Rapid Detection Methods: An Industry Necessity, A Laboratory Challenge!

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August 18, 2010

OVERVIEW

- SHORT Introduction to *Vibrios*
- PCR lesson and options
- Laboratory implications of molecular methods; checklists and methods

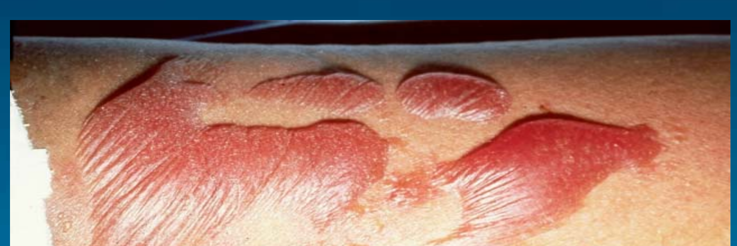
Vibrio vulnificus: An Introduction

- CDC (1997-2004): *Vibrio* most frequently isolated genus in Gulf coast waters. (CDC, 2005)
- *Vibrio vulnificus*
  - Leading cause of mortality from seafood consumption (fatality rate > 50%)
  - Low infectious dose; most severe foodborne infections- but <u>only in </u>susceptible persons
  - 80% of infections between May and October

Motes et al, 1998; Hlady et al, 1993; Kelley et al, 1997

Wound infection vs. septicemia

- Wounds from exposure to shellfish or seawater
- Septicemia follows ingestion of raw oysters
- Post-harvest processing (PHP) of oysters mandated by FDA?
- Rapid detection is crucial for the consumer and the industry



Post-Harvest Processing of Oysters

- PHP- summer months

  *For cooking? For shucking? For PHP?
- Demonstrate reduction of *V. vulnificus* by achieving 3.52 log reduction
- After validation of method, "vibrio reduced". Verified monthly.

The <u>Past</u> and the Future for the Shellfish Industry

- PHP to reduce *V. vulnificus* in 25% of oyster shellstock from Gulf Coast (2003)
- PHP for *V. parahaemolyticus* (2005)
- California bans untreated oysters (2005)
- Monitor harvest waters for Vibrios (2007)
- Demonstrate 60% reduction in *V. vulnificus* disease by 2010 (2007)

The Past and the Future for the Shellfish Industry

- Rescinded Ban? well….
- Time/temperature controls
- What's are we doing?

What Is In Place Right Now?

- *Vibrio* infections have continued to occur in spite of the efforts of regulatory agencies, industry and the scientific community.
- 2007, national surveillance system for *V. vulnificus*. CDC collaborated with Alabama, Florida, Louisiana, Texas, and Mississippi to monitor cases of *V. vulnificus* infection in the Gulf Coast region; nationally notifiable.
- 2010, Integrated (Federal/State) Food Safety System: Reportable Food Registry

Detection Options

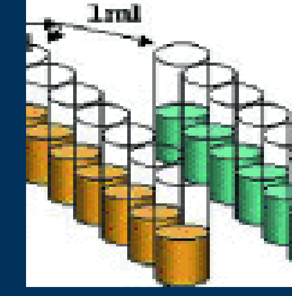
- Biochemicals… BAM
- DNA Probe
- PCR options, either approved or in the pipeline (hint: multiplex)…

Validating Methods: PHP for industry

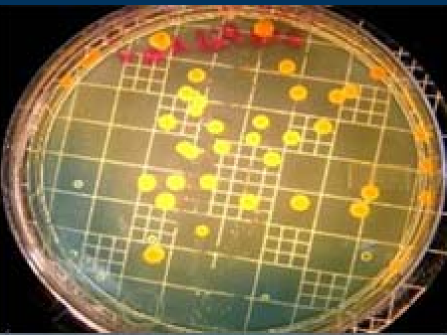
- FDA-MPN (FDA, 2004)
    1. Homogenized and inoculated into MPN tubes (24hrs)
    2. Streaked on selective media (24hrs)
    3. Picked and spotted onto nutritive media (24hrs)
    4. Lifted and pro-k (24 to 48hrs)
    5. Hybridized (<24 hrs).

- This process is as tedious as it sounds!!! So scientists at UF did something about it…

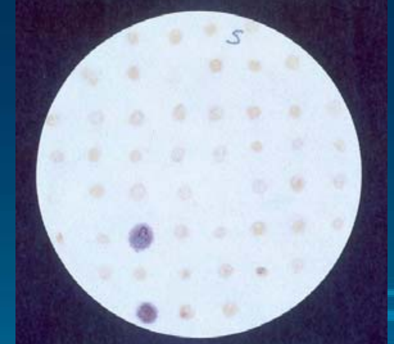
QPCR Application for MPN



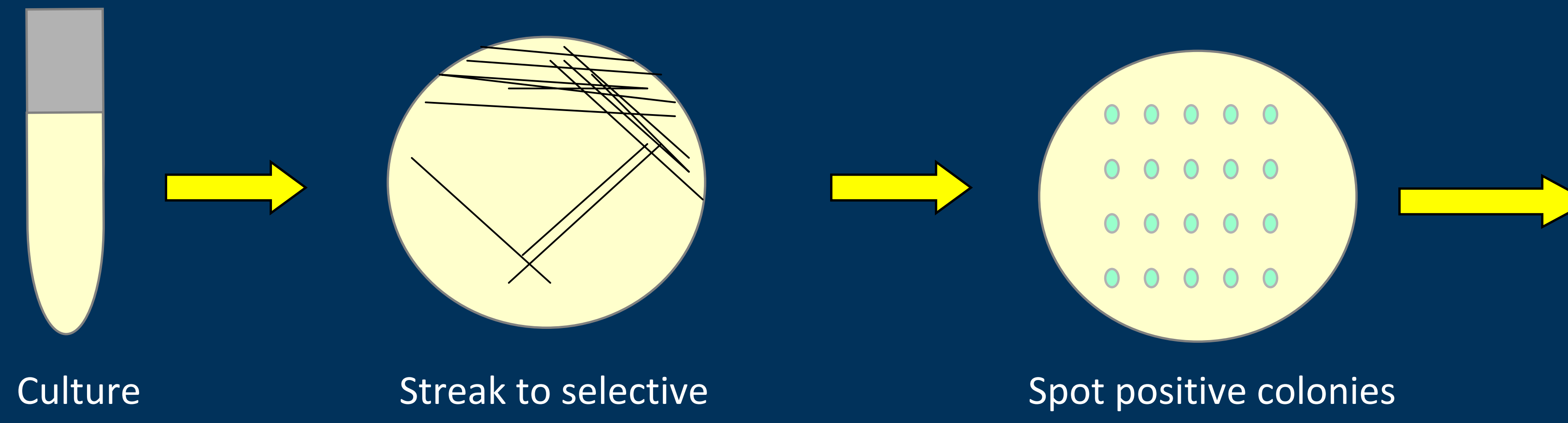
1. Homogenize oysters and dilute
2. Replicates (n=3-10) of APW enrichment tubes for MPN (Day 1)
3. Streak to selective agar (Day 2)
4. Pick colonies (Day 3)
5. Identify colonies by DNA probe (Day 4)



1. OR…. PCR



Colony Blot Hybridization



Probed Filter with positive and negative colonies

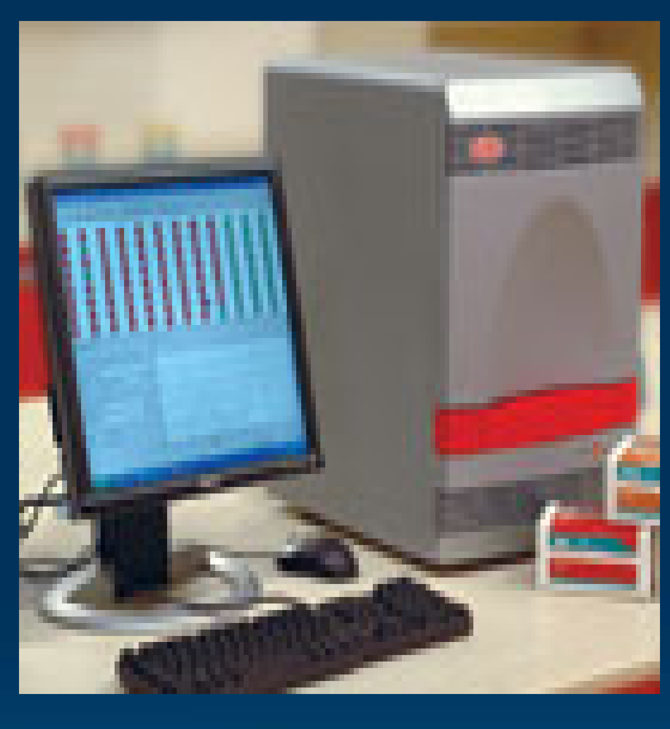
- Purple (+)
- Yellow (-)

- VERY EXPENSIVE!
- Can be subjective...

Yes we can...........PCR

- Mastermix: primers, taq, dNTP's (ACTG's), Sybr green dye 1, water and salt
- Standard curve to quantify with known concentrations of *V.v.*
- Result in CT value and Melt temp.
- WE HAVE A CHECKLIST COMING AND A METHOD IN NSSP!!!

…Confirm positive APW directly by Q-PCR



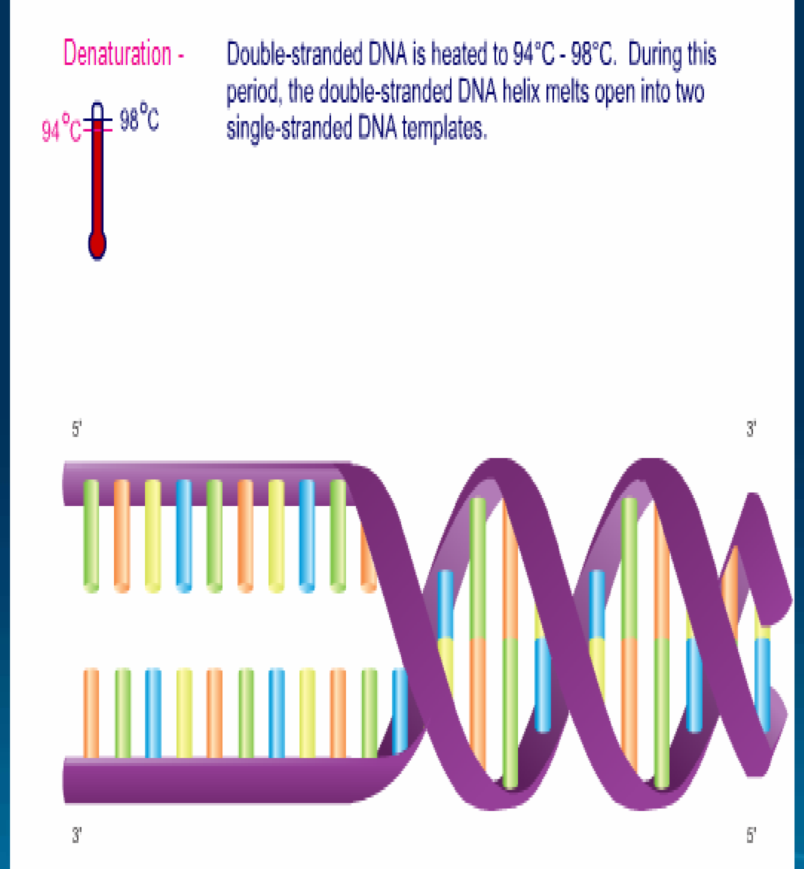
DuPont Qualicon Q7

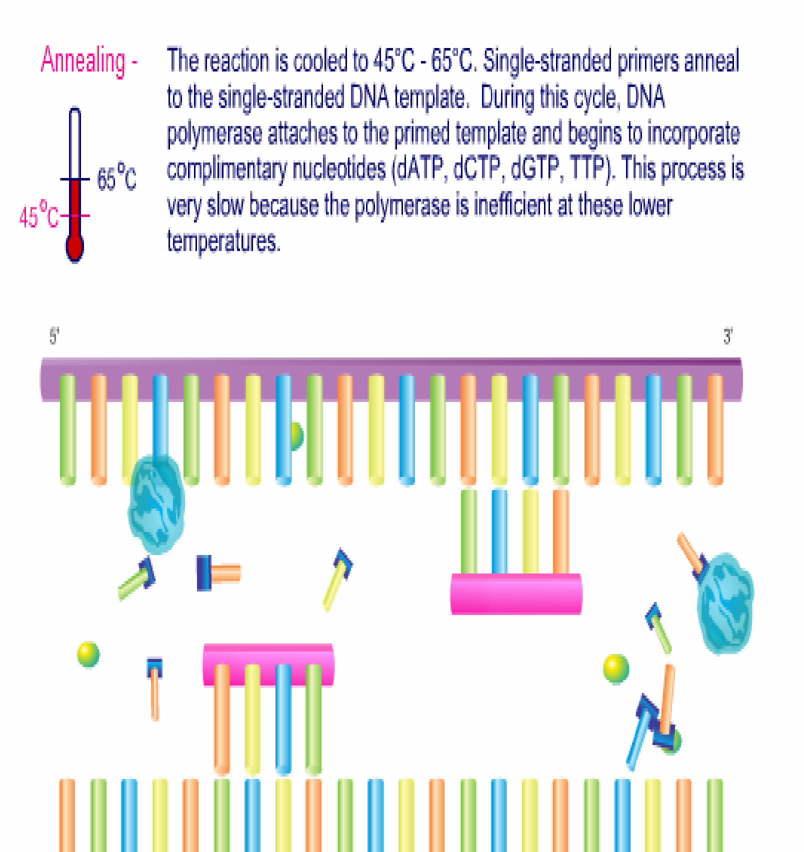
Cost?

Cepheid Smartcycler

How PCR is done!

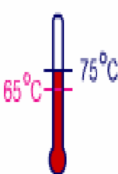
http://www.sigmaaldrich.com/lifescience/molecularbiology/pcr/learning-center/sybr-green-animation.html





How PCR is done: part 2

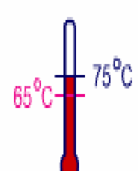
Extension - SYBR Green I binds all newly synthesized double-stranded DNA complexes and fluoresceses. The fluorescence accumulates as cycling of PCR continues and is measured at the end of each PCR cycle. The intensity of fluorescence generated by SYBR Green I above background level (C_T value) is measured and used to quantitate the amount of newly generated double-stranded DNA strands.

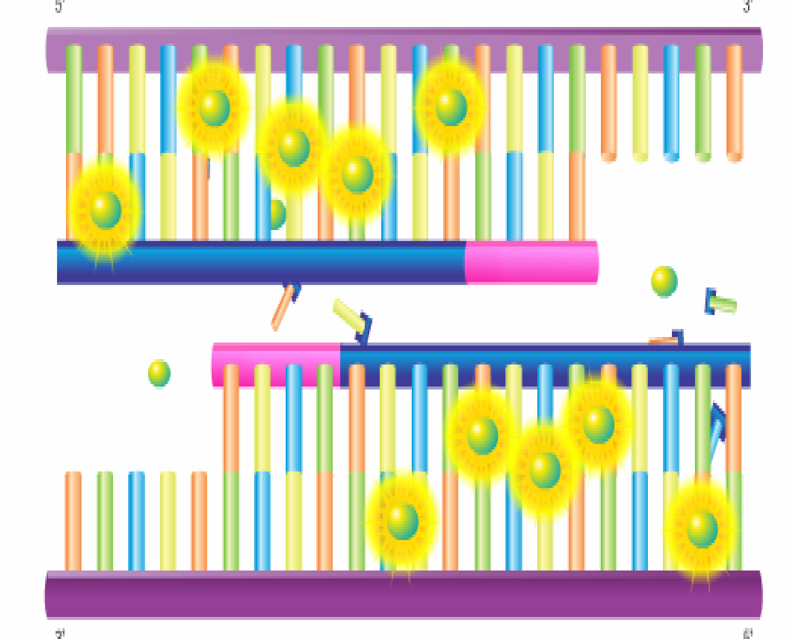


5' 3'

3' 5'

Replicated DNA - After repeating the denaturation, annealing, and extension cycles approximately 35-40 times, you are ready to begin analysis. The C_T values can be used to quantitate starting amounts of DNA or to establish a standard curve for gene expression studies or other comparative analysis.



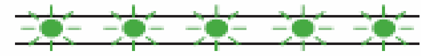


Sybr method: Cepheid®

- Method utilized in *V. vulnifucs* SYBR method
- ISSC interim approval

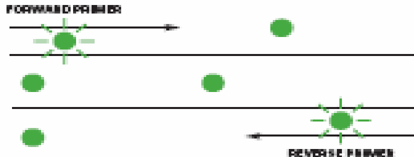
SYBR® GREEN I DYE ASSAY CHEMISTRY

1. **Reaction setup:** The SYBR® Green I Dye fluoresces when bound to double-stranded DNA.

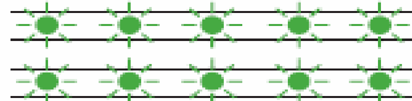


2. **Denaturation:** When the DNA is denatured, the SYBR® Green I Dye is released and the fluorescence is drastically reduced.

3. **Polymerization:** During extension, primers anneal and PCR product is generated.

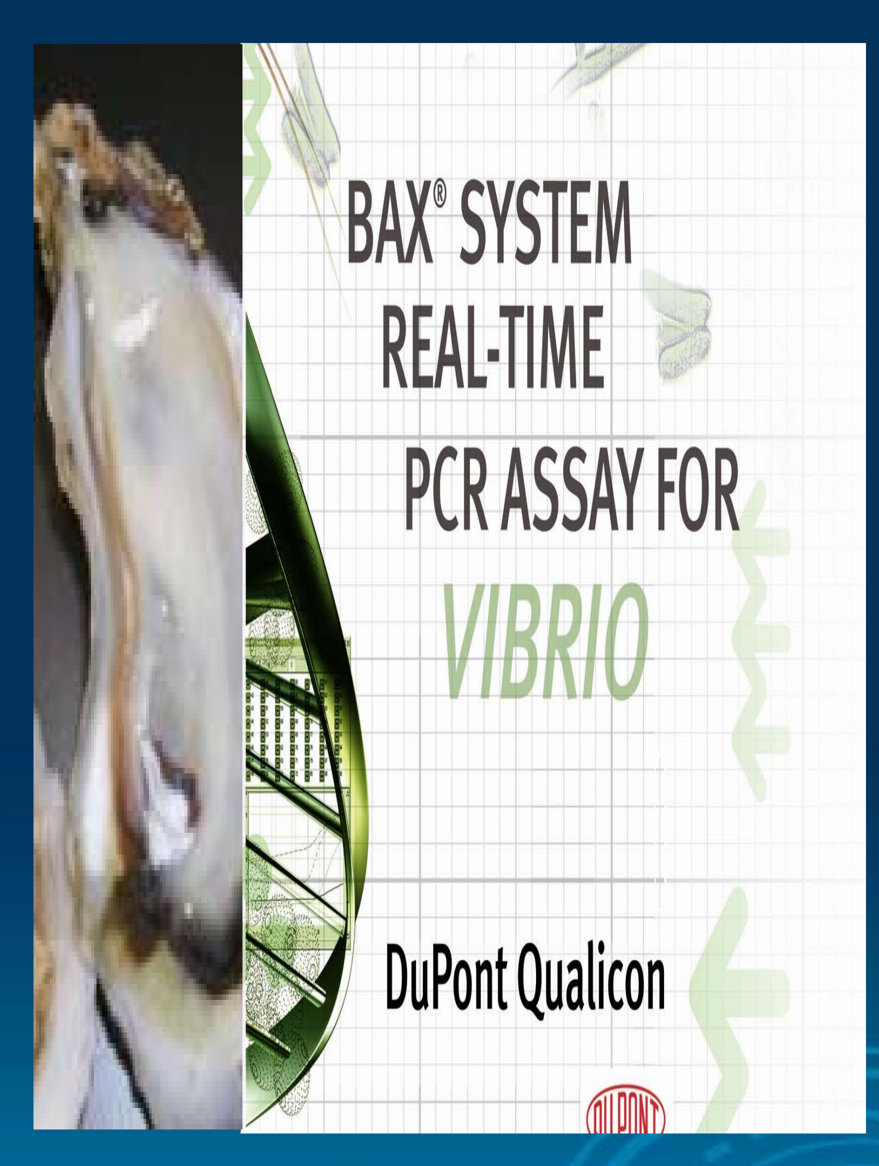


4. **Polymerization completed:** When polymerization is complete, SYBR® Green I Dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the 7900HT system.

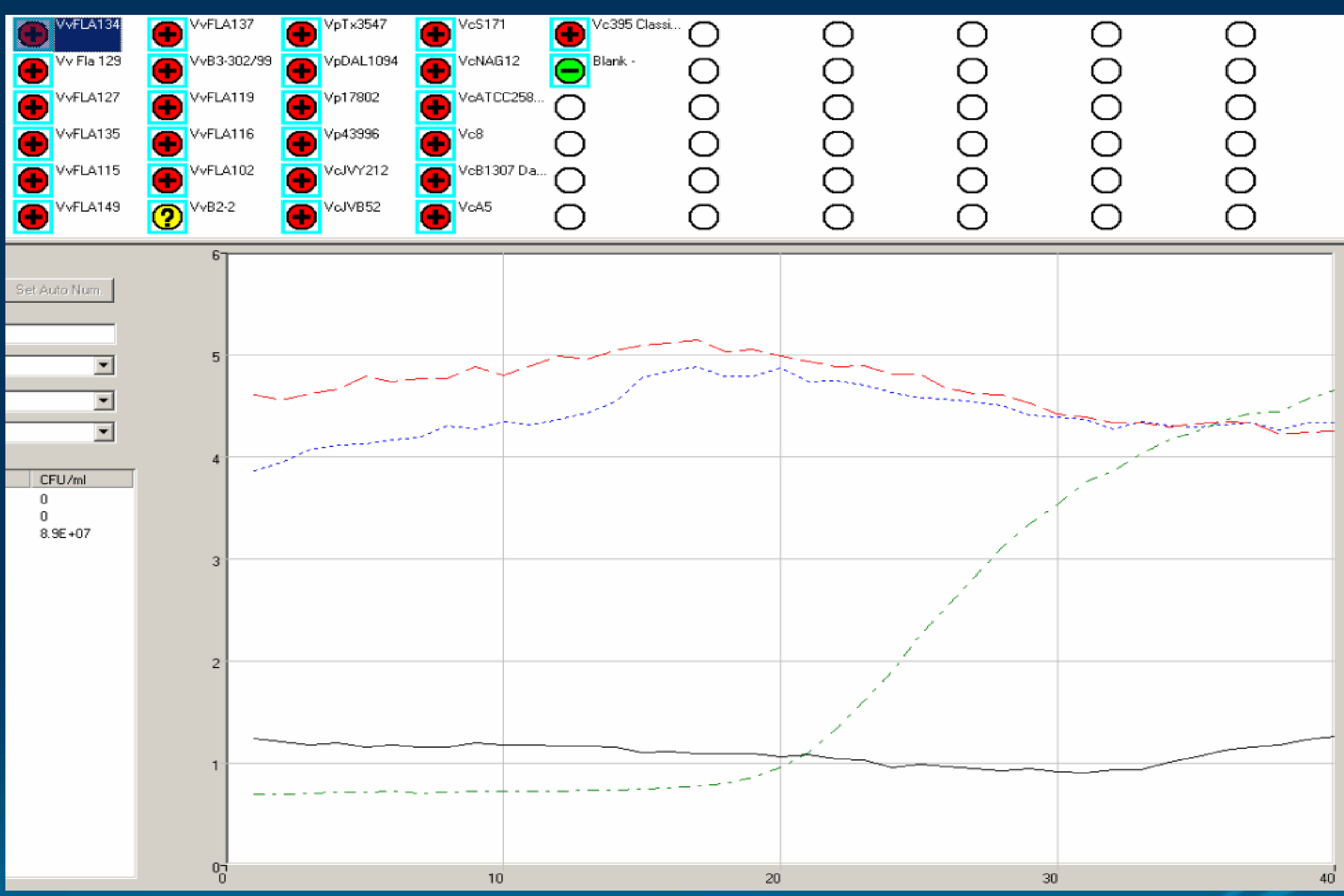


DuPont method currently under consideration

- Frequently utilized, probes for quantitative PCR.
- Mutiplex; *V.v. V.p. V.c.*
- TaqMan-based QPCR
- Simplified extraction Internal control standard
- AOAC approved to 10^4 CFU/ml
- Protocol submitted to ISSC



Vibrio Multiplex (Dupont Qualicon)



Checklist Information

- Critical Information:
  - All procedures, with materials, methods, calibrations and directive regarding interpretations of data.
  - Quality information is similar, points of interest for PCR
  - Sybr method standard curve and interpretation… DuPont method no interpretation.
  - Commercial kit.

NSSP/BAM methods…. How to implement in your lab (AKA what the checklist says)

- Must have a PCR area that does not contain live cultures
- Must have cold storage available
- Either platform is costly!
- Cepheid: thermocycler, centrifuge, heat block etc
- DuPont: thermocycler and heatblocks included. No centrifugation…

Validating and verifying methods the easy way!!

- Quantitative PCR was evaluated, as it may increase both speed with which samples may be processed without sacrificing sensitivity.
- Homogenized and inoculated into MPN tubes (24hrs) or possible to run the sample without enrichment! Extract and run PCR (<24 hrs)

I Want to Validate a Method. What Do I Do Next?

- New guidance documents under review to standardize submission.
- Schedule an evaluation for the laboratory involved in the microbiological testing
- Call and ask!!

Validation Results

- Results of a side by side sample comparison support application of Q-PCR technology for validation oyster processing protocols

- Quantitative PCR was applied to most probable number (QPCR-MPN) for validation of PHP methods for reduction of *Vibrio vulnificus* in oysters.

So What???

- Industry needs an efficient method of detection, especially with trend of PHP mandates.
- Laboratories will be implementing molecular methods and training and guidance will be available through FDA and LEO's
- Checklists are currently in final revisions and will be submitted to ISSC in 2011 for acceptance.
- Molecular methods will allow industry an

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QUESTIONS????

Lizzie Evans: