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Using Today's Data to Close the Beach Today. Quantitative Polymerase Chain Reaction (QPCR) rapid beach closings tool.

Richard A. Haugland

USEPA, Office of Research and Development,
National Exposure Research Laboratory

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How Does Quantitative PCR Analysis Work?

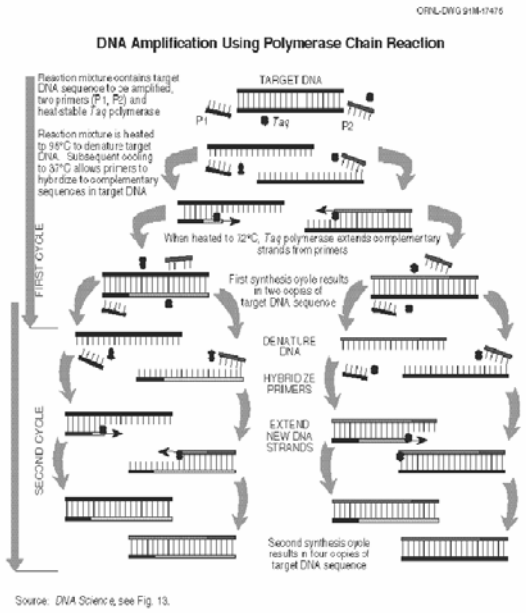
Principles of QPCR Analysis

PCR : POLYMERASE CHAIN REACTION

- PCR is now a widely used laboratory method for detecting specific DNA (or RNA) sequences that can originate from specific organisms, e.g. fecal indicator bacteria

- It does this by making copies of these sequences (amplification) in large enough numbers (e.g. Millions) to allow their detection - usually after the amplification is completed.

- Quantitative PCR (QPCR) differs from conventional PCR by detecting these copies with a fluorescent probe directly in the instrument as the reaction proceeds - for this reason it is also often called real time PCR.

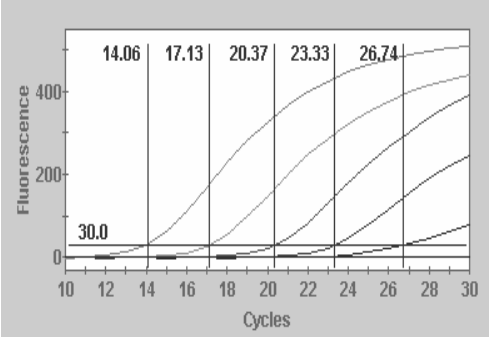


Source: *DNA Science*, see Fig. 13.

QPCR Growth Curves

- The quantitative capability of real time PCR stems from the direct correlation that has been shown between the starting number of target sequence copies in the sample and the number of amplification cycles required for the instrument to first detect an increase in probe fluorescence associated with the generation of new copies.

- The cycle numbers where the probe fluorescence curves cross a threshold value (red line near the bottom of the figure) that is significantly above the background fluorescence (purple line at the very bottom) are automatically reported by real time PCR instruments.

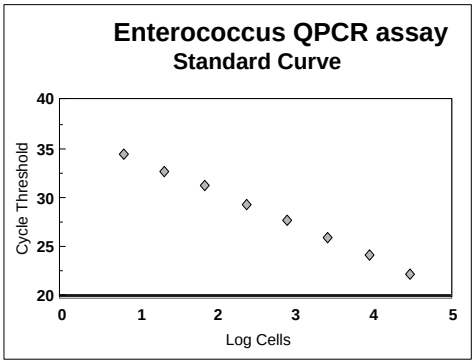


10000 pg DNA
1000 pg DNA
100 pg DNA
10 pg DNA
1 pg DNA
No DNA

*Cycle Threshold: Cycle # at which growth curve = 30 fluorescence units (significantly above background)

-Assuming that the recovered quantity of DNA sequences from the target organisms is consistent in the sample extraction process, this same relationship will also hold true for target cell numbers versus cycle threshold values.

- Hence, log-linear standard curves, such as the one shown in this figure, can be generated for the quantitation of target organisms in similarly processed test samples.



10 Steps and 2 Hours to Recreational Water Quality Results

Procedures for Quantitative PCR analysis of fecal indicator bacteria

Step 1. Collect water sample

Step 2. Filter water sample

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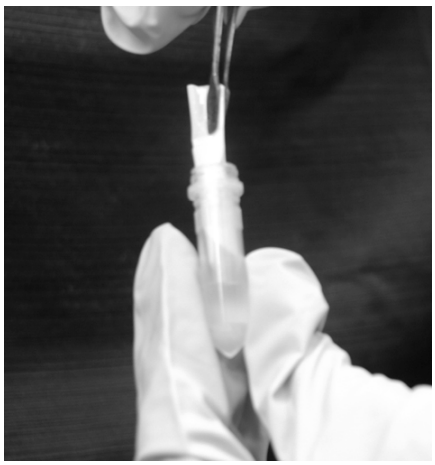
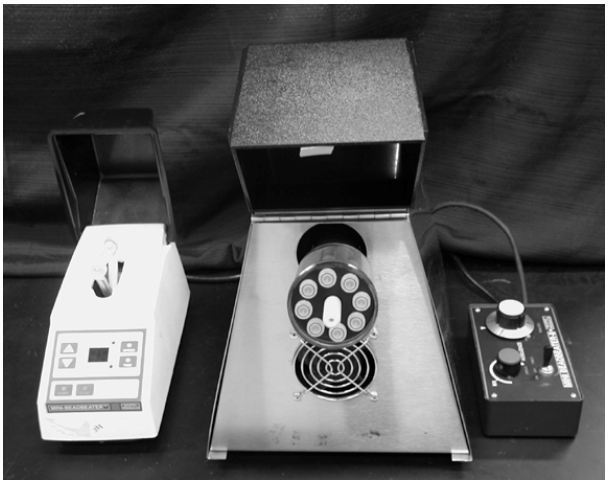
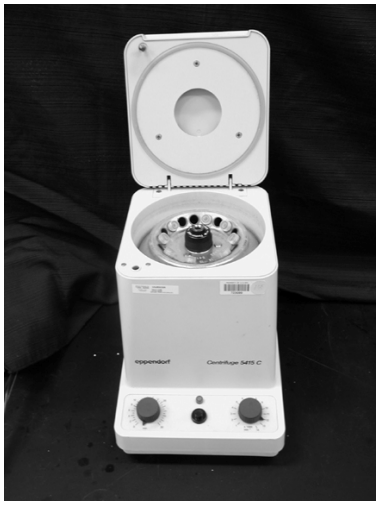
Step 3. Transfer filter to extraction tube containing glass beads, buffer and positive control DNA



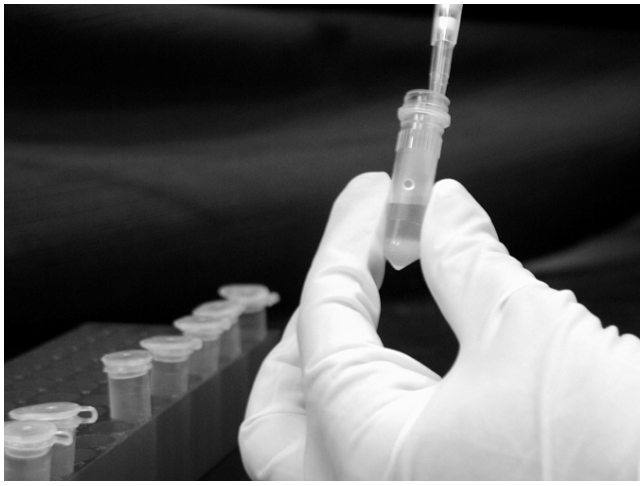
Step 4. Bead mill filter membrane for 1 min to break cells and release DNA



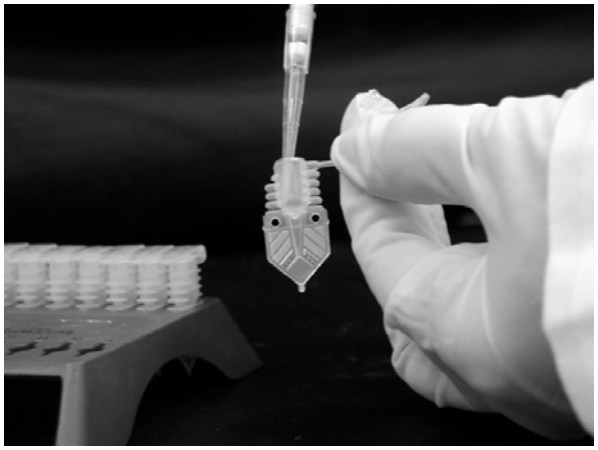
Step 5. Centrifuge briefly



Step 6. Recover liquid sample with released DNA from extraction tube



Step 7. Transfer sample to reaction tube containing PCR reagents



Step 8. Place reaction tube in real-time thermal cycler

Step 9. Run reaction in thermal cycler

Step 10. Import run data into spreadsheet and calculate target cells in sample

Sample	Entero CT	Control CT	dCT	Calib. dCT	ddCT	Ratio	Calib. cells	QPCR cells
5A	22.82	26.43	-3.61	-4.97	1.36	0.39	1.03E+005	40126.98
5B	23.56	27.23	-3.67	-4.97	1.30	0.41	1.03E+005	41831.00
5C	22.87	27.09	-4.22	-4.97	0.75	0.59	1.03E+005	61244.17
2A	33.58	28.74	4.84	-4.97	9.81	0.00	1.03E+005	114.74
2B	32.87	28.56	4.31	-4.97	9.28	0.00	1.03E+005	165.68
2C	33.61	28.99	4.62	-4.97	9.59	0.00	1.03E+005	133.65

Epidemiological and Environmental Assessment Studies

QPCR vs. Membrane Filtration Results

U.S. EPA, N.E.E.A.R. Study

- **Two Freshwater Beaches in 2003:**
  - **West Beach, Indiana Dunes National Lakeshore, Lake Michigan**
  - **Huntington Beach, Bay Village OH, Lake Erie**
- **Two Freshwater Beaches in 2004:**
  - **Silver Beach, St. Joseph, MI, Lake Michigan**
  - **Washington Park Beach, Michigan City, IN, Lake Michigan**
- **New water monitoring protocol:**
  - **6-9 sampling locations / beach**
  - **Three sampling visits / day**
  - **2-3 days / week (weekends and holidays)**
  - **8-10 weeks / beach**
- **Survey of swimming-associated health outcomes**
- **Two analytical methods for enterococci in water samples (QPCR and EPA Method 1600 membrane filtration)**

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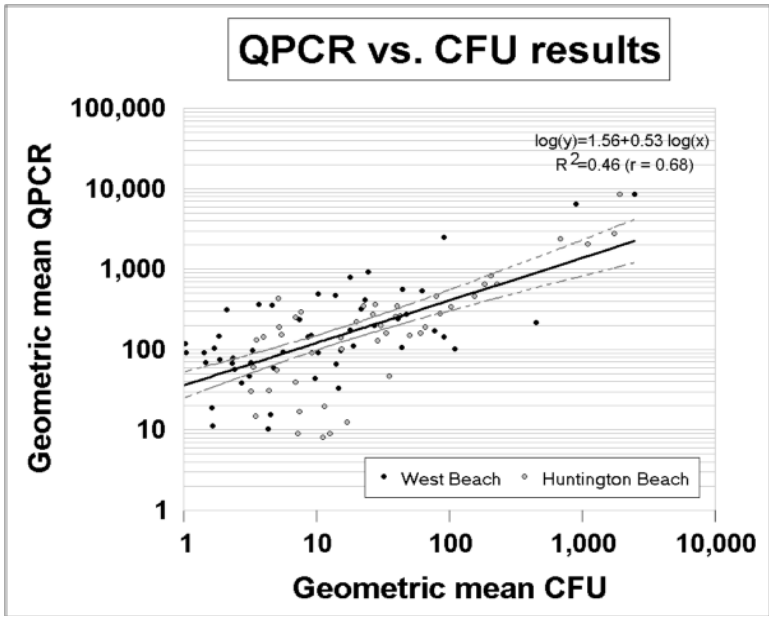

RESEARCH & DEVELOPMENT

Building a scientific foundation for sound environmental decisions

Southern California Coastal Water Research Project, Mission Bay Study, 2003

- **6 Marine Beaches within an Embayment**
- **Water monitoring protocol similar to EPA study:**
  - **2 - 5 sampling locations / beach**
  - **Two sampling visits / day**
  - **2-3 days / week (weekends and holidays)**
  - **10 weeks / beach**
- **Survey of swimming-associated health outcomes**
- **Same analytical methods as EPA study – QPCR and Membrane Filtration for enterococci in water samples**

2004 Rapid Methods Comparison Study

Organized by Southern California Coastal Water Research Project

Study Design

1. **Tested four potential rapid methods (including QPCR) for enterococci in parallel with standard MF and MPN methods performed by several Southern CA water testing laboratories.**

2. **Matrices were offshore marine water samples spiked with "known" enterococci CFU numbers from sewage or urban runoff and several ambient marine and freshwater samples with high expected enterococci numbers.**

3. **Potential interferences from humic acids and suspended solids present in some samples**

4. **Results for rapid methods reported at 2, 4, 6 and 8 hrs.**

23 ***Summary of enterococci results from QPCR and MF methods***

Sample Type/Target CFU	Mean QPCR	Mean MF	STD QPCR	STD MF
Offshore Seawater	4	2	8	4
Offshore Seawater w/sewage/35 CFU	93	215	22	113
Offshore Seawater w/sewage/104 CFU	255	459	78	183
Offshore Seawater w/sewage/1000 CFU	1486	5620	317	2604
Offshore Seawater w/sewage and humic acids/104 CFU	1023	1034	595	448
Offshore Seawater w/sewage and humic acids/1000 CFU	5629	10720	4895	4554
Offshore Seawater w/urban runoff/35 CFU	80	37	47	13
Offshore Seawater w/urban runoff/104 CFU	578	80	88	39
Offshore Seawater w/urban runoff/1000 CFU	539	74	445	40
Yorktown Drain	133	9	54	5
Doheny Beach	5634	4860	895	1309
San Juan Creek- Doheny Beach	7477	8453	3564	2521
Santa Ana River at OCSD Plant	675	508	189	106
Nearshore Seawater w/sewage and suspended solids/ 104 CFU	1665	128	1412	37
Nearshore Seawater w/sewage and suspended solids/ 1000 CFU	3854	1112	1941	203

STD : Standard Deviation

Recent & Future Improvements to QPCR Method

- New Control Assays for *Enterococcus*
- Assays for New Indicator Organisms
- Automation

New Control Assays for Enterococcus

- **OBJECTIVE: to improve precision and accuracy of *Enterococcus* QPCR measurements by developing assays that more accurately identify and compensate for interferences* caused by the water sample matrices**

  ***Major potential interferences come from PCR inhibitors and variable cell lysis and DNA recovery during extraction of the samples**

- **CONTROL ASSAY 1: Assay for synthetic DNA target with high sequence similarity to *Enterococcus* target DNA.**
  - **Improved test for PCR inhibitors in sample extracts**
- **CONTROL ASSAY 2: New assay for a species closely related to *Enterococcus* that can be added to samples before DNA extraction.**
  - **Improved compensation for variable cell lysis and DNA recovery**

New Indicator Organism Assays

- ***Bacteroides*: another group of bacteria that has great potential as indicators of fecal pollution due to their high numbers in the GI tract and short half-life in the environment.**
  - **QPCR assay developed for *Bacteroides*.**
  - **Organisms not detected in a significant percentage of beach water samples - possibly due to low sensitivity of the assay (~100-fold less sensitive than Enterococcus assay).**
  - **New QPCR reagent currently being tested that increases the sensitivity of the *Bacteroides* assay to a similar level as the assay for *Enterococcus.***
  - **New reagent also significantly shortens time for both assays.**
  - **Assays for other common GI tract organisms that may serve as indicators of fecal pollution are also in development.**

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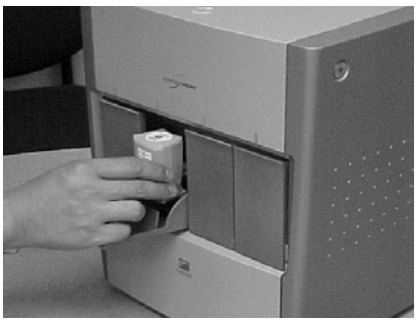
Automation

GenXpert Automated Quantitative PCR System, Cephied, Inc.



Sample Prep < 5 min, Amplification and Detection < 25 min

Conclusions

• The QPCR method for enterococci may be currently useful as an early warning system for high fecal pollution levels but confirmation with accepted methods is still required at this time

• QPCR results to date show promising correlation with swimming related illness rates. Results from ongoing epidemiological studies may lead to the development of new criteria for beach closings based on same-day measurements by this method