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Quantifying Environmental Microorganisms and Viruses Using qPCR

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Overview

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Quantitative polymerase chain reaction (qPCR), also known as real-time PCR, is a widely-used molecular technique for enumerating microorganisms in the environment. Prior to this approach, quantifying microorganisms was limited largely to classical culture-based techniques. However, the culturing of microbes from environmental samples can be particularly challenging, and it is generally held that as few as 1 to 10% of the microorganisms present within environmental samples are detectable using these techniques. The advent of qPCR in environmental microbiology research has therefore advanced the field greatly by allowing for more accurate determination of concentrations of microorganisms such as disease-causing pathogens in environmental samples. However, an important limitation of qPCR as an applied microbiological technique is that living, viable populations cannot be differentiated from inactive or non-living populations.

This video demonstrates the use of qPCR to detect pepper mild mottle virus from an environmental water sample.

Principles

The basic principles behind qPCR is the same as regular PCR – repeated cycles of primer annealing to template, elongation of PCR product, and denaturation of product from template, leading to the exponential amplification of a target sequence of interest, known as the “amplicon”, from a pool of starting material. The innovation of qPCR is in the addition of fluorescent chemicals into the reaction, which allows the synthesis of PCR product at each cycle to be directly visualized in “real time” by specialized thermocyclers, making it possible to quantify the amount of template sequence in the original sample. The quantity is usually measured in terms of the threshold cycle (C_t, also known as quantification cycle or C_q), which is the PCR cycle at which the amount of fluorescent products exceeds the background level.

Quantification can be relative, where the C_t value of one sequence is compared to that of another standard or control sequence. Alternatively, if a series of DNA of known quantity is run alongside the samples in the reaction, a “standard curve” comparing fluorescence value to DNA amount can be produced, and allows the sample DNA to be quantitated absolutely.

In one qPCR method, a short stretch of DNA, known as a probe, is designed against a specific target sequence of interest. The probe is chemically attached to a fluorescent dye as well as a “quencher” molecule that suppresses the fluorescence signal from the dye when in close proximity. The polymerase enzyme, which synthesizes the DNA product, has a DNA-degrading activity that would cause the fluorescent molecule to be released from the probe, thus separating the dye from the quencher and allowing the fluorescence signal to be detected. Fluorophore levels are quantitatively measured after each PCR cycle, with increasing signal strength correlating to higher levels of amplified target sequences (termed “amplicons”) present within the environmental sample.

Procedure

1. Sample Collection

1. Collect soil using an auger or shovel to a determined depth. If collecting soil from the rhizosphere, only collect soil within 7 mm around plant root, by hitting excess soil off the root and scraping desired soil into a collection barrel.
2. Place a sterile Nalgene bottle into dipping stick. Hold the end of the stick and collect water by submerging bottle. Place bottle into a cooler with ice.
3. Transfer samples to the laboratory.

2. Nucleic Acids Extraction

1. To isolate microorganisms from the collected samples and to extract DNA and/or RNA from them, please see the JoVE Science Education video on community nucleic acid extraction.

3. Reverse Transcription

1. If the genetic material being assayed is RNA, it must be used to generate complementary DNA (cDNA) via reverse transcription before proceeding to PCR. For details, please refer to JoVE Science Education video on RT-PCR.

4. Setting up qPCR

1. Retrieve reagents stored at -20 °C, and thaw them on ice or at room temperature inside a clean hood. Reagents used in this example include the qPCR reaction mix (dependent on the qPCR machine used; contains DNA polymerase), forward and reverse primers, and the TaqMan

probe. The primer and probe sequences are designed with sequences specific to the organism that is being enumerated. Refer to current literature to find sequences of interest. In this example, the Roche Light Cycler 480 Probes Mix reagent system will be used. Pepper mild mottle virus will be enumerated in the water sample.[1,2] See **Table 1** for primer and probe sequences.
2. Thaw extracted (c)DNA from samples and the positive control DNA (which consists of organism-specific sequences cloned into bacterial plasmids) at room temperature.
3. Prepare a 96-cell table template that resembles the 96-well qPCR plate. Label each cell with the reaction that will be loaded onto the plate. Include reactions for each sample and standard in triplicate, as well as for the positive control and negative control, such as a no-DNA reaction.
4. Calculate the reagent volumes needed for a reaction “master mix”, which includes all of the reagents that are constant among the reactions, based on manufacturer’s instructions and the literature. Prepare enough master mix for triplicate reactions for all samples plus controls, and an additional 10% to account for pipetting error. For a sample master mix recipe, refer to **Table 2**.
5. Working inside a clean hood, once all reagents are completely thawed, add calculated amount of each reagent into a 1.5-mL low-binding microfuge tube to create master mix. Vortex and briefly minicentrifuge each reagent before adding. Change pipette tip between each reagent to prevent contamination and ensure correct concentrations. After all reagents have been added, vortex and minicentrifuge tube with the master mix to ensure homogeneity.
6. Aliquot the appropriate volume of master mix into each designated well in the 96-well PCR plate.
7. Add the appropriate volume of (c)DNA sample, positive control plasmid, and negative control (molecular grade water) into designated wells.
8. Once all samples and controls have been added, seal plate with sealing foil. Use a sealing tool to push air out from underneath the foil and prevent bubbles. Tear off edges of the foil carefully.
9. Centrifuge the sealed 96-well plate in a centrifuge with a plate-holder to gather mixture at the bottom of each well. Make sure to use a counterweight plate to ensure that the centrifuge will be properly balanced while rotating. Pulse centrifuge up to 1000 rpm, then let the centrifuge slowly stop without brakes.

5. qPCR Operation

1. Place sealed 96-well plate into qPCR machine. Ensure that machine indicates it is ready to start.
2. Follow qPCR machine instructions to properly input all information needed by software, then set qPCR machine to run.
3. After machine completes run, the software will be able to use the known concentrations of the positive control to calculate the quantity of cDNA in each reaction. The quantity of virus in the original sample can then be calculated, based on the dilution, filtration, concentration, amplification, and/or extraction processes performed to obtain the DNA sample.

Reagent	Sequence (5’ → 3’)	Volume (µL / well)	Final Conc.
Forward Primer	GAGTGGTTTGACCTTAACGT-TTGA	2.25	900 nM
Reverse Primer	TTGTCGGTTGCAATGCAAGT	2.25	900 nM
Probe	FAM-CCTACCGAAGCAAATG-BHQ1	1.0	200 nM

Table 1. Sample primer and probe sequences for detecting pepper mild mottle virus.

Reagent	Volume (µL / well)	Number of Wells	Master Mix Volume (µL)
LC 480 Mix	12.5	26	325
Molecular H_2O	4.5		117
Forward Primer	2.25		58.5
Reverse Primer	2.25		58.5
Probe	1.0		26
Total	22.5		585

Table 2. Reagent volumes for individual reaction and master mix.

Applications and Summary

The ability to quantify targeted genomic segment copies using the qPCR technique is of importance in a number of scientific fields. Example applications include:

(1) Enumerating pathogens in water, soil, food, surfaces, *etc*.

Real-time PCR is utilized to enumerate pathogens in various environments. During outbreaks, water and soil samples can be analyzed for the pathogen of interest to find the source causing spread. The source can then be further analyzed to enumerate the concentration of the pathogen and determine the amount of contamination. For example, during an outbreak of norovirus on a cruise ship that has caused severe gastroenteritis, vomiting, and diarrhea to among passengers, water and food samples may be subjected to real-time PCR to identify the source of the virus, *e.g.*, water that was not properly treated and contained high fecal contamination.

(2) Measuring the reduction of pathogenic microbes by wastewater treatment

Raw sewage water contains an abundance of disease-causing microorganisms and therefore must be treated in order to protect public health. Water samples can be collected at different points along a wastewater treatment train, and analyzed using qPCR to determine the reduction in levels of pathogenic microorganisms including viruses. The calculated reductions then provide valuable information as to the effectiveness of wastewater treatment processes and potential water reuse applications.

(3) Measuring functional gene markers in the environment

Microbial communities are subject to changes in membership and fluctuations in activity due to environmental pressures. These shifts can be monitored via analysis of functional genes that might be activated by particular environmental stressors. Real-time PCR can be used to quantify the expression of these genes in samples to monitor changes in microbial community activity. For example, qPCR allows microbial ecologists to quantify the expression of genes activated for biodegradation pathways in the presence of man-made contaminants present in soils.

References

1. Zhang, T., Breitbart, M., *et al.* RNA Viral Community in Human Feces: Prevalence of Plant Pathogenic Viruses. *PLoS Biology*. **4**, e3 (2005).
2. Haramoto, E., *et al*. Occurrence of Pepper Mild Mottle in Drinking Water Sources in Japan. *Applied Environmental Microbiology*. **79**, 7413-7418 (2013).

