qPCR: Basic Principles and Range of Applications

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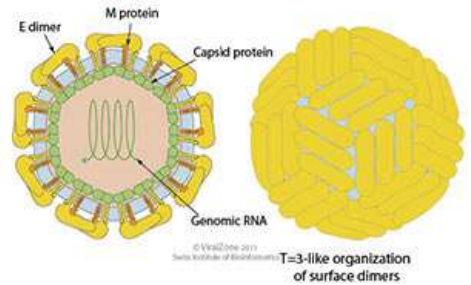
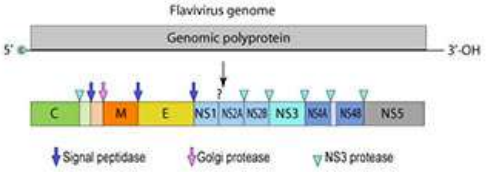
Orange County, California





Because oysters contain not only pearls.

foodproof®
Norovirus Detection Kit

- Fast multiplex real-time PCR
- Based on ISO TS 15216
- Differentiates between GI and GII
- Process control included
- Matrices validated include shellfish, berries, leafy greens, ground beef, etc.

Quantitative PCR

Pros:

- Rapid (less than 2 hours)
- High throughput capacity
- Highly specific, capacity for direct pathogen quantification
- Multiple targets can be measured at once
- Can be user-friendly and highly cost effective

Cons:

- Only semi-quantitative unless you attend to proper controls
- Has potential for cross-reactivity if not designed correctly
- Detects both viable and non-viable cells
- Requires capital costs, calibration of equipment, and devoted space

Specific examples of applications

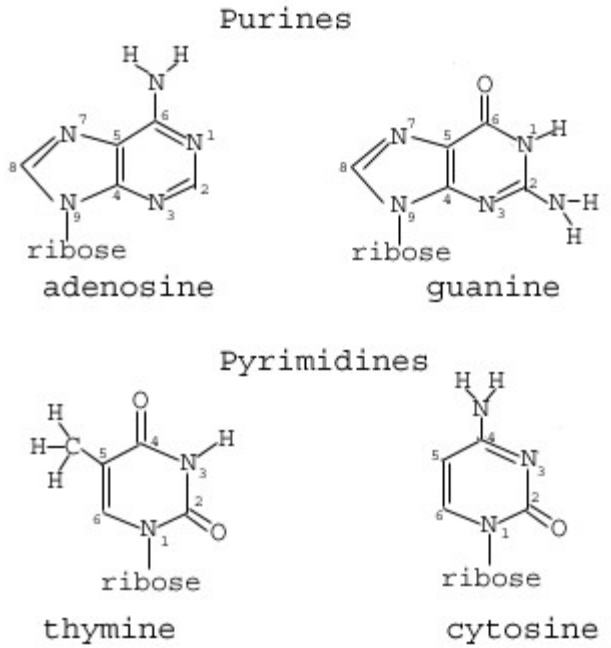
- Clinical microbiology: HIV, HepA, HepB, HepC, antibiotic resistant bacteria, *Mycoplasma pneumoniae*, herpes simplex virus
- Food microbiology: Salmonella, Listeria, E. coli (ETEC and subtypes),Shigella, Vibrio, Campylobacter
- Food quality: GMO, mixed meat testing, animal feed additives
- FDA FSMA Produce Safety Rule, E. coli, Salmonella, Listeria
- Genetic variation, inherited traits, cancer diagnoses
- Prenatal and post-natal diagnostics: non-invasive, Streptococcus B, genetic disease testing, C. diff
- Clinical standardization and accreditation by CAP

Quantitative PCR for environmental monitoring

- Many applications for environmental monitoring
- Fecal indicator bacteria quantification, EPA-approved (now: EPA 1609 and 1611, future: Methods B and C)
- Viral and bacterial pathogen quantification from water and shellfish (norovirus, Vibrio sp.)
- Pathogen and indicator quantification in drinking water, reclaimed waters, stormwater, beach waters, seafood and shellfish, irrigation water, produce wash water
- Discrimination of pathogens from harmless populations for protection of public health, reopening for closures

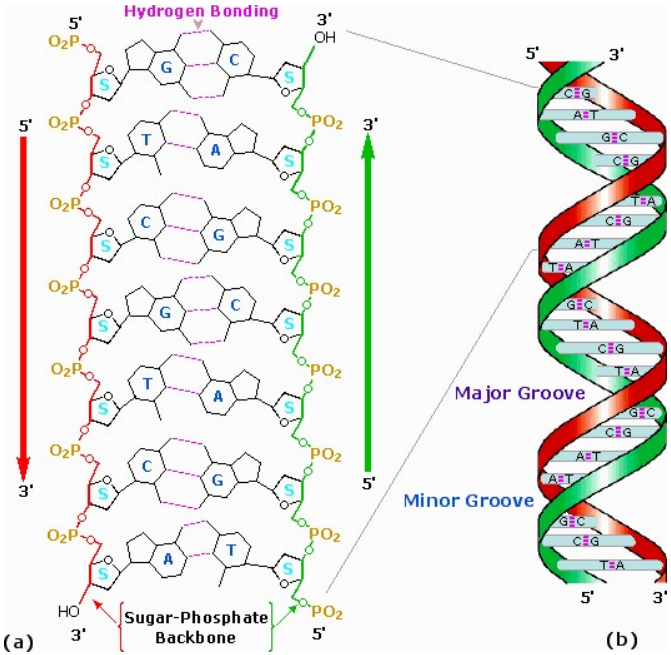
DNA key to qPCR

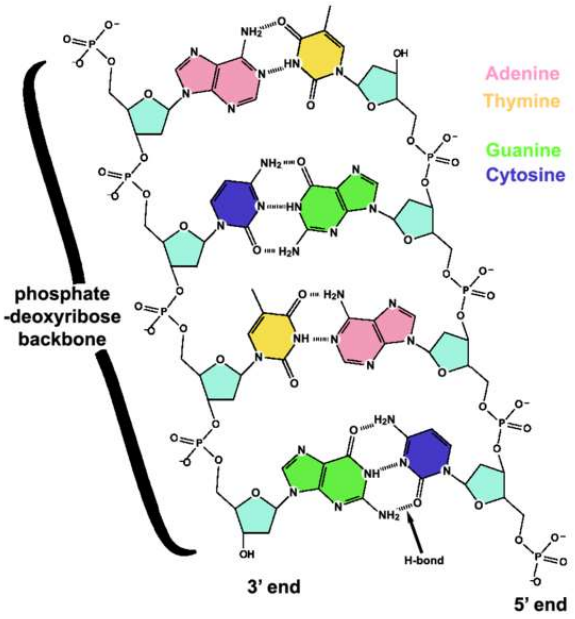
- Hooked to ribose sugar
- Guanine (G) pairs with Cytosine (C)
- Adenine (A) pairs with Thymine (T)
- A – T pair, 2 H bonds, G – C pair has 3 H bonds
- H bonds between the bases dictate the characteristic melting and annealing temperatures in PCR
- DNA has key structural characteristics



Structure of DNA

- Double stranded
- 5’ to 3’ orientation on strand runs in the opposite direction as the corresponding strand

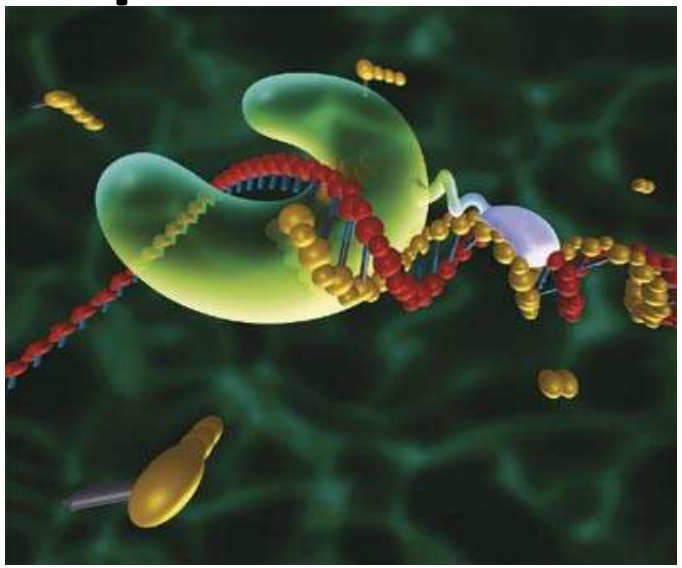




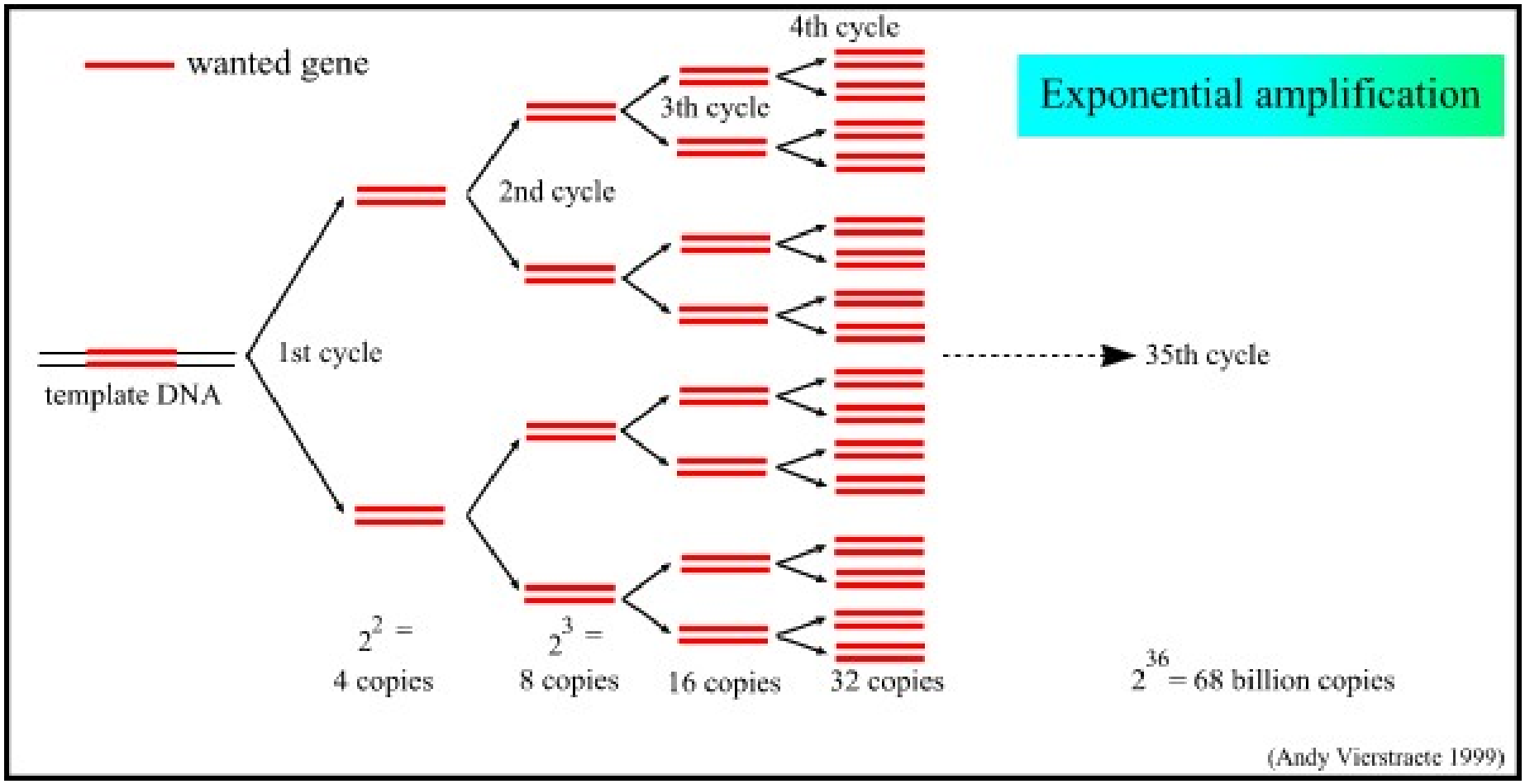
- Note hydrogen bond differences between A – T pairs versus G – C pairs

Basic necessities for qPCR?

- qPCR machine/basic sampling processing equipment
- PCR requires nucleotide building blocks (A, T, C, G, called dNTPs)
- Taq Polymerase –thermal stable polymerase with exonuclease function
- Optimal pH, buffers, nucleotides and metal cofactors – Mg^{++}, K^{+}) needed for the polymerase to work properly
- Purified template DNA – extracted from bacterial cells (such as *Enterococcus* or *E. coli*) or environmental samples
- Standardized, high levels of quality control



PCR is exponential

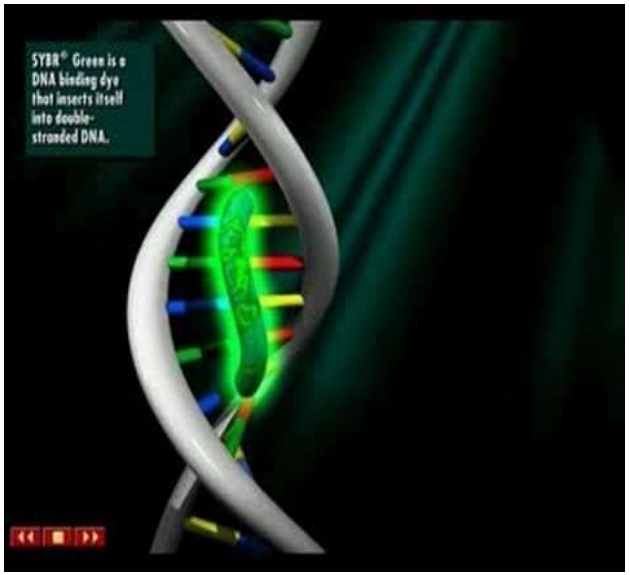


Concerns for Environmental Monitoring

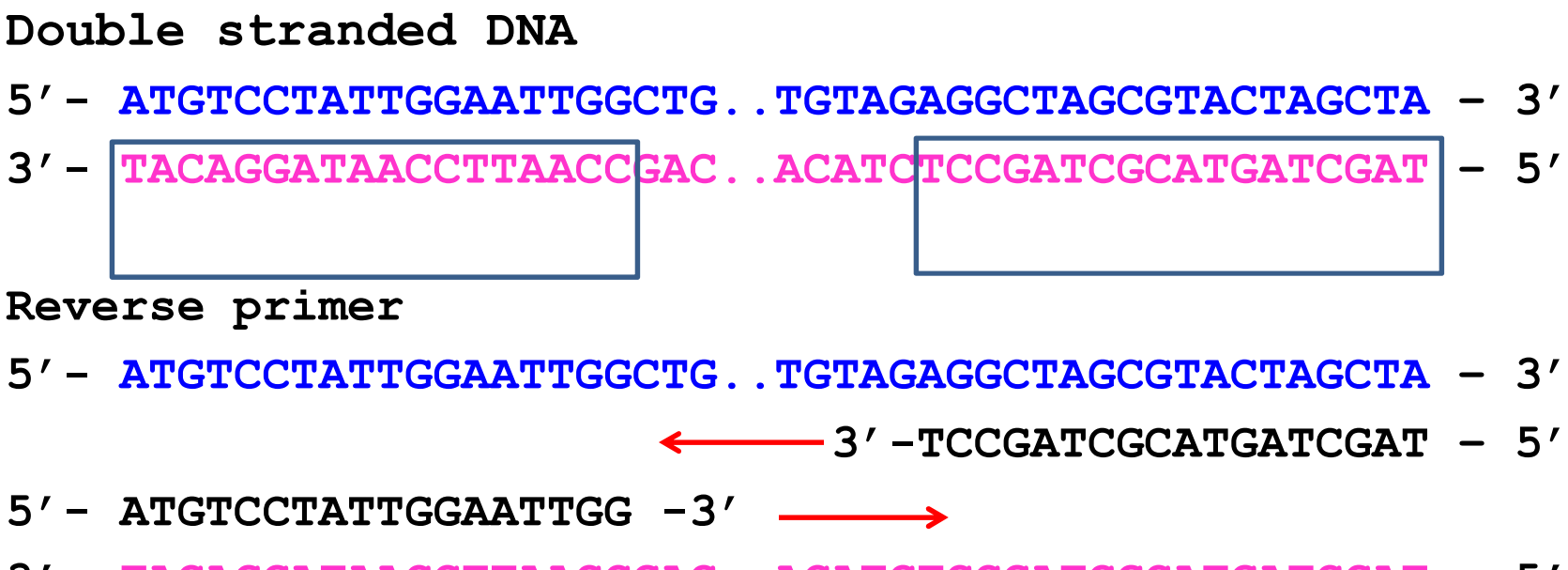
- Primer -small segment single stranded DNA which binds to single template DNA strand
    - Provides double stranded start point for polymerase
    - Base pair composition of primer determines where the primer binds
    - Primer/probe design matters!
- Thermocycler conditions that permit DNA separation (~94 to 95^{o}C, produces single stranded template), annealing, (temp that allow optimal binding of the primers ~40-65 ^{o}C, depending on primer base pair composition), and extension, optimal functioning of the thermostable polymerase (~72 ^{o}C), 40 cycles
- Standards (also termed calibrators), cells? Viruses? DNA? Synthesized oligonucleotides? Plasmid DNA?
- Controls, homemade? Purchased?

“Quantitative” qPCR

- Differences from conventional PCR only in that you include a way to follow the amount DNA (fluorescence) produced at each cycle
- Two types, “intercalator or dye-based” (e.g. SYBR Green: intercalates into double stranded DNA), and probe-based (Taqman®, Scorpions®, Molecular Beacons®)
- Both rely on quantification based on a standard curve created by target cells, target DNA, or cloned copies of the target DNA
- Internal control (IC) and specimen processing controls (SPC) often employed to assess recovery and detect inhibition
- QA/QC vital, MIQE guidelines (Bustin et al. 2009)



Primer Pair Design



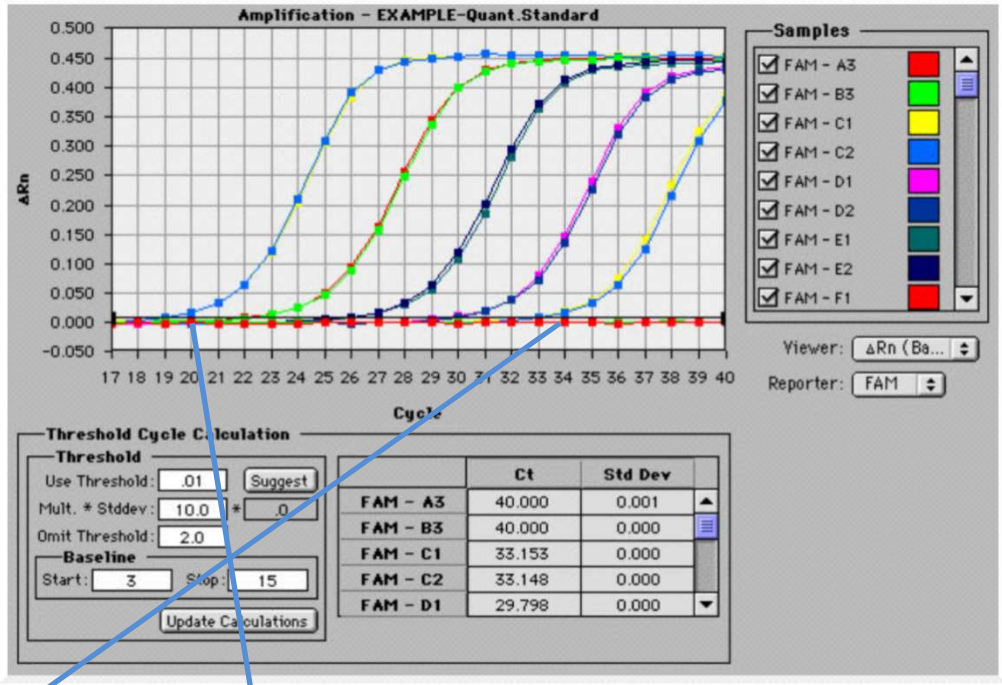
- Primer and probe design is very important
- Major driver of quality control issues
- Learn to be critical of inadequate designs, much free information on line

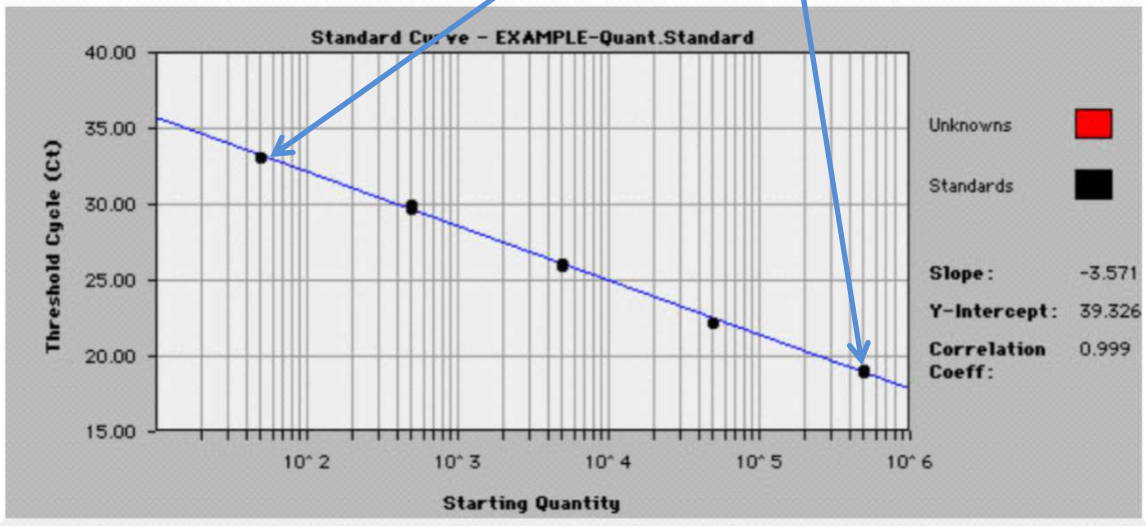
Standard Curve

Where fluorescence crosses specific threshold=
Ct (cycle threshold)
Slope= -3.32, y intercept

High initial concentration of target= low Ct value

Low initial concentration of target= high Ct



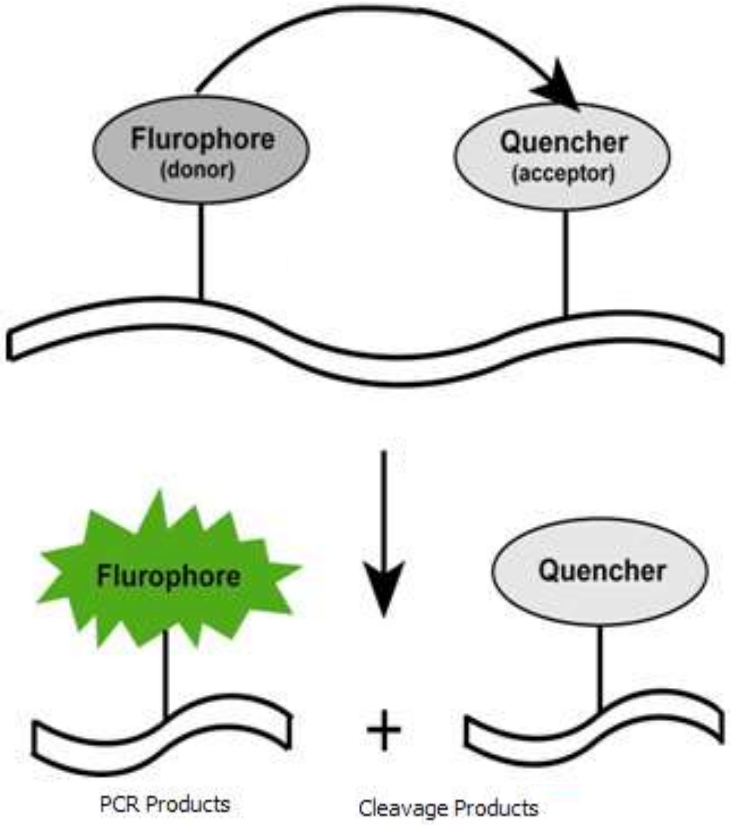


Standard Curve Critical Guidelines

- Accurate pipetting is required because the standard curve target material (cells or DNA) must be diluted over several orders of magnitude
- Pipet calibration
- Standard curve criteria for clinical applications
- Four serial log dilutions, duplicate, with amplification efficiencies ranging from 90-110%
- R2 of generated data >0.985

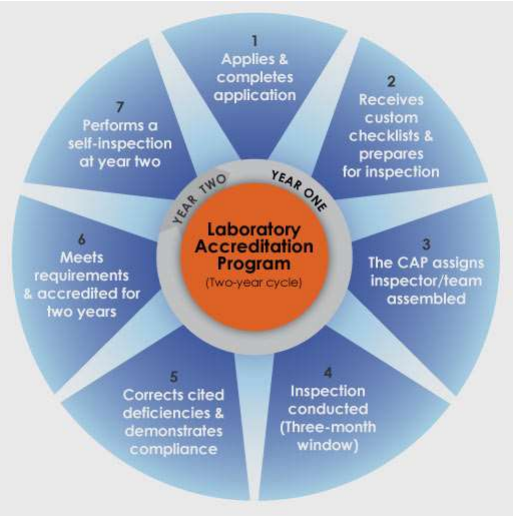
What are the main chemistries?

- Taqman
- Molecular beacons
- Scorpion
- All utilize fluorescence resonance energy transfer (FRET)
- When fluorophore and quencher are within a certain distance of each other, the quencher quenches the fluorescent signal
- Intellectual property space
- Many options, different licensing costs and ways cost is handed down to user



qPCR: the details

- Not all real-time qPCR is quantitative
- Environmental monitoring can benefit from the development of accreditation in the clinicial and food safety arenas
- Accreditation at multiple levels
- College American Pathologists: Inspections, laboratory training, proficiency testing, QA/QC, pee based
- University medical centers, physician office laboratories
- FDA method approval laboratory guidance
- Good Laboratory Practice
- MIQE guidelines



How are we different?

- Environmental water is a highly variable matrix
- Can contain compounds and organisms that interfere with the PCR reaction
- These are called inhibitors and include humic acids, organic substances, impure DNA, particles, attached organisms
- Processing controls used to normalize performance from one assay to another
- Salmon sperm control has been selected by EPA for (SPC) use in quantifying inhibition in water samples, not optimal
- Inhibitors diluted out by small scale dilution
- Cost, smaller markets, cascading testing
- Standardization of required controls will be a major issue

qPCR in Practice

- Key components to success
- Pipetting accuracy
- Proficiency assessments prior to sample analyses always preferable to post-analysis troubleshooting
- Always run positive controls to see if reagents working properly (false negatives)
- Always run negative controls – both no DNA for your reagents and blank extraction controls when processing environmental samples (false positives)
- Standard curves, best friend
- Standardization will be major key to data interpretation

Conclusions

- qPCR is an excellent tool, can be used for a wide array of applications
- Molecular assays will revolutionize environmental monitoring and specifically water quality in the coming decade
- Materials and costs have already dropped and kits are becoming more user-friendly
- There are guidelines, validation tools, training materials and programs available

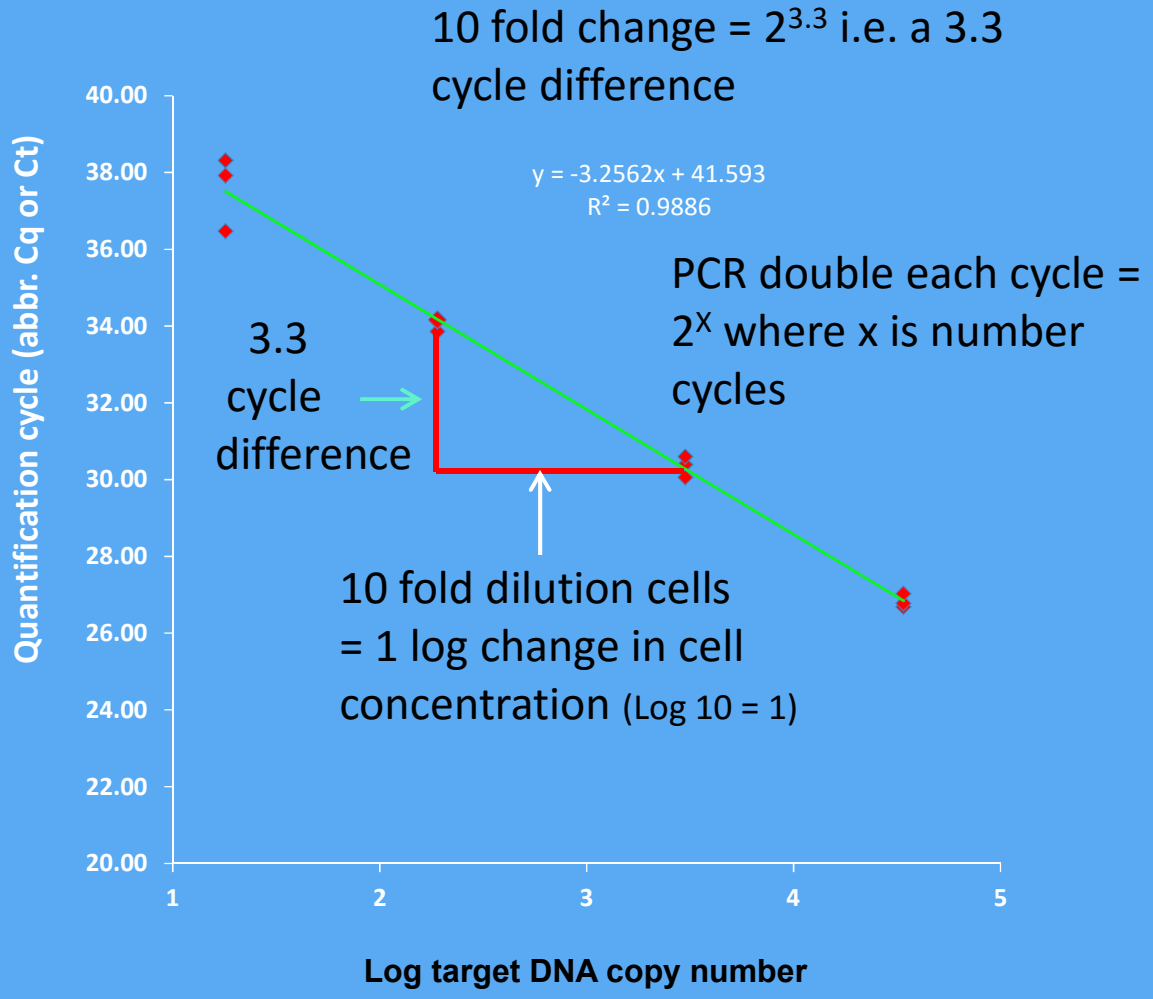
Standard curve slope

Determine the number of target sequences using standard methods

Dilute the target DNA 1:10 over a four to five-fold dilution series

Run qPCR reactions

Plot Ct values versus the log target DNA copy number



Calculate regression line - Slope should be -3.2 to -3.4.

Cell based standard curve

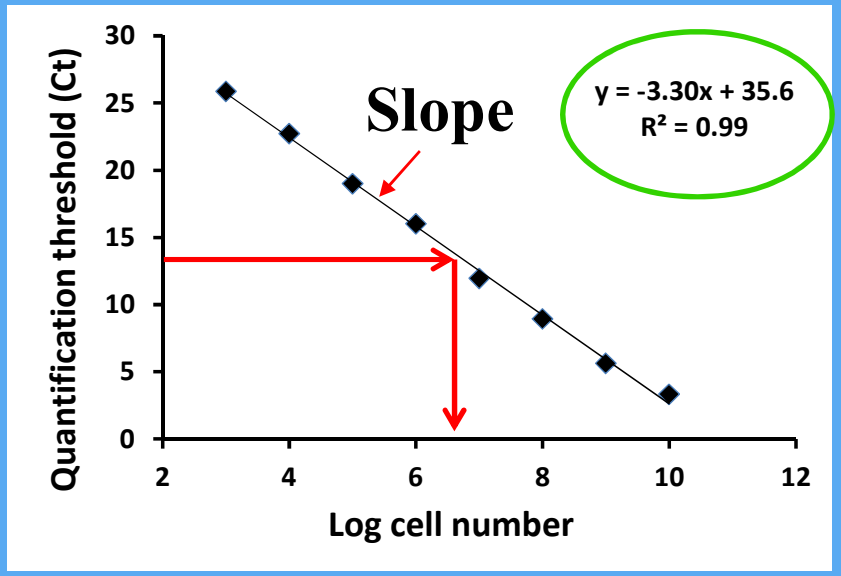
Determine the cell number (e.g. Enterococcus) using standard methods

Dilute the cell 1:10 over a four to five-fold dilution series

Extract DNA

Run qPCR reactions

Plot Ct values versus the log cell number



1. **Y Cq = (slope) * log cell number + y intercept**
2. **Y Cq- y intercept = (slope) * log cell number**
3. **(Y Cq- y intercept) / (slope) = log cell number**
4. **10^ log cell number**

$y = -3.32x + 38.5 \quad R^2 = 0.995$

Determine the PCR efficiency

- **A common statistic that is calculated is the efficiency of the reaction.**
- **The formula for Efficiency = $10^{(1/-slope)} - 1$**

eg. with slope = -3.32

$= 10^{(0.3012)} - 1$

$= 2.000 - 1$

$= 1.00$

% Efficiency = 1 *100 = 100%

Amplification Factor (AF) – if have perfect amplification this would be 2, i.e. product would double each cycle (2^X), where x is the cycle number. If inhibited, AF will be < 2.

- **Ideally should range between 95% and 105% efficiency = slopes of between -3.2 to -3.4**