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 positive samples that test positive. Specificity refers to the proportion of negative samples that test negative. The ideal assay has high sensitivity and high specificity, with few false positives and few false negatives.

1.2. This method is essential for determining if a marker performs as expected and should be added to the laboratory's capabilities. It provides valuable information about potential cross-reactivity, the prevalence of the marker in the feces of Florida animals, and the ability to detect low levels of material in filtered water samples.

2. TEST FECAL SAMPLES

2.1. 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 pets, zoos, wildlife rescues, farms, etc. Aliquot and store fecal library samples at -80°C in 2 mL tubes.

2.2. Collect fecal samples from other 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 that potentially cross-react and these should be tested as well.

2.3. Weigh out ~0.25g feces per sample and purify with Qiagen PowerSoil DNA Extraction kit. Refer to SOP PCR-4.2 for details.

2.4. Run DNA from positive and negative fecal samples on qPCR plate. Collect additional samples as needed if results are inconclusive.

2.5. Dilute positive and negative fecal samples in PBS and filter 100 mL fecal slurry. Weigh each sample aliquot in 2 mL tube before dilution.

2.5.1. Take a fecal sample aliquot (~1 g of sample) and add 1 mL PBS. Use a pipette tip or plastic applicator to break up the sample and bring into solution.

2.5.2. Add this fecal slurry to a 90 mL dilulok bottle. Mix thoroughly by shaking to ensure homogenization.

2.5.3. Transfer 1 mL of slurry to a 99 mL dilulok bottle. Mix thoroughly by shaking.

2.5.4. Transfer 1 mL of slurry to a new 99 mL dilulok bottle. Mix thoroughly and filter entire 100 mL slurry.

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

3. TEST WATER SAMPLES

3.1. 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.

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

3.2.1. The expected positive and negative water samples can 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 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.

3.3. Filter 100-200 mL of each water sample and collect two filters per sample.

3.4. Extract samples using both extraction methods, crude and purified.

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

4. CALCULATE SENSITIVITY AND SPECIFICITY

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).

4.1.1. True positives are positive controls that had amplification for gene of interest.

4.1.2. True negatives are negative controls that had no amplification for gene of interest.

4.1.3. False positives are negative controls that had amplification for gene of interest.

4.1.4. False negatives are positive controls that had no amplification for gene of interest.

4.2. Calculate the sensitivity of the marker based on these results.

4.2.1. $Sensitivity = \frac{TP}{TP+FN}$

4.3. Calculate the specificity of the marker based on these results.

4.3.1. $Specificity = \frac{TN}{TN+FP}$

4.4. 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

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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-4.2 *Purification of Filtered Samples Using the Qiagen DNeasy PowerLyzer PowerSoil Kit*

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