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

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How Does Quantitative PCR Analysis Work?

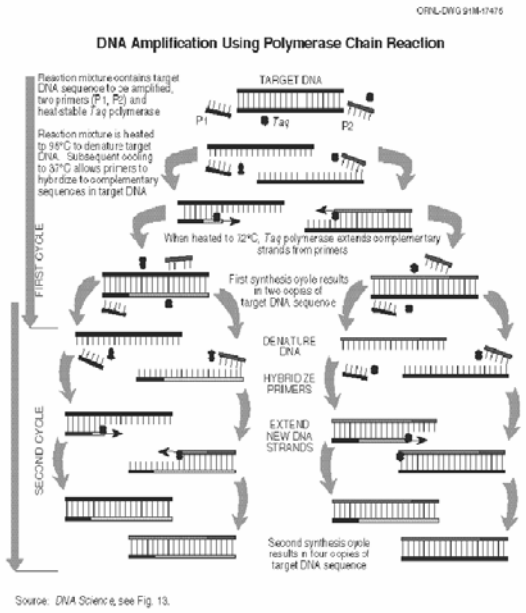
Principles of QPCR Analysis

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.

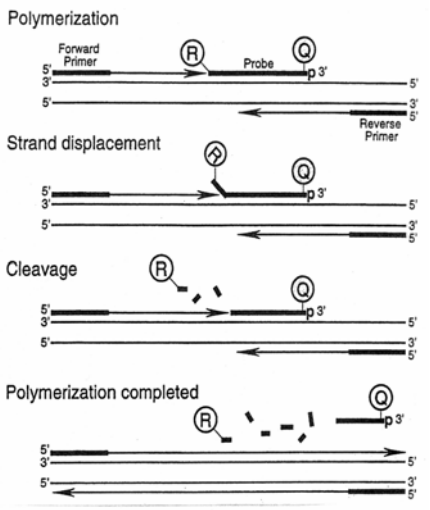
- Several types of chemistries have been developed for this direct detection of PCR-copied sequences. One of the most popular is the TaqMan™ system illustrated here.

- In the TaqMan™ system, as each new copy of the target sequence is made – a hybridization probe which binds to the sequence is simultaneously hydrolyzed by the polymerase enzyme

- This causes two fluorescent dyes at either end of the probe to become separated and eliminates the Q dye quenching effect on the fluorescence of the reporter or R dye.

- For each new copy of the sequence that is made, the fluorescence of one reporter dye molecule becomes detectable by the instrument.

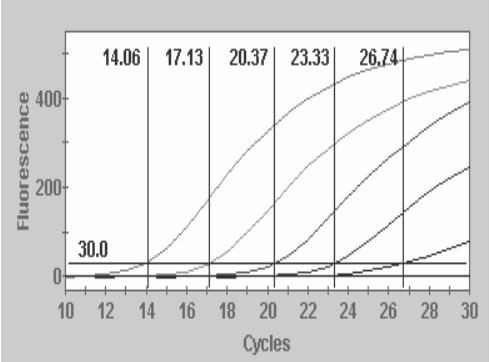
TaqMan™ Sequence Detection System



QPCR Growth Curves

- The quantitative capability of this system 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 reporter dye fluorescence associated with the generation of new copies.

- The cycle numbers where the reporter dye 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.

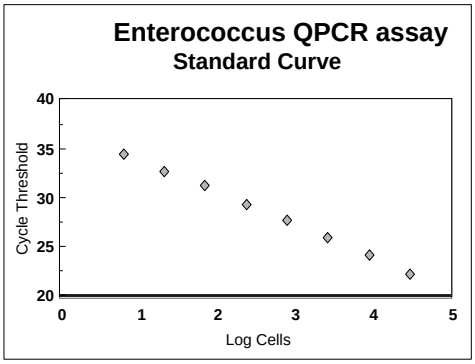


100 pg DNA
10 pg DNA
10 ng DNA
1 pg DNA
1 ng DNA
no DNA

*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.



Calculation of target organism cells in test samples from TaqMan assay cycle threshold results using the comparative cycle threshold method

Target cells in sample	Sample type	C_T	ΔC_T ($C_{T,test}$-$C_{T,calib}$)	Measured cells in test sample ($2^{-\Delta CT}$ x cells in calibrator)
20000	Calibrator	19.8	----	----
Unknown	Test	22.9	3.1	0.11 x 20000 = 2200
Unknown	Test	26.2	6.4	0.012 x 20000 = 240

assuming amplification efficiency = 2

- Information from the standard curve and results from a single calibrator sample containing known target cell numbers - that is extracted and run with the test samples - can also be used to determine target cell numbers in the test samples using a simple calculation called the comparative cycle threshold method as illustrated here.

Example of $\Delta\Delta C_{T,}$ comparative cycle threshold method calculation

Target Cells in sample	Sample type	$C_{T,„target}$	$C_{T,ref}$	ΔC_T	$\Delta\Delta C_T$	Measured cells in test sample ($2^{-\Delta\Delta CT}$ x cells in calib.)[a]
20000	Calib.	21.4	18.3	3.1	----	----
Unknown	Test	23.9	17.4	6.5	3.4	0.089 x 20000 = 1800
Unknown	Test	27.5	17.7	9.8	6.7	0.0096 x 20000 = 190

[a] assuming amplification efficiency = 2

-Results from similar analyses of a positive control DNA that is added to the calibrator sample and each of the test samples can be used to detect inhibition of the PCR by the test sample and also to correct the measurements for variations in DNA recovery during the extraction process using the delta delta CT method shown here.

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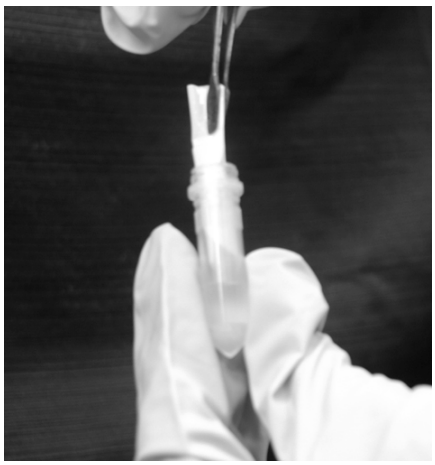
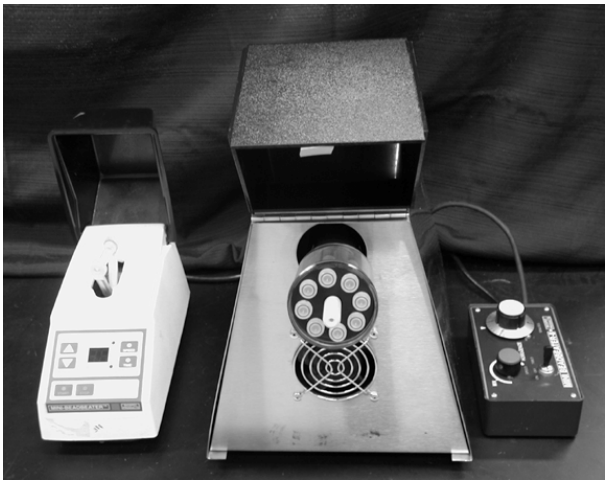
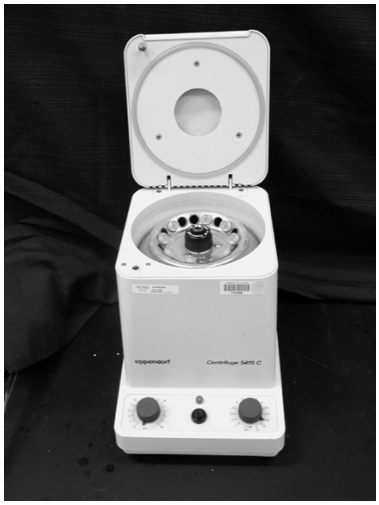
Step 3. Transfer filter to extraction tube containing glass beads, buffer and positive control DNA



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



Step 5. Centrifuge briefly



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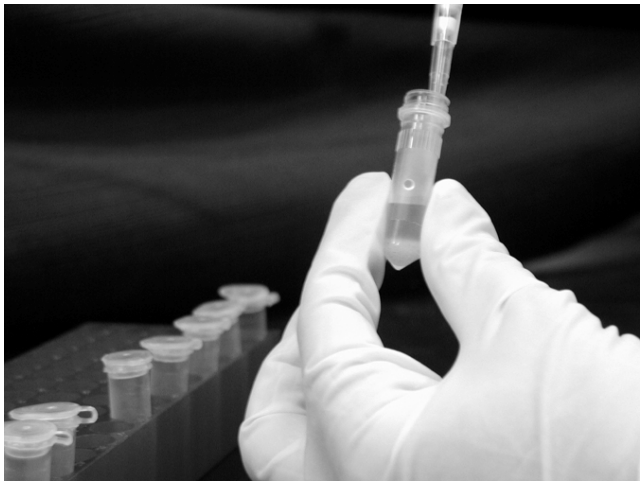
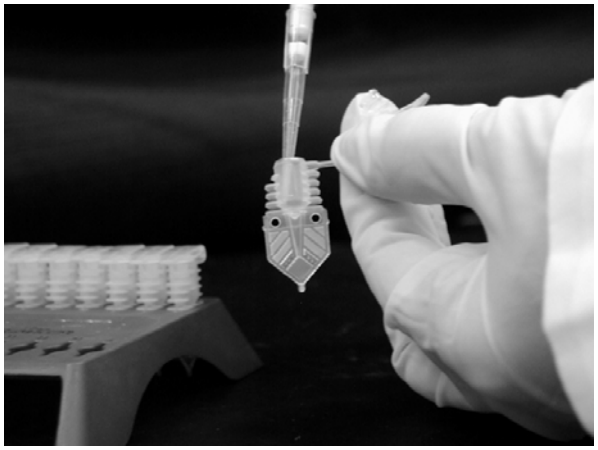
Step 6. Recover liquid sample with released DNA from extraction tube



Step 7. Transfer sample to reaction tube containing PCR reagents



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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

ORD Beaches Epidemiology Study

2003 QPCR vs. Method 1600 Results

Study Design

- **Two Beaches (Freshwater)**
- **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 water samples (QPCR and Membrane Filtration)**

Summary of Results from Two Beaches

	WEST BEACH		HUNTINGTON BEACH	
Enterococci/100 mL	**MF**	**QPCR**	**MF**	**QPCR**
Geometric Mean of all Sampling Visits*	**9**	**143**	**27**	**159**
C.V. Between Sampling Visits**	**0.93**	**0.63**	**0.84**	**0.75**
C.V. Within Sampling Visits	**0.36**	**0.88**	**0.66**	**0.84**

***One sampling visit corresponds to the geometric mean of results from all sampling locations for a particular time and day**
**** Coefficient of variation (= Standard deviation in original units/mean)**

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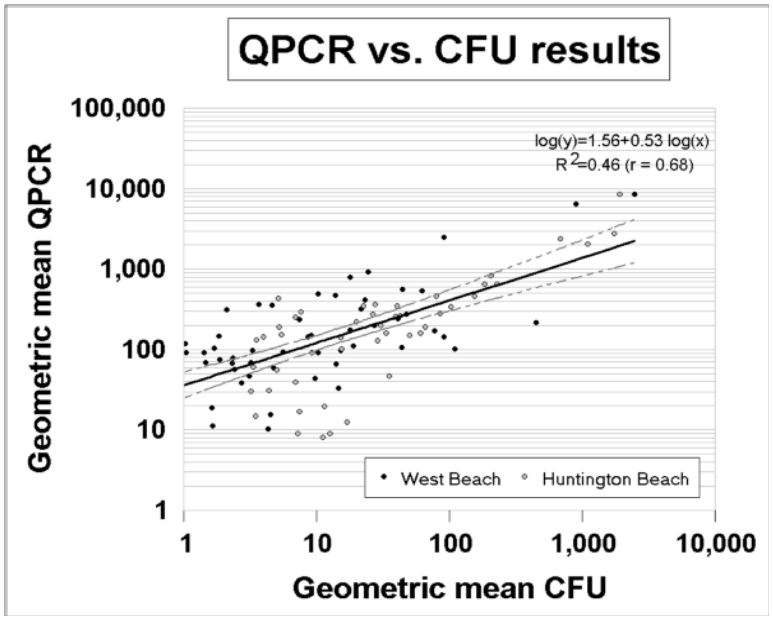

RESEARCH & DEVELOPMENT

Building a scientific foundation for sound environmental decisions

2004 Rapid Methods Comparison Study

Organized by Southern California Coastal Water Research Project

Study Design

1. **Tested four potential rapid methods (including QPCR) 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.**

26 ***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

Future Advances

- Sensitivity/Interferences
- Bacteroides assay

Sensitivity/Interferences

Problem:

1. **95% detection limit of QPCR method for Enterococcus is ~ 2 cells/sample or ~20 cells/sample for standard 10-fold diluted extracts of recreational waters. Elimination of high levels of PCR inhibitors requires further dilutions of extracts, e.g. 50 or 100-fold, which could decrease sensitivity to unacceptable levels.**

Future solutions:

1. **In ORD Beach Study analyses to date < 5% of sample extracts required additional dilutions (50-fold). PCR inhibition may therefore not be a major problem for most recreational water analyses.**

2. **DNA purification? Currently used by many labs. Advantages: should eliminate need for extract dilution and hence increase sensitivity. Disadvantages: adds time and expense to overall method, DNA losses during purification can offset sensitivity gains. Further testing needed.**

Bacteroides assay

Problem:

1. **The genus Bacteroides is 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. A new QPCR assay for these organisms is also being used in the ORD Beach Study.**

2. **In analyses to date, these organisms have not been detected in a significant percentage of beach water samples - probably due to low sensitivity of this assay (>100-fold less sensitive than Enterococcus assay).**

Future solution:

1. **A new QPCR reagent is currently being used that increases the sensitivity of the Bacteroides assay to a similar level as that of Enterococcus assay – also increases speed of both assays.**

Conclusions

- QPCR results to date show good correlation with health data and also with results of the current MF method (particularly at high pollution levels)

- The QPCR method may be useful at this time as an early warning system but confirmation with other methods is still recommended

- Results from ongoing epidemiological studies may lead to the development of new criteria for beach closings based on same-day measurements by this method