Data Analysis and Calculations

Overview: This section describes a method for determining the ratio of the target sequence quantities recovered from a test (water filtrate) sample compared to those recovered from identically extracted calibrator samples using an arithmetic formula, referred to as the ΔΔCT comparative cycle threshold calculation method. The ΔΔCT relative quantitation method also normalizes these ratios for differences in total DNA recovery from the test and calibrator samples using qPCR analysis CT values for a reference sequence provided by the SPC DNA. These ratios are converted to absolute measurements of total target sequence quantities recovered from the test samples by multiplying them by the average total number of target sequences that are normally recovered from a constant number of target organisms that are added to all calibrator samples. The complete procedure for determining target sequence quantities in water samples is detailed below.

Generation of CT value vs. target sequence number standard curve: Three replicate serial dilutions of a DNA standard, should be prepared to give concentrations of $4 \times 10^4$, $4 \times 10^3$, $4 \times 10^2$, $2 \times 10^2$ and $1 \times 10^2$ lsrRNA gene sequences per $5 \mu$L (the standard sample volume added to the PCR reactions) and the replicates of each dilution pooled.

Aliquots of each of these dilutions should be stored at 4°C in low retention microcentrifuge tubes and can be reused for repeated qPCR analyses.

qPCR analyses of these diluted standards using the Enterococcus primer and probe assay should be performed at least three separate times in duplicate.

CT values from these composite analyses should be subjected to regression analysis against the log10-transformed target sequence numbers per reaction.

Amplification factors (AF) used for subsequent comparative cycle threshold calculations can be calculated from the slope value of this curve by the formula $AF = 10^{(1 / (-)\text{slope value})}$.

An example calculation using the slope value from the example regression is shown below:

$AF = 10^{(1 / 3.4777)} = 1.94$

Calculation of average target sequence recovery in calibrator sample extracts: A minimum of nine calibrator sample extracts should initially be prepared from at least three different freezer stored aliquots of each cultured *E. faecalis* stock suspension.

Dilutions of each of these calibrator sample extracts equivalent to the anticipated dilutions of the test samples used for analysis (*e.g.*, 1, 5 and/or 25 fold) should be analyzed with the Enterococcus primer and probe assay.

The average CT value from these analyses should be interpolated on the standard curve generated from the DNA standard to determine the average number of target sequences per $5 \mu$L of extract used in the reactions.
Average calibrator target sequences/5 μL extract = \(10^{\frac{(25.21-38.44)}{-3.477}}\) = 6382

*Note:* Four places should be kept from this calculation for the following calculation (i.e., 6382.6983). Dividing this value by 5 gives the average calibrator target sequences/μL extract which can be multiplied by the total volume of the extract at the applicable dilution level (e.g., 600 μL of original extract volume x 5 = 3000 μL for a 5 fold diluted sample) to determine the average total quantity of target sequences recovered in the calibrator sample extracts. An example of this calculation using the average calibrator target sequences/reaction value determined immediately above is shown below:

Average target sequences = 6382 target sequences x 3000 μL total extract volume

Calibrator extract 5 μL extract= 3,829,619

**Calculation of target sequence quantities in test samples:** A minimum of three fresh calibrator samples should be extracted and analyzed at least on a weekly basis and preferably on a daily basis in association with each batch of water sample filtrates.

QC analysis of the analysis results from these calibrator extracts should be performed.

CT values from the Enterococcus target sequence and salmon DNA Sample Processing Control (SPC) qPCR assays for both the calibrator and test samples are used in the ΔΔCT comparative cycle threshold calculation method to determine the ratios of target sequences in the test and calibrator sample extracts and these ratios are converted to absolute measurements of total target sequence quantities recovered from the test samples.

Subtract the SPC assay CT value (CT, SPC) from the target assay CT value (CT, target) for each calibrator sample extract to obtain ΔCT value and calculate the average ΔCT value for these calibrator samples. Subtract the SPC assay CT value (CT, SPC) from the target assay CT value (CT, target) for each water sample filtrate extract to obtain ΔCT values for each of these test samples. *Note:* if multiple analyses are performed on these samples, calculate the average ΔCT value. Subtract the average ΔCT value for the calibrator samples from the ΔCT value (or average ΔCT value) for each of the test samples to obtain ΔΔCT values. Calculate the ratio of the target sequences in the test and calibrator samples using the formula:

\[ AF^{\Delta\Delta CT} \]

where \(AF =\) amplification factor of the target organism PCR assay. Multiply the ratio of the target sequences in the test and calibrator samples by the average target sequences/calibrator extract to determine absolute numbers of total target sequences/extract for each of the test samples.

*Note:* This calculation can be applied without modification to the analyses of diluted extracts if both the test sample and calibrator extracts are equally diluted and equal volumes of diluted extracts are analyzed.

The geometric mean of the measured target sequences and associated coefficients of variation in multiple water samples can be determined from individual sample CT values using the following procedure:

Use ΔCT value for each individual water sample extract and the mean calibrator ΔCT value to calculate the measured target sequence numbers in each water sample extract. Calculate the log10 of the measured target sequence numbers in each water sample (log N) Calculate the mean
(M) and standard deviation (S) from the values of log N obtained in the previous step for all of the water sample extracts. Calculate the geometric mean as 10M. The implied coefficient of variation (CV) is calculated, based on the log normal distribution, as the square root of \( \frac{10V}{0.434 - 1} \), where \( V = S^2 \).

**Reporting Results:** Where possible, duplicate analyses should be performed on each sample. Report the results as *Enterococcus* (large subunit ribosomal RNA gene) target sequences per volume of water sample filtered.

*Modified from USEPA, Method A.*