JoVE: Science Education

Quantification of Organism Nucleic Acids via PCR

--Manuscript Draft--

Manuscript Number:	10186
Full Title:	Quantification of Organism Nucleic Acids via PCR
Article Type:	Manuscript
Section/Category:	Manuscript Submission
Corresponding Author:	Ian Pepper UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Ian Pepper
First Author Secondary Information:	
Order of Authors:	Ian Pepper
	Bradley Schmitz
	Luisa Antionette Ikner
	Chuck Gerba
Order of Authors Secondary Information:	

Photo or Graphic File
Click here to download Photo or Graphic File: Pepper_qPCR_table1.docx

Reagent	Sequence (5' → 3')	Volume (ul / well)	Final Conc.
LC 480 Mix	[redacted]	12.5	1x
H_2O		4.5	-
Forward Primer	GAGTGGTTTGACCTTAACGTTTGA	2.25	900 nM
Reverse Primer	TTGTCGGTTGCAATGCAAGT	2.25	900 nM
Probe	FAM-CCTACCGAAGCAAATG-BHQ1	1.0	200 nM

Table 1

Photo or Graphic File
Click here to download Photo or Graphic File: Pepper_qPCR_table2.docx

Reagent	Volume (ul / well)	N	Master Mix Volume (ul)
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

PI Name: Bradley W. Schmitz, Dr. Luisa A. Ikner, Dr. Charles P. Gerba, and Dr. Ian L. Pepper
Environmental Science Education Title: Quantification of Organism Nucleic Acids via PCR

Overview:

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 because the concentrations of microorganisms such as disease-causing pathogens in samples can be more accurately determined. 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.

One type of qPCR process is performed by attaching fluorescent reporter probes onto target gene segments of interest as they are amplified over the course of several varying temperature gradient cycles. During the course of what is termed in PCR as the "extension" step, the fluorescent component of the probe (known as the "fluorophore") is severed by the polymerase enzyme, resulting in the release of a fluorescent signal that is detected by the qPCR machine. Fluorophore levels are quantitatively measured after each cycle, with increasing signal strength correlating to higher levels of amplified target sequences (termed as "amplicons") present within the environmental sample.

This video demonstrates the processing of an environmental water sample, including nucleic acid extraction, and application of the fluorescent reporter probe qPCR process for the detection of pepper mild mottle virus.

Procedure:

1. Sample Collection

    1.1. Collect soil using an auger or shovel to a determined depth. If collecting soil from the rhizosphere, only collect soil within 7mm around plant root, by hitting excess soil off the root and scraping desired soil into a collection barrel.

    1.2. 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.

    1.3. Transfer samples to the laboratory.

2. Extract Nucleic Acids

2.1. Extract DNA from organisms by lysing the cells of bacteria/protozoa. Extract RNA or DNA from viruses by lysing protein capsids. This process is explained in more depth in a separate video.

3. Reverse Transcriptase Polymerase Chain Reaction

3.1. If trying to enumerate a virus that is RNA based, the nucleic acid must be converted into cDNA via RT-PCR in order to be detected via downstream methodologies. Refer to RT-PCR video.

4. Thaw qPCR reagents inside a clean hood at room temperature. (Stored at -20 ^{o}C)

4.1. Open freezer containing reagents that are stored at -20 ^{o}C.

4.2. Place reagents in sterile rack inside clean hood to thaw at room temperature. 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.

4.2.1. Roche Light Cycler 480 Probes Mix (varies depending on machine used)

4.2.2. 10 uM Forward Primer (organism sequence specific)

4.2.3. 10 uM Reverse Primer (organism sequence specific)

4.2.4. 5 uM TaqMan Probe (organism sequence specific)

5. Thaw extracted (c)DNA from samples and cloned positive control plasmids. Place in a designated DNA hood. Do NOT place in the clean hood, in order to prevent contamination with reagents.

5.1. Take samples out of -20 ^{o}C freezer and place them in a rack to thaw at room temperature. Take plasmids out of freezer and place them in rack to thaw.

5.1.1. Standard Plasmid (10^3 organism sequence specific cloned positive control).

5.1.2. RNA organism: thaw cDNA product from RT-PCR reaction.

5.1.3. DNA organism: thaw DNA product from extraction process.

6. Return to clean hood with thawed reagents and prepare reagent rack. Prepare 1.5 ml DNA LoBind Eppendorf tube. Tube must be DNA LoBind to prevent nucleic acids from attaching to plastic. Place tubes in order in which they will be added into mixture.

6.1. Collect Eppendorf tube from package, do not touch tube to prevent contamination.

6.2. Label the cap of the tube “MM” for “master mix”.

6.3. Place small tube containing molecular grade DNA/RNA free water into rack.

6.4. Place tube in rack in front of reagents, and place reagents in order in which they will be added into the tube.

6.4.1. Tube > H_2O > LC Mix > Forward Primer > Reverse Primer > Probe

7. Fill out a 96 well plate sheet that resembles the qPCR plate. The axis are labeled according to the plate to ensure each well is labeled correctly.

7.1. Label duplicate wells for each sample.

7.2. Label duplicate wells for positive control. Most common control is 10^3 concentration.

7.3. Label well for negative control.

8. On the 96 well plate sheet, calculate final master mix volume needed to be obtained in order to fill each well. In this case, we are calculating for 22.5 ul to master mix, plus 2.5 ul sample nucleic acid to be placed into each well. This volume may be adjusted to 20 ul master mix and 5 ul nucleic acids depending on sensitivity and volume of the assay.

8.1. Count the number of wells that will be filled with qPCR master mix and add an extra 10 percent to ensure enough mix is created in case of error:

8.1.1. s = number of wells labeled, including positive and negative controls

8.1.2. N = number wells

$$N = s + s(.10)$$

$$N = 23 + 2.3$$

$$N = 26 \text{ wells}$$

8.2. Refer to literature to determine the concentrations for each reagent needed in qPCR reactions.

8.2.1. In this example, we will be enumerating Pepper Mild Mottle Virus. (Zhang et al. 2006 PLoS Biol., PMMV-FP1-rev: Haramoto et al. 2013 Appl. Environ. Microbiol)

table 1

PCR: 95 °C, 5 seconds → 60 °C, 60 seconds [68 bp]

8.3. Calculate amount of each reagent that will be added to the Eppendorf tube to create the proper concentration master mix. Multiply each reagent volume by the number of wells (N).

8.3.1. N = number of wells calculated in previous step

Table 2

9. Add calculated amount of each reagent into 1.5 ml Eppendorf DNA LoBind tube to create master mix. Each reagent must be completely thawed. Vortex and minicentrifuge each reagent for one second before adding into master mix. Change pipette tip between adding each reagent to prevent contamination and ensure correct concentrations.

    9.1. Vortex, minicentrifuge, and add molecular grade water into Eppendorf tube.

    9.2. Vortex, minicentrifuge, and add lighcycler 480 master mix into Eppendorf tube.

    9.3. Vortex, minicentrifuge, and add forward primer into Eppendorf tube.

    9.4. Vortex, minicentrifuge, and add reverse primer into Eppendorf.

    9.5. Vortex, minicentrifuge, and add probe into Eppendorf tube.

    9.6. After all reagents added, vortex and minicentrifuge Eppendorf tube to create homologous master mix throughout the entire tube.

10. Aliquot 22.5 ul master mix into every designated well in the 96 well PCR plate.

    10.1. After adding master mix to 96 well plate, move plate into DNA hood where the samples and positive plasmid are thawing.

11. Add 2.5 ul samples into designated wells in 96 well plate. Ensure samples are thawed.

    11.1. DNA based organism = add 2.5 ul extraction product

    11.2. RNA based organism = add 2.5 ul cDNA product from RT-PCR

12. Add 2.5 ul positive control plasmid into designated wells in 96 well plate. Positive control concentration is best at 10^3 per ul dilution.

13. Add 2.5 ul molecular grade H_2O into negative control well in 96 well plate.

14. Seal 96 well plate with Lightcyler 480 sealing foil.

    14.1. Use sealing tool to push air out from underneath the foil and prevent bubbles.

    14.2. Tear off edges of the foil carefully by pushing the white strips downward along the perforated edges.

15. Centrifuge the sealed 96 well plate to gather mixture at the bottom of each well.

    15.1. Place the 96 well plate inside a centrifuge holder and balance the weight with another holder to ensure that the centrifuge will be evenly balanced while rotating.

    15.2. Pulse centrifuge up to 1000 rpm, then let the centrifuge slowly stop without brakes.

16. Run qPCR function in machine (Lightcycler 480) to enumerate nucleic acid concentration present within each well.

16.1. Place sealed 96 well plate into qPCR machine (Lightcycler 480). Ensure that machine indicates it is ready to start. In this case, the Lightcycler 480 machine will have two green lights indicating both software and hardware are ready.

16.2. Follow qPCR machine protocol to properly insert all information needed by software, then start qPCR function to complete the experiment.

16.2.1. In this case, we are enumerating Pepper Mild Mottle Virus, so we would follow protocol from literature (95 ^{o}C, 5 seconds → 60 ^{o}C, 60 seconds [68 bp]).

16.3. After machine completes run, write down concentrations detected by machine and calculate the concentration of organism present in original sample by multiplying by dilution, filtration, concentration, amplification, and/or extraction processes used before starting qPCR.

Applications:

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

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, an outbreak on a cruise ship has caused severe gastroenteritis, vomiting, and diarrhea to passengers. Fecal and vomit samples are analyzed, indicating high concentration of norovirus. To determine the source, water and food samples are subjected to real-time PCR indicating high concentrations in water that was not properly treated and contained high fecal contamination.

Determining severity of contamination

In areas that are reporting water contamination from fecal-borne pathogens, real-time PCR can be utilized to enumerate the concentration of pathogens in the water. Higher pathogen concentrations correlate to higher fecal contamination, which gives indication on the severity of the problem.

Measuring the Reduction Pathogenic Viruses in Wastewater

Raw sewage water contains an abundance of disease-causing microorganisms, and therefore must be treated in order to ensure 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.

Measuring Functional Gene Markers in the Environment

Microbial communities are subject to changes in membership and fluctuations in activity due to environmental pressures and changes. These shifts can be monitored via analysis of functional genes that might be activated given particular environmental stressors. Real-time PCR can be used to quantify the expression of these genes in samples in effort to monitor changes in 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.