

Rapid Methods for the Detection of General Fecal Indicators

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Notice: Although this work was reviewed by EPA and approved for publication, it may not necessarily reflect official Agency policy.

BEACH Act of 2000

Specified that EPA should develop:

- **appropriate and effective indicators for improving detection in a timely manner of pathogens in coastal waters**
- **appropriate, accurate, expeditious and cost-effective methods for the timely detection of pathogens in coastal waters**



Main Problem:

- Current culture-based methods only tell us what the water quality was yesterday



Consequences:

- Human exposure to unsafe swimming conditions
- Unnecessary beach closings

EPA Approach to a Solution:

- Develop and/or identify available rapid analytical methods for monitoring fecal pollution indicators
- Demonstrate correlation between results of new method(s) and swimming-related illness rates in multiple epidemiological studies

Methods Used in Previous Epidemiological (NEEAR) Studies

Method	Epi Studies	Conclusion/Disposition
<i>Enterococcus spp.</i> by culture on mEI medium (EPA method 1600)	2003, 2004 Freshwater 2005, 2007 Marine	Evaluation ongoing but not a rapid method
<i>Enterococcus spp.</i> by rDNA qPCR (Ludwig & Schleifer assay)	2003, 2004 Freshwater 2005, 2007 Marine	Adequate sensitivity Evaluation ongoing
<i>Enterococcus spp.</i> by antibody capture and fluorescence (Raptor)	2004 Freshwater	Insufficient sensitivity Discontinued
<i>Enterococcus spp.</i> by antibody capture and fluorescence (Luminex 100)	2004 Freshwater 2005 Marine	Insufficient sensitivity Discontinued
<i>Bacteroidales spp.</i> by rDNA qPCR (Kate Field lab assay)	2004 Freshwater	Insufficient sensitivity Discontinued
<i>Bacteroidales spp.</i> by rDNA qPCR (Sieftring et al. modified Kate Field lab assay)	2005, 2007 Marine	Adequate sensitivity Evaluation ongoing
<i>Clostridium spp.</i> by rDNA qPCR (Modified Rinttilä et al. assay)	2007 Marine	Adequate sensitivity Evaluation ongoing
<i>E. coli</i> by <i>uidA</i> gene qPCR (Modified Rachel Noble lab assay)	2007 Marine	Insufficient sensitivity Modifications being evaluated
Human-associated <i>Bacteriodes</i> by rDNA qPCR (Modified Rachel Noble lab assay)	2007 Marine	Sensitivity may be adequate Further evaluation TBD
Male-Specific F+ coliphage by Enrichment and Latex Agglutination/Typing Assay with antibody detection (David Love assay)	2007 Marine	Not a rapid method (currently takes 6 hr or more) Further evaluation TBD

RESEARCH & DEVELOPMENT

Building a scientific foundation for sound environmental decisions

Key Features of qPCR Methods



- Established TaqMan® qPCR primer/probe assays:
 - Ludwig and Schleifer (2000) Systematic Applied Microbiology 23, 556: Primers and probe homologous to 23S ribosomal RNA gene sequence from all *Enterococcus* species
 - Sieftring et al. (2008) Journal of Water and Health 6: 235: Primers and probe homologous to 16S ribosomal RNA gene target sequence from majority of *Bacteroidales* species
 - Rintillä et al. (2004) Journal of Applied Microbiology 97, 1166: Primers homologous to 16S ribosomal RNA gene target sequence from “*Clostridium perfringens* group” (~40 species). Probe developed by EPA.

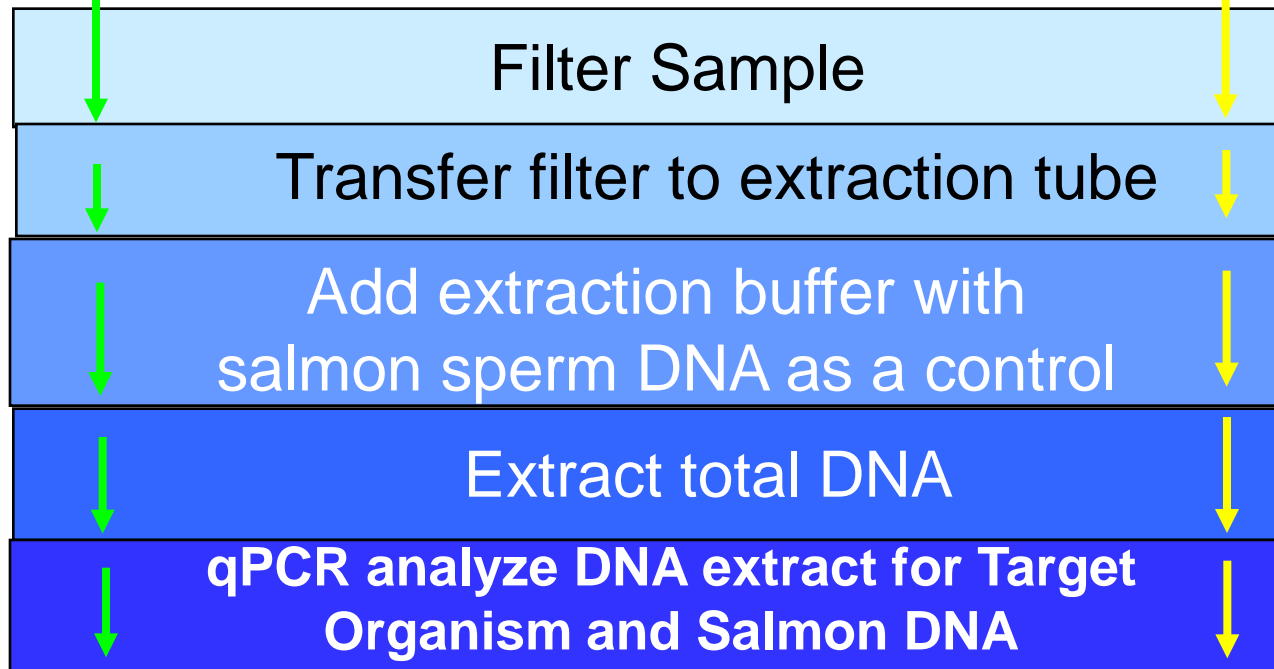
- Easy to use positive control consisting of salmon sperm DNA
 - Haugland, et al. (2005) Water Research 39, 559: Primers and probe homologous to Internal Transcribed Spacer Region 2 of ribosomal RNA genes of *Oncorhynchus keta* (chum salmon)
 - Salmon DNA added to extraction buffer, qPCR assay used to determine sample-related effects on DNA recovery and/or PCR inhibition

Relative quantification of target organisms by Comparative C_T Method

Calibrator sample contains a known number of target organism cells that is processed and analyzed in same manner as test samples.

**Calibrator sample: 10² - 10⁵ cells
in buffer or water**

**Test sample: 50 - 100 ml of surface
water or treated waste water**



Calculation of calibrator cell equivalents (CCE) in test water sample by comparative C_T method

Sample type	C_{TEnt}^a	C_{TRef}^b	ΔC_T ($C_{TEnt}-Ref$)	$\Delta\Delta C_T$ ($\Delta C_{TTest}-Calib$)	Ratio test/calib ($E+1$) ^(-$\Delta\Delta C_T$)	CCE in test sample (Ratio x Calib cells)
Calibrator (10^5 cells)	25.21	30.45	-5.24	----	----	----
Test	32.53	31.13	1.40	6.64	0.01	$0.01 \times 10^5 = 1000$

^aTarget organism assay (Ent)

^bSalmon DNA assay (Ref)

E: amplification efficiency from slope of standard curve (= 1 in this example)

1. Subtract C_{TRef} from C_{TEnt} for both calibrator and test samples = ΔC_T
2. Subtract ΔC_T Calibrator from ΔC_T Test sample = $\Delta\Delta C_T$
3. Calculate $(E+1)^{(-\Delta\Delta C_T)}$ = ratio of target sequence copies (TSC) in test sample/calibrator sample
4. Multiply ratio of TSC in test sample/calibrator samples by known cell #'s in calibrator sample = CCE in test sample



Advantages/Disadvantages of the Current qPCR Methods

- Advantages
 - *Enterococcus* method used with success in all NEEAR epi studies to date
 - Relatively simple and cost-effective approach to qPCR analysis
 - Whole cell calibrators adjust for variations in DNA recovery from cells as well as PCR conditions
 - Salmon DNA assay provides control for DNA recovery and/or PCR inhibition in each sample
 - CCE estimates may be useful for indicating relative cell densities of different target organisms in samples
- Disadvantages
 - CCE estimates invite comparisons with culture results that may not be appropriate
 - Use of crude DNA extracts limits sample volumes that can be analyzed and thus method sensitivity
 - Current salmon DNA positive control assay is more sensitive to PCR inhibition than target organism assays

On-going Refinements in qPCR Methods



- Evaluation of Standardized Reagents
 - Bioballs™ or equivalent for standardized calibrator samples
 - Customized DNA standards
 - Customized Internal Amplification Controls for PCR inhibition
 - Improved sample processing controls
- Sample purification and concentration approaches
 - - to improve method sensitivity
- Testing of additional qPCR platforms
- Methods for quantifying target sequences rather than cell equivalents in samples
 - - that still incorporate advantages of calibrator based approach
- Methods for defining uncertainty in qPCR analytical results

Ongoing and Upcoming CPSP Projects



- **Study the effects of sample holding time, storage and preservation on qPCR target sequence integrity (Project 16)**
- **Reanalyze archived filters from all epi studies for multiple indicators (general and human-associated) by refined qPCR methods (Project 22)**
- **Validate qPCR method(s) for most promising indicator(s) in single and multiple lab studies (Project 17)**
- **Determine relative persistence of qPCR signals from different indicators in treated and ambient waters (Project 8)**

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- Thank you for your attention.
- Questions?