Calculating limit of detection (LOD) and limit of quantification (LOQ) for qPCR markers

1. INTRODUCTION

1.1. This method describes how to calculate the limit of detection (LOD) and limit of quantification (LOQ) for qPCR markers undergoing validation. The LOD is the lowest measured concentration of a target DNA fragment that can be detected with 95% confidence. This minimum measured concentration should be greater than zero and statistically different from a method blank in the tested replicates. The LOQ is the lowest amount of an analyte that can be reliably detected and quantified.

1.2. These calculations are essential for determining if a marker can be identified at sufficiently low concentrations. Bacterial DNA is often highly diluted in environmental samples, and molecular assays need to be sensitive enough to reliably detect and quantify these low amounts of material.

2. PREPARE SAMPLES

2.1. Test positive fecal samples to identify the sample with the highest concentration of target qPCR marker. Use this sample for the LOD and LOQ determination.

2.1.1. Weigh out a fecal sample aliquot (~1 g of sample) in 2 mL tube and record the weight. Add 1 mL of PBS and bring sample into solution.

2.1.2. Add this fecal slurry to a 90 mL dilulok bottle. Mix thoroughly by shaking to ensure homogenization. This will be the initial diluted fecal sample.

2.1.3. Transfer 1 mL of the initial diluted sample to a 99 mL dilulok bottle. Mix thoroughly by shaking. This will be the 1/100 diluted fecal sample.

2.1.4. Transfer 10 mL of the 1/100 diluted sample to a 90 mL dilulok bottle. Mix thoroughly by shaking. This will be the 1/1000 diluted fecal sample.

2.1.5. Collect 3 filters of 25 mL each per concentration (100-fold and 1000-fold).

2.1.6. Extract DNA from the filters using crude extraction protocol (SOP PCR-4.1) and run qPCR plate including the following controls: standard curves for SKETA and assay being validated; NTC controls for SKETA and validation assay; method blank; extraction blank; and extraction control.

2.1.7. If applicable, a bacterial culture containing the target organism can be used instead of fecal samples.

2.2. Determine approximate expected concentration in Target Sequence Copies (TSC)/5 µL of your initial diluted fecal samples by calculating concentrations of the 100-fold and 1000-fold diluted samples. Choose the sample with the highest concentration to use in step 2.3.

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2.3. Determine how many dilutions to perform and which concentrations to collect.

2.3.1. The LOD determination requires a minimum of three concentrations for the calculations. Testing four concentrations is preferred to cover a broader range and increase chances of accurately calculating LOD. Ten filters are collected per concentration.

2.3.2. Dilutions are typically 5-fold or 10-fold. Smaller dilutions can also be used and will allow the analyst to more accurately identify the LOD but will likely require additional concentrations to cover the appropriate range.

2.3.3. The goal is to have at least 1 concentration with 10/10 detects and at least 1 concentration with 1-9 detects. It is helpful to aim for the most concentrated sample to amplify ~C_q 30-32.

2.3.4. Example concentrations to collect:

2.3.4.1. 10-fold dilutions: E2, E1, E0, E-1

2.3.4.2. 5-fold dilutions: 5E1, 1E1, 2E0, 4E-1

2.3.5. More concentrated samples will be collected to serve as standards for quantifying target concentration. Use samples in the E3-E5 TSC/well range and test at least 2 concentrations that do not appear very turbid, as turbid samples are more likely to be inhibited.

2.4. Perform 5-fold or 10-fold dilutions to cover the appropriate range and filter samples (SOP PCR-4.0). The total volume for each dilution is determined by the number of filters collected.

2.4.1. For higher concentrations serving as standards, collect 3 filters of 25 mL each per concentration. For LOD determination, collect 10 filters of 100 mL each.

2.4.2. Filter an additional 50 mL of PBS to serve as a method blank.

2.5. Extract DNA from all filters using the crude extraction protocol (SOP PCR-4.1). Include an extraction blank and extraction control.

3. RUN SAMPLES

3.1. Run one plate containing all the samples and controls.

3.1.1. Include the following controls: standard curves for SKETA and assay being validated, NTC controls for SKETA and validation assay, method blank, extraction blank, extraction control.

3.1.2. Add each more concentrated standard sample to 4 wells, three containing the master mix for the validation assay and one containing the master mix for SKETA.

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Add the 3rd well spike to one well. Name these samples by concentration and letter (E5A, E5B, E5C, etc).

3.1.3. Add each LOD sample to one well containing the master mix for the validation assay. Due to space limitations, SKETA is not run for these samples. Since these samples are highly diluted, they are unlikely to be affected by inhibition. Name these samples by concentration and letter (E2A-E2J, etc.).

3.2. Analyze plate and determine if controls are acceptable.

3.2.1. Look at standard curve and blanks. Efficiency and R^2 values should be within appropriate range, 90-120% for efficiency and $\geq$0.99 R^2. Blanks should have no amplification. If there is any amplification, samples should be remade.

3.2.2. Check higher standards for inhibition and to see if they are in linear range of detection. SKETA and 3rd well spike values should be between 50-175% recovery and the samples should amplify within the range of the standard curve.

3.2.2.1. If E5 and E4 or E4 and E3 samples were collected, values should be approximately 10-fold apart. If one concentration does not meet criteria but another does, only the passing concentration should be included for further analysis.

3.3. If controls and higher standards are acceptable, determine the true value for each concentration. The low levels of target material cannot be accurately quantified, so the StepOnePlus or QuantStudio3 results for these samples are not used to determine an accurate concentration.

3.3.1. Take the average concentration of reliable high standards and use this as the true value.

3.3.2. Calculate copy number for all the other samples based on the dilutions performed. Make sure to account for differences in volume filtered (25 mL for high standards and 100 mL for lower dilutions).

3.4.Determine the number of detects for each concentration. A sample is considered a detect if there is any amplification.

4. CALCULATE LOD AND LOQ

4.1. Calculate LOD using Excel add-on software XLSTAT. Plot a sigmoidal curve and determine concentration at which marker will be detected 95% of the time. Refer to user guide on XLSTAT website for additional information.

4.1.1. Open Excel and input the following information in a table: concentration, units, number of detects, total number of samples per concentration. See example below.

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Concentration	Units	Detects	Total
66.9	TSC/5 µL	10	10
13.4	TSC/5 µL	10	10
2.7	TSC/5 µL	9	10
0.5	TSC/5 µL	5	10

4.1.2. Click on XLSTAT tab and launch software by selecting “Start XLSTAT”.

4.1.3. Select XLSTAT/Dose/Dose effect analysis and a dialog box appears. Select the appropriate data in the table (including headers) for each variable.

4.1.3.1. Select “Detects” column for Response Variable.

4.1.3.2. Choose Sum(Binary) for Response Type. Check Quantitative box and select data from “Concentration” column.

4.1.3.3. Select Model:Probit. “Sheet” and “Variable labels” should be selected.

4.1.3.4. Check “Observation weights” box and select data from “Total” column.

4.1.3.5. Under Options tab, Confidence interval (%) should be 95 and “Take the log” should be checked.

4.1.4. Click OK and results are displayed in a new sheet with a variety of tables and statistics. Use these to determine 95% LOD.

4.1.4.1. Scroll to the bottom and you will see the Logistic regression of Detects by Log (Concentration), which shows the sigmoidal curve fitted to the data, and the Probability analysis with the fitted model (Variable Detects). The probability analysis shows the concentration expected to be detected at probabilities ranging from 1-99%. See example below.

Probability	Concentration
0.01	0.029
0.05	0.068
0.10	0.106
0.20	0.181
0.30	0.268
0.40	0.374
0.50	0.511
0.60	0.698
0.70	0.975
0.80	1.440

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0.90	2.475
0.95	3.871
0.99	8.955

4.1.4.2. Look at the concentration that corresponds to a 95% probability of detection (0.95). This is the LOD for a reaction, in TSC/5 µL. LIMS will calculate the LOD in units of TSC/100 mL based on the amount filtered, volume of extract, and dilution factor.

4.2. Calculate the LOQ using one of two methods.

4.2.1. Method 1: Use the LOD to determine the LOQ. The LOQ is equal to 3-5x the LOD. Current practice is to use 5x LOD.

4.2.2. Method 2: The LOQ is determined by standard deviation of spiked samples. Perform fecal sample dilutions, filtration, extraction, and qPCR as in LOD section. Filter at least 5 replicates at each concentration and determine standard deviation of C_q values at each concentration. The LOQ is the lowest concentration where the standard deviation is <0.5 C_q.

5. REFERENCES

5.1. DEP SOP PCR-4.0 *Preparation of Samples for qPCR Analysis*

5.2. DEP SOP PCR-4.1 *Crude Lysate Extraction Method for qPCR Analysis*

5.3. XLSTAT website article: https://help.xlstat.com/customer/en/portal/articles/2062251-dose-effect-analysis-in-excel-tutorial?b_id=9283

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