

# JoVE: Science Education

## Utilizing Polymerase Chain Reaction to Detect Microorganisms in Environmental Samples

--Manuscript Draft--

Manuscript Number:	10081
Full Title:	Utilizing Polymerase Chain Reaction to Detect Microorganisms in Environmental Samples
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 Antoinette Ikner Chuck Gerba
Order of Authors Secondary Information:	

**PI Name:** Bradley W. Schmitz, Dr. Luisa A. Ikner, Dr. Charles P. Gerba, and Dr. Ian L. Pepper

**Environmental Science Education Title:** Utilizing Polymerase Chain Reaction to Analyze Microbial Genetic Information in the Environment to Detect Microorganisms in Environmental Samples

**Overview:**

Polymerase chain reaction (also known as PCR) is a tool used to detect microorganisms that are present in soil, water, and atmospheric environments. By amplifying specific sections of DNA or RNA, PCR can facilitate the detection and identification of targeted microorganisms down to the species, strain, and serovar/pathovar level. The technique can also be utilized to characterize entire communities of microorganisms in samples.

The culturing of microorganisms in the laboratory using specialized growth media is a classical technique and remains in use for the detection of microorganisms in environmental samples. Many microbes in the natural environment, while alive, maintain low levels of metabolic activity and/or doubling times and are thus referred to as viable but non-culturable (VBNC) organisms. The use of culture-based techniques alone cannot detect these viable but non-culturable microbes and, therefore, does not provide a thorough assessment of microbial populations in samples. The use of PCR allows for the detection of culturable microbes, VBNC organisms, and those that are no longer alive or active, as the amplification of genetic sequences does not generally require the pre-enrichment of microorganisms present in environmental samples. However, PCR cannot differentiate the aforementioned states of viability and activity found in samples. When combined with one or more culture-based techniques, the viability or certain subsets of microorganisms may still be determined.

The detection of human pathogenic microorganisms in the environment is a particular challenge, as they may be present in low numbers, yet are still capable of causing illness in humans. For example, risk assessment of human noroviruses indicated that ingestion of as few as 10 to 100 viruses can initiate acute gastroenteritis. The Nested-PCR technique facilitates the detection of low concentrations of microorganisms by employing a two-step PCR utilizing specific primers. The first PCR assay is performed using primers that are designed to anneal and initiate amplification for a random subset of microbes. The second PCR is performed using a specific primer that only anneals to the target microorganism. When conducted properly, Nested-PCR allows for pathogens of interest that are present at low concentrations in the natural environment to be amplified to higher levels that are then detectable.

**Procedure:**

1. Sample Collection

**Commented [DM1]:** Comments from Production:  
**Scope:** As this is an Environmental video, de-emphasize the actual PCR procedure, and focus on the other steps: sample collection and processing, designing the primer, troubleshooting, etc.  
  
**Overview:** In the same vein as the procedure, expand on the concept of VBNC. Put the writeup in the broad scope of metagenomics.

1.1. Collect soil by using an auger or shovel up to a determined depth. If collecting soil from the rhizosphere, only collect directly from around the plant root by hitting the soil off the root and into a collection barrel.

1.2. Collect the water sample by dipping a sterile Nalgene bottle into the water while holding the end of the dipping stick.

## 2. Extract Nucleic Acids

2.1. Extract DNA and RNA from organisms by lysing the cells of bacteria or protein capsids of viruses.

2.2. Refer to the extraction video: this entire process is expanded upon in a separate video.

## 3. Prepare Nucleic acids

3.1. After extraction, have the DNA ready in labeled tubes. If the extract needed to be frozen overnight or longer periods of time for storage, thaw the DNA tubes at room temperature.

3.2. If organisms contain RNA (viruses) or RNA is the focal point of the project, perform Reverse-Transcription PCR to create cDNA. This needs to happen before the reaction PCR procedure can take place.

## 2.4. Conventional Polymerase Chain Reaction

2.1.4.1. Place the PCR enzyme (10x Ex Taq Polymerase) in ice and thaw the other reagents (2.5 mM dNTPs and primers) inside a designated "clean" hood at room temperature.

2.1.4.1.1. The enzyme is stored at -20 °C, but never freezes. It is temperature sensitive, so it must be kept cool and not exposed to ambient temperature.

2.2.4.2. Calculate the reagent mixture volume needed (Table 1).

2.2.1.4.2.1. Multiply the volume of each reagent needed in one reaction, by the number of samples/tubes to be processed. Make sure to account for positive and negative controls in the calculations. Add an additional 10% to final PCR reaction volume to ensure enough mixture is created in case of error.

2.2.1.1.4.2.1.1. Primer volumes depend on assays for specific organisms. Refer to outside literature to obtain proper and accurate PCR mixture volumes.

Example: 3 samples + 1 positive control + 1 negative control = 5 reactions.

2.3.4.3. Prepare a LoBind Eppendorf tube. LoBind prevents reagents from binding to plastic. Make sure to have a large enough tube to hold the entire volume of the master mix.

2.4.4.4. When reagents thaw, add the calculated volumes (one by one) to the LoBind Eppendorf tube.

2.4.1.4.4.1. Gently vortex and spin (mini-centrifuge) each reagent before adding.

2.5.4.5. Prepare an 8-tube strip, with tube "1" positioned on the left and tube "8" on the right. Designate a tube for each sample, including positive and negative controls.

2.6.4.6. Dispense 45  $\mu$ L PCR mixture into each tube on the strip.

2.7.4.7. Move to a new hood designated "template hood" for adding sample cDNA into tubes.

2.7.1.4.7.1. Note that nucleic acid material should never be exposed in or near the "clean" hood. This is to avoid possible contamination in the master mix.

2.8.4.8. Add 5  $\mu$ L cDNA template from the samples into the 8-tube strips containing the PCR mixture. Add the 5  $\mu$ L positive template into the positive control tube. Add 5  $\mu$ L molecular grade H<sub>2</sub>O into the negative control tube. The final volume of each tube should be 50  $\mu$ L.

2.8.1.4.8.1. 45  $\mu$ L PCR mixture + 5  $\mu$ L template.

2.9.4.9. Place the cap securely on the 8-tube strip. Label to minimize contamination and confusion.

2.10.4.10. Centrifuge the 8-tube strip for a few seconds using a mini-centrifuge.

2.11.4.11. Place the 8-tube strip in a PCR machine (thermal cycler).

2.12.4.12. Perform the PCR amplification function in the machine (Figure 21). This function typically consists of the following:

2.12.1.4.12.1. Denaturation at 94 °C for 3 minutes.

2.12.2.4.12.2. 30 – 40 cycles of amplification (denaturation at 94 °C for 3 minutes, annealing at 50 – 60 °C, and extension at 72 °C).

2.12.3.4.12.3. Final extension at 72 °C for 7 minutes.

### 3.5. Prepare Agarose Gel

~~3.1.5.1.~~ Weigh 1 g agarose into a 125 mL Erlenmeyer flask.

~~3.2.5.2.~~ Add 100 mL lithium borate (LB) buffer into the flask, and swirl the flask by hand.

~~3.3.5.3.~~ Place the flask in a microwave oven at high power for 1 min.

~~5.4.~~ Remove from the microwave and swirl by hand to make sure all the agarose has melted. If the agarose hasis not completely melted, microwave for another minute.

~~3.4.5.5.~~ Microwave at high power again, this time for 30 s. Remove and swirl. Then, microwave a third time, again for 30 s. Remove the flask and swirl.

~~3.5.5.6.~~ Cool the flask with the agarose mixture ~~cool~~ to 50 °C by rotating under a cold water faucet with the cap tightly secured.

~~3.6.5.7.~~ Add 1 µL ethidium bromide (EtBr) using a designated micropipette for EtBr only.

~~3.6.1.5.7.1.~~ EtBr is carcinogenic, so personal protective equipment (goggles, lab coat, EtBr resistant gloves) must be worn.

~~3.7.5.8.~~ Pour the molten gel into an electrophoresis gel casting tray (**Figure 32**). Make sure that no bubbles are trapped within the agarose.

~~3.9.5.9.~~ Place a comb into the gel and clamp securely (**Figure 43**).

~~3.9.5.10.~~ Wait approximately 20 – 30 min for the gel to solidify.

~~3.10.5.11.~~ Remove the comb carefully; do not cause any tears in the gel. The comb creates wells in the gel to accommodate space for adding samples.

### 4.6. Gel Electrophoresis

~~4.1.6.1.~~ Place the solidified agarose gel into the electrophoresis chamber.

~~4.2.6.2.~~ Add a liquid LB buffer into the chamber until the gel is barely submerged.

~~4.3.6.3.~~ Label a fresh set of microfuge tubes according to samples.

~~4.3.1.6.3.1.~~ Alternative: parafilm surface with a designated spot for each sample (**Figure 54**).

4.4.6.4. Add 10x orange loading dye and samples to create 2X concentration.

4.4.1.6.4.1. 1  $\mu$ L of loading dye for every 4  $\mu$ L of sample.

4.5.6.5. Place 2  $\mu$ L 10X orange loading dye into each tube.

4.6.6.6. Add 8  $\mu$ L DNA product from the 8-tube strip into the designated tubes, and aspirate using the pipette to mix the loading dye and sample.

4.7.6.7. To a 6<sup>th</sup> tube, add 2  $\mu$ L loading dye and 8  $\mu$ L 123 base pair (bp) DNA ladder.

4.8.6.8. Load 10  $\mu$ L from each microfuge tube into the designated wells in the agarose gel (**Figure 65**). Carefully, load the wells with a pipette tip submerged at an angle.

4.9.6.9. Connect the electrodes to the electrophoresis chamber and turn on the power source for 20 – 25 min. DNA is negatively charged, so it “runs” toward the positive electrode. Therefore, connect the positive electrode to the opposite side of the chamber, where the wells were loaded.

4.9.1.6.9.1. 225 V for the small chamber or 275 V for the large chamber) for 20 – 30 min. Small bubbles should be visualized moving up the sides of the chamber.

## 5.7. Visualizing Gel

5.1.7.1. Carefully transport the gel into the transilluminator or visual imager and turn on the UV light to visualize the DNA bands on the gel.

5.2.7.2. Analyze the band size and the positions on the gel. Compare the band positions of the samples to the positive control to determine if the organism template DNA is present.

5.2.1.7.2.1. Stronger/larger bands indicate more DNA product at that location.

5.2.2.7.2.2. Large DNA segments are not able to transport through gel easily. Therefore, the bands closer to the wells on the gel contain larger DNA segments.

5.2.3.7.2.3. Small DNA segments are able to travel through the gel quickly. As the bands get farther away from the wells, the DNA segment size decreases.

5.2.4.7.2.4. Samples containing target DNA have similar band segment sizes/positions on the gel as the positive control. This indicates that the organism is present (**Figure 76**).

**Commented [BS2]:** 4.4.1 and 4.4.2 are the most important aspects when dealing with PCR, so as long as these are explained via a voiceover or talent recording this should be good

6.8. Nested (2<sup>nd</sup>) PCR. This procedure involves performing PCR again, using the 1<sup>st</sup> PCR product and more specific primers to amplify the DNA for more precise genetic analysis.

### Applications:

PCR is a molecular technique that allows researchers to detect, identify, amplify, and analyze microorganisms in the environment. Specific primers multiply target sequences so that genetic information can be studied in greater detail. In the environment, genetic information is difficult to obtain and diluted within heterogeneous mixtures. PCR can isolate and multiply target DNA so it is not disrupted and no longer mitigated.

PCR can be employed independently to quickly determine the presence or absence of pathogens or harmful genetic material in the environment (**Figure 87**). For example, sequences specific to the brain-eating amoeba, *Naegleria fowleri*, will amplify DNA and exhibit strong bands on a gel if the organism is present in a sample, or will not amplify and not produce a band on the gel if the organism is absent. This allows scientists to determine if a pathogen associated with human health risks are present in the environment. If a single organism is not the main interest, but rather genes associated with toxins, PCR can determine the presence or absence of specific genetic material linked to toxins.

Within research, PCR can be operated as a confirmation procedure. If a culture method cannot differentiate which organisms are present from an environmental sample, then PCR can distinguish which organism is specifically associated with the results. Also, PCR can provide a results balance, as it can confirm trends or patterns noticed throughout samples processed using other techniques.

As science and technology progress, PCR is harmonized with more advanced techniques. PCR is no longer limited to double-stranded DNA, but can also analyze single-stranded RNA or DNA with the implantation of reverse transcription PCR (RT-PCR). Genetic material is no longer limited to just presence-absence, as quantitative PCR (qPCR) enumerates the concentration for cDNA present within a sample. Most recently, the advancement in sequencing technologies, allows PCR products to be analyzed one dNTP at a time to encrypt the exact arrangements of all genetic material present in an environmental sample. This information allows for more in-depth analysis on microbial populations, relationships, and interactions.

### Legend:

Figure 1: The PCR cycle. Denaturation (Step 1), annealing (Step 2), and extension (Step 3).

Figure 12: 8-tube strip containing amplified PCR product.

Figure 23: Pouring the molten gel into the casting tray.

Figure 34: Gently placing the comb into position.

Figure 45: Preparing the dye and sample mixture on parafilm surface.

**Commented [JR3]:** It looks like there was a whole subset of steps for Nested 2<sup>nd</sup> PCR that Bradley deleted. I assume he meant to leave this step in, so I've left it as is.

(Table 2 was in this section, which is why it has been dropped.)

**Commented [JR4]:** Figure 1 and Table 2 were dropped. Since Figure 1 was deleted, all the other figures needed to be renumbered. I've attached the files with the new, correct numbers.

Figure 56: Loading mixture into the wells on the gel.

Figure 67: Visualizing band segments on agarose gel following electrophoresis. The DNA ladder (1) provides sample banding to analyze the sizing and approximate concentration for DNA strands within samples. Negative control (2) does not contain any genetic material. Positive control (3) spiked with target DNA to indicate size and location of bands for sample comparison. Samples 4, 6, 8, and 9 exhibit similar band segmentation as positive control, therefore indicating that these samples contain target genetic material. It can be inferred that the organism is present in the environments from these samples.

Figure 78: A young scientist preparing samples for DNA amplification by PCR.

Table 1: PCR Mixture Calculation.

**References:**

Pepper, Ian L., and Charles P. Gerba. "Contact Slide Assay." *Environmental Microbiology A Laboratory Manual*. 2nd ed. N.p.: Elsevier, 2004. 19-25. Print.

Pepper, Ian L., Charles P. Gerba, and Terry J. Gentry. "Earth Environments." *Environmental Microbiology*. 3rd ed. N.p.: Elsevier, 2014. 59-88. Print.



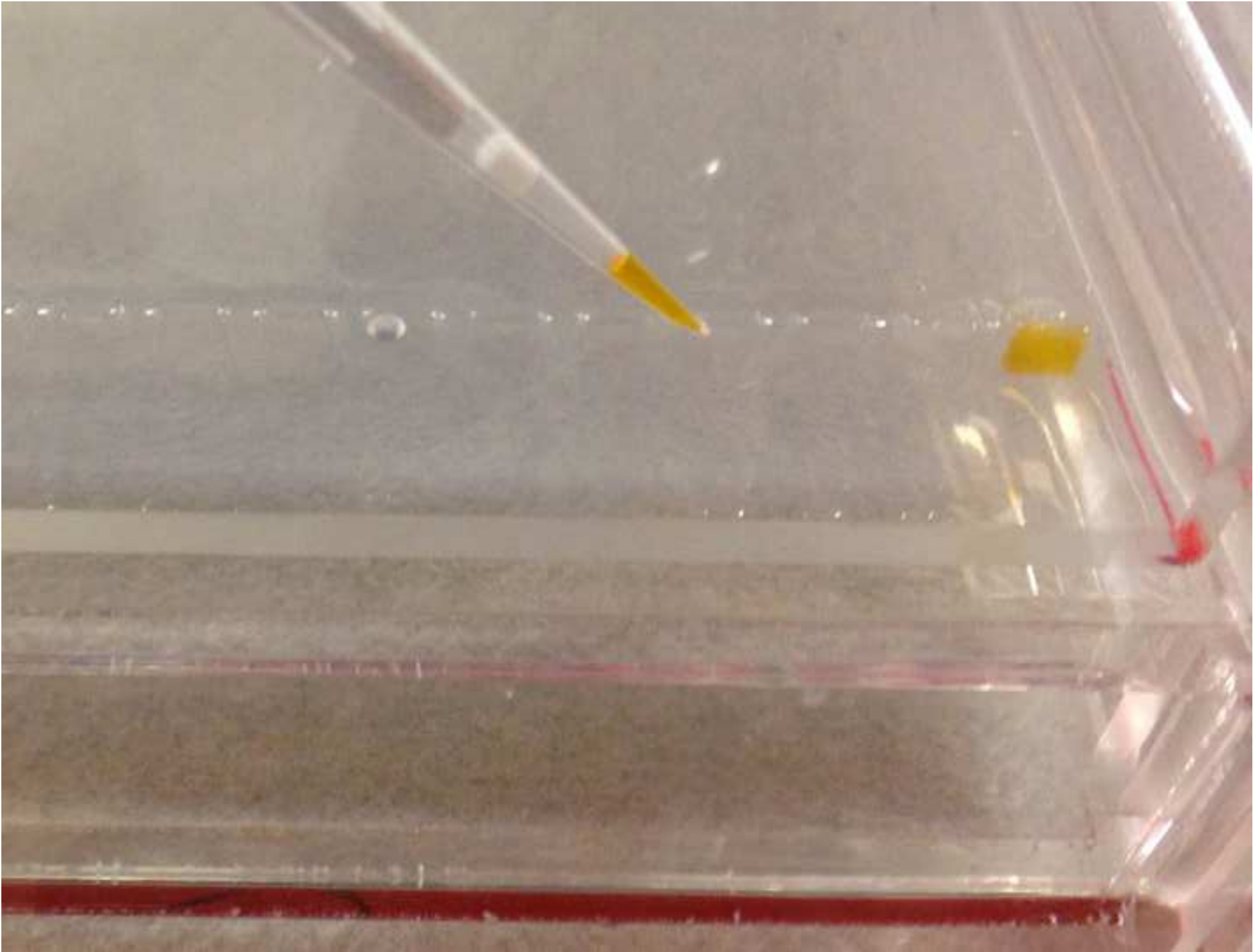


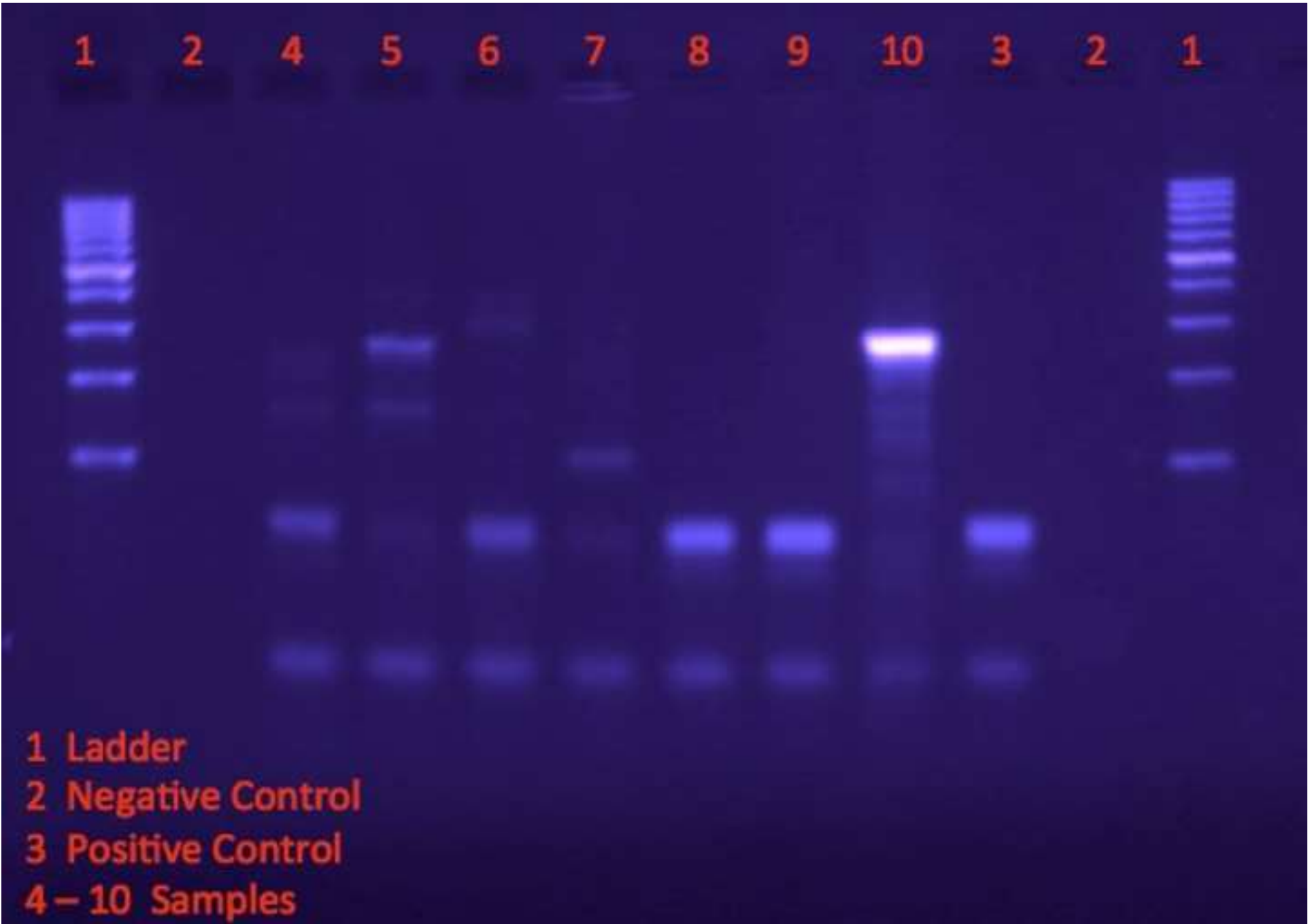














Component	Volume per Tube (μL)	Volume for 5 tubes (μL)	Final Concentration
10x Ex <i>Taq</i> buffer	5.0	25	1x
2.5 mM dNTPs	4.0	20	0.2 mM
Forward Primer*	2.0	10	400 nM
Reverse Primer*	2.0	10	400 nM
Molecular H <sub>2</sub> O	31.75	158.75	-
Ex <i>Taq</i>	0.25	1.25	2.5 U
PCR Mixture	45	225	

\*Primer volumes vary depending on organism assay. Adjust the volume of molecular grade water to make the final volume 45 μL. Volumes of other components should not vary.