
Temperature is raised to separate DNA strands

Annealing
Temperature is decreased to allow primers to bind to DNA template

Extension
Polymerase extends DNA strand

Exponential Amplification
Process is repeated, and the region of interest is amplified exponentially

1st cycle → 2nd cycle → 3rd cycle → 4th cycle → 5th cycle

$2^1 = 2$ copies

$2^2 = 4$ copies

$2^3 = 8$ copies

$2^4 = 16$ copies

$2^5 = 32$ copies

HOW DOES IT WORK?

PCR Reaction

- DNA, Primers
- Temperature
- Taq Polymerase
- dNTP
- Mg²⁺

Temperature Profile

- Stages of the reaction
- Number of cycles

Polymerase chain reaction - PCR

The diagram illustrates the PCR process in three stages: 1. Denaturation at 94-96°C, where the original DNA is heated to separate into two single strands. 2. Annealing at 48°C, where DNA primers bind to the single strands. 3. Elongation at 72°C, where Taq polymerase synthesizes new DNA strands by adding nucleotides to the primers. The legend indicates: Denaturation at 94-96°C (red circle), Annealing at 48°C (blue circle), and Elongation at ca. 72°C (green circle).

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!!!!!!

The gel electrophoresis image shows a DNA ladder (M) with bands at 2000, 1000, 750, 500, 250, and 100 base pairs. Lane 1 shows a band at approximately 500 bp, and lane 2 shows a band at approximately 250 bp.

WHAT IS QPCR?

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

The qPCR workflow diagram shows a cycle of: Run (Real-Time PCR Instruments) -> Analyze (PCR Analysis Software) -> Setup (PCR Reagents, PrimePCR Assays, PCR Plastics) -> Run. The central text is 'qPCR workflow'.

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

The diagram illustrates the Taqman approach in four stages: 1. Polymerization: A forward primer (5' to 3') and a reverse primer (3' to 5') bind to a target DNA strand. A probe with a 5' fluorophore (R) and a 3' quencher (Q) also binds to the target. 2. Strand Displacement: The forward primer is extended by DNA polymerase, displacing the probe. 3. Cleavage: The probe is cleaved by the polymerase, separating the fluorophore from the quencher. 4. Cycle Complete: The fluorophore is released and fluoresces, while the quencher remains bound to the probe fragment.

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

Excitation

Channel	Reporter dye	Excitation (nm)
Channel 1	FAM	480-490
Channel 2	HEX	515-525
Channel 3	Texas Red	560-590
Channel 4	Cy5	620-650

Detection

Channel	Reporter dye	Emission (nm)
Channel 1	FAM	515-525
Channel 2	HEX	560-590
Channel 3	Texas Red	615-625
Channel 4	Cy5	675-695

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.

Fig. 1. Real-time PCR Amplification using HotStart-IT™ Probe qPCR Master Mix (PN 757160).


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: ACACCACGCCCAAAAGTAC
 - Reverse: CCGTTCATGATGGGATAATG
- 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

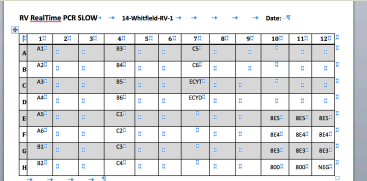
- Negative controls
 - Water
 - Sample tissue
- Positive controls
 - Infected animal
 - Virus culture
 - DNA



- Important to guard against both contamination and inhibition


REPLICATION

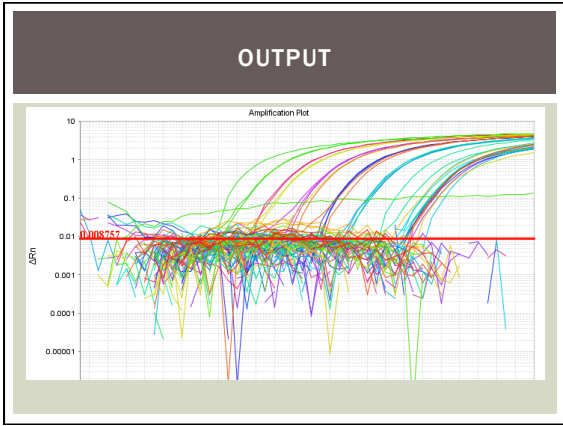
- Often each sample (and standard) is run in triplicate
 - Ensures confidence in negative results and positive values
- Some laboratories use less (duplicate or singlicate)
 - Cheaper
 - Higher sample size can make up for error

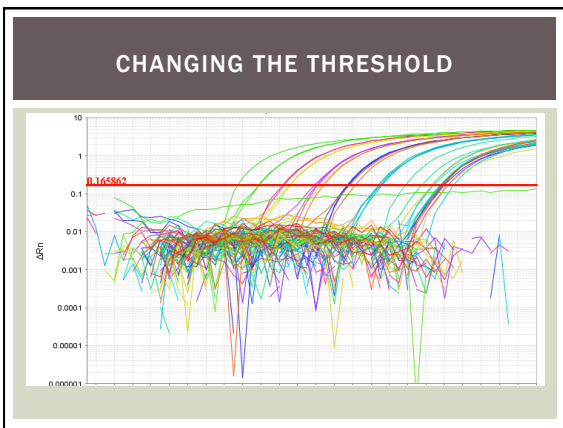


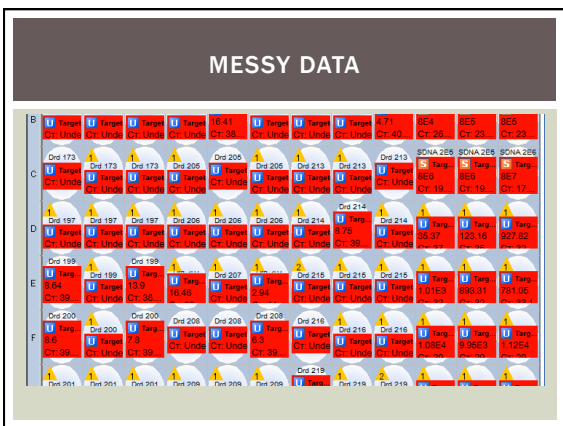
ANALYSIS

- 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?









QUESTIONS?

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