DETERMINING SENSITIVITY AND SPECIFICITY OF qPCR MARKERS

1. INTRODUCTION

1.1. This method describes the steps to calculate the sensitivity and specificity of qPCR markers undergoing validation. Sensitivity refers to the proportion of true positive samples that test positive in the assay. Specificity refers to the proportion of true negative samples that test negative in the assay. The ideal assay has both high sensitivity and specificity, with few false positives and false negatives.

1.2. This method is essential to evaluate new qPCR assay performance before use in the DEP laboratory. It provides valuable information about potential cross-reactivity, the prevalence of the marker in the feces of samples collected for validation from animals, birds etc. and the ability to detect low levels of marker gene in environmental water samples.

2. TESTING FECAL SAMPLES

2.1. Sample Collection –

2.1.1. Target Species: Collect fecal samples from 5–10 animals of the target species to serve as positive controls. These should be collected from multiple locations, if possible. Samples can be collected from wildlife, pets, zoos, wildlife rescues, farms, etc. Aliquot the fecal library samples in 2 mL tubes and store at -80°C.

2.1.2. Non-Target Species: Collect fecal samples from non-target animals to test for cross-reactivity and to serve as negative controls. It is best to collect samples from animals that share a similar environment (farm animals, household pets), diet (herbivores, omnivores, carnivores), or genetic makeup (dog, fox, wolf). Publications referencing the marker often mention animals with potential cross-reactivity, and these should be tested as well.

2.2. Preparation and Analysis of Fecal Samples –

2.2.1. Weigh out approximately 0.25 g feces per sample and record the weight. Purify the fecal DNA using the Qiagen PowerSoil Pro kit. Refer to SOP PCR-4.2 for details.

2.2.2. Analyse fecal DNA from target and non-target species with qPCR. Collect additional fecal samples as needed if results are inconclusive. Make sure to check for signs of inhibition in the 3rd well spike and surrogate wells.

2.3. Preparation and Analysis of Diluted Fecal Samples –

2.3.1. In parallel, dilute positive and negative fecal samples in PBS and filter 100 mL of fecal slurry using the following procedure:

2.3.1.1. Weigh out approx. 1 g of fecal sample in a 2 mL tube or 15 ml conical tube and record the weight.

2.3.1.2. Add 1 mL of PBS and use a pipette tip or plastic applicator to break up the sample and bring it into solution.

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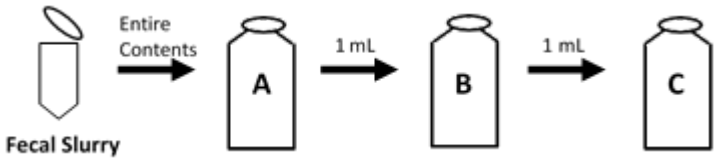
2.3.1.3. Dilution of Samples – See figure below for details.

2.3.1.4. Label 3-99 ml dilulok bottles: A, B, and C.

2.3.1.5. Add this fecal slurry (2.3.1.2.) to a 99 mL dilulok bottle labeled “A”. Mix thoroughly by shaking to ensure homogenization.

2.3.1.6. Transfer 1 mL of the slurry from bottle “A” to the 99 mL dilulok bottle labeled “B”. Mix thoroughly by shaking.

2.3.1.7. Transfer 1 mL of the slurry from “B” to the 99 mL dilulok bottle labeled “C”. Mix thoroughly and filter entire 100 mL slurry from bottle C according to SOP PCR-4.0.



2.3.1.8. Extract the DNA using the crude extraction protocol (SOP PCR-4.1) and run on qPCR plate.

3. TESTING WATER SAMPLES

3.1. Sample Collection –

3.1.1. Collect water samples that are expected to be positive for target DNA of interest. For example, a beach with a large seagull population, a pond located within a cattle farm, and a lake with a recent raw sewage spill might serve as positive samples for seagull, cow, or human fecal markers, respectively.

3.1.2. Collect water samples that are expected to be negative for target DNA of interest.

NOTE: The expected positive and negative water samples may be collected specifically for the purposes of validating a new marker. Alternatively, previously collected samples (from routine monitoring or a specific project) can be used if information about the potential sources of fecal contamination is known. Wastewater samples can serve as positive controls for human-associated markers and can be used to test for cross-reactivity with other animal markers.

3.2. Preparation and Analysis of Water Samples –

3.2.1. Filter 100-200 mL of each water sample, according to SOP PCR-4.0. Collect two filters per sample.

3.2.2. Extract samples using both extraction methods, crude and purified. See SOPs PCR-4.1 and PCR-4.2 for details.

3.2.3. Run positive and negative water samples on a qPCR plate.

4. CALCULATE SENSITIVITY AND SPECIFICITY

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4.1. Analyze data from all plates and determine the number of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN) (See table below).

	Control Type	Amplification in Gene of Interest?
True Positive	Positive	Yes
True Negative	Negative	No
False Positive	Negative	Yes
False Negative	Positive	No

4.2. Calculate the sensitivity and specificity of the marker based on the results, using the following equations:

$$\text{Sensitivity}=\frac{TP}{TP+FN} \qquad \text{Specificity}=\frac{TN}{TN+FP}$$

4.3. Compare these values to the published literature and determine if marker is acceptable for the laboratory's use. A general guideline is to aim for >0.8 for both sensitivity and specificity. These values can vary widely between markers and geographic range. For some markers, it may be acceptable to be highly specific but not very sensitive at the individual level. The marker may perform well in the environment when detecting fecal contamination from a population of animals.

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. DEP SOP PCR-4.2, *Purification of Filtered Samples Using the Qiagen DNeasy PowerSoil Pro Kit*

Appendix of Significant Changes

08/15/2023 Reformatted SOP and added reference to DEP SOP PCR-4.0. Added figure to Section 2 and table to Section 4.

08/11/2025 Reformatted sections of the SOP.

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