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Amplifying RNA-Based Environmental Microorganisms via Reverse Transcriptase Polymerase Chain Reaction

--Manuscript Draft--

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Environmental Science Education Title: Amplifying RNA-Based Environmental Microorganisms via Reverse Transcriptase Polymerase Chain Reaction

Overview:

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) involves the same process as conventional PCR, cycling temperature gradients to amplify nucleic acids. However, conventional PCR amplifies deoxyribonucleic acids (DNA), whereas RT-PCR exponentially increases the amount of ribonucleic acids (RNA) through the formation of complementary DNA (cDNA). This transforms single-stranded nucleic acids into double-stranded structures, enabling RNA-based organisms to be analyzed utilizing methods and technologies designed for DNA.

Conventional, nested, and quantitative PCR (qPCR) methodologies rely on double-stranded nucleic acid templates, therefore, inhibiting their capabilities to amplify RNA present in the environment. However, RT-PCR provides a means for single-stranded RNA and mRNA structures to be converted by specialized enzymes, known as reverse transcriptase, that reverse transcribe RNA into cDNA (**Figure 1**). After the double-stranded configuration is amplified, the nucleic acid can be implemented as the starting template into further downstream assays.

Many viruses house RNA as their genomic material, requiring RT-PCR as the pretreatment before further molecular assays. Several RNA-based viral pathogens, such as *Norovirus*, and indicator organisms, such as Pepper Mild Mottle Virus (PMMoV), do not have culture-based detection methods for quantification. Therefore, assays for these viruses rely on RT-PCR as a precursor to PCR or qPCR for their detection and enumeration in the environment. Without RT-PCR, microbiologists would not be able to assay and research numerous RNA-based viruses that pose risks to human and environmental health.

RT-PCR can also be employed as a tool to measure microbial activity, as messenger RNA (mRNA) is the single-stranded template for protein translation. Whole community activity can be analyzed by using a fusion primer during RT-PCR, which generates cDNA from mRNA that can be used in further assays to determine the gene expressions within an environmental sample. This indicates which genes and microbes are synthesizing into proteins within the environment in order to perform functions necessary for metabolic pathways and survival.

Reverse transcription can be controlled to amplify desired nucleic acid products. Random primers are initiation sequences incorporated into RT-PCR to start amplification of most RNA present. These primers are able to attach to nucleic sequences that are broadly found throughout many types of microbes. This enables the reverse transcriptase enzyme to polymerize the genetic material for numerous microbes, so the sample can be analyzed for multiple organisms and/or their relative abundance in the environment.

However, specific primers initiate cDNA amplification for precise sequences found in only one or a few microbes. This allows singular analysis without interfering nucleic acids.

Procedure:

1. Sample Collection.

    1.1. Soil Sample.

        1.1.1. Collect soil samples from multiple depths of a soil profile using an auger or shovel.

        1.1.2. Sieve soil through 2 mm to remove gravel and rock.

    1.2. Water Sample.

        1.2.1. Collect 1 L of water in Nalgene bottle.

2. Nucleic Acid Extraction.

    2.1. Extract RNA from viruses, as capsids are broken, which allows nucleic acid to be accessed.

        2.1.1. Obtain a commercial extraction kit.

            2.1.1.1. Soil – MoBio Power Soil RNA Isolation Kit.

            2.1.1.2. Water – MoBio Power Water RNA Isolation Kit or Zymo ZR Viral DNA/RNA Extraction Kit.

    2.2. Extract mRNA from eukaryotes and prokaryotes via cell lysis.

        2.2.1. Grow colonies on a Petri dish containing proper substrate for desired microbe growth.

        2.2.2. Add colony to sterile tube containing 500 μL molecular grade water.

        2.2.3. Vortex to suspend pellet.

        2.2.4. Add locking cap to tube and boil for 10 min using a hot plate.

        2.2.5. Vortex for 1-2 min to shear DNA.

        2.2.6. Centrifuge for 5 min at max speed to pellet cell debris.

        2.2.7. Transfer the top 100 μL of supernatant to a clean tube.

2.2.8. Dilute DNA to 1:10 and 1:100.

3. Reverse Transcriptase Polymerase Chain Reaction.

3.1. Thaw reagents inside a "clean" hood at room temperature (stored at -20 ^{o}C).

3.1.1. 25x dNTP, 10x Reverse Transcription Buffer, 10x Random Primer, Multiscribe Reverse Transcriptase (50 U/μL), and Rnase Inhibitor.

3.2. Calculate the final cDNA volume needed to be obtained from each sample.

3.2.1. C = cDNA volume needed per sample.

3.2.2. 2 = duplicates for each organism being analyzed.

3.2.3. n = number of organism species, plus one extra, to be analyzed.

3.2.4. r = volume of cDNA per reaction utilized during future analysis.

3.2.5. Add an additional 10% and round up to the nearest ten.

$$C = 2\ n\ r$$

Example: 4 RNA-based viruses need to be amplified into cDNA for future qPCR analysis that utilizes 2.5 μL per reaction.

cDNA per sample = 2 duplicates x 5 virus types x 2.5 μL per reaction = 25 μL

25 μL + 2.5 μL (10%) = 27.5 μL ≈ 30 μL cDNA per sample

3.3. Calculate the master mix volume that needs to be created.

3.3.1. Split the previously calculated volume cDNA in half to create 1:1 ratio of master mix to sample extract.

3.3.2. e = number of sample extracts to be processed + one positive control.

3.3.3. m = master mix volume needed for each sample.

3.3.4. M = total master mix volume needed.

3.3.5. Add an additional 10% and round up to the nearest ten.

cDNA per sample = 30 μL = 15 : 15 ratio

15 µL master mix : 15 µL sample extract

$$m = 15\ \mu L$$

Example: There are 10 sample extracts to be processed for future qPCR analysis.

$$M = e\ m$$

Master Mix volume = 11 extracts x 15 µL master mix per sample = 165 µL

165 µL + 17 µL (10%) = 182 µL ≈ 185 µL Total Master Mix needed

3.4. Calculate the factor by which reagents are to be multiplied by to create the master mix volume needed.

3.4.1. M = total master mix volume needed.

3.4.2. v = total component volume of master mix for one reaction.

3.4.2.1. One reaction requires 10 µL master mix.

3.4.3. x = factor to multiple reagents by.

$$M = v\ x$$

$$185\ \mu L = 10\ x$$

$$x = 18.5\ \mu L$$

3.5. Calculate the volume of each reagent needed to create the total master mix volume required for the cDNA amplification of all samples. Multiply the reagent volume needed in one reaction by the factor calculated from the previous step (**Table 1**).

3.6. Prepare 1.5 ml LoBind Eppendorf tube. LoBind prevents binding to plastic.

3.7. When reagents thaw, add calculated volumes to LoBind Eppendorf tube.

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

3.7.2. Change pipette tip between adding each reagent to prevent contamination and to ensure precision when adding volumes.

3.7.3. Put reagents back into storage (-20 ^{o}C) after adding into master mix.

3.8. After all reagents have been added into the 1.5 ml Eppendorf tube, vortex and spin to create a heterogenous mixture.

3.9. Prepare 8-tube strip, with tube "1" positioned on the left and tube "8" on the right.

3.9.1. If preparing more than 8 samples/tubes, multiple strips are needed.

3.9.2. Designate a tube for each sample, including negative controls with only H_2O.

3.10. Aliquot calculated amount of master mix required into each tube on the strip.

3.10.1. Amount to be added was calculated in Step 3.4.3.

cDNA per sample = 30 µL
15 µL master mix : 15 µL sample extract

Aliquot 15 µL master mix into each tube.

3.11. Thaw extracts and add calculated amount into each sample's designated tube.

cDNA per sample = 30 µL
15 µL master mix : 15 µL sample extract

Aliquote 15 µL extract into each tube.

3.12. Cap strip securely on 8-tube strip, and mini-centrifuge to ensure all liquid is collected at the bottom of each tube (**Figure 2**).

3.13. Place 8-tube strip securely in thermal cycler. Press down to ensure tubes are secured.

3.14. Run thermal cycler for High Capacity Reverse Transcripase (**Table 2**).

3.15. When thermal cycler is finished, the tubes contain cDNA product.

3.16. Analyze product via gel electrophoresis to ensure cDNA was created.

3.17. Store cDNA at -20 ^{o}C, until it is later processed via PCR or qPCR.

Applications:

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) is necessary to create double-stranded complementary DNA (cDNA) from a single-stranded RNA template. This enables RNA-based microorganisms to be analyzed utilizing assays that require double-stranded templates. Once the double-stranded product is created, PCR assays can determine the

presence or absence of RNA-based microorganisms within an environmental sample. This enables further downstream analysis to determine microbial ecology, health risks, and environmental risks.

RT-PCR can be utilized to amplify mRNA as a means to observe which genes are being expressed abundantly in an environment. This provides information on which protein and functions microbes rely on to survive in the environment. Gene expression can highlight microbial pathways that breakdown contaminants, such as hydrocarbons or chlorinated solvents, and these microbes can be implemented for bioremediation.

Risk assessment incorporates RT-PCR in order to analyze human and environmental health risks. Many viral pathogens' morphology simply contains RNA nucleic acids within a protein capsid. Therefore, most assays are not able to analyze these viruses. However, RT-PCR provides a means for further downstream assays to be employed to determine risk exposure. Combining RT-PCR with qPCR allows RNA viruses to be enumerated within samples, so that human and environmental exposure can be calculated for the overall Quantitative Microbial Risk Assessment (QMRA).

Legend:

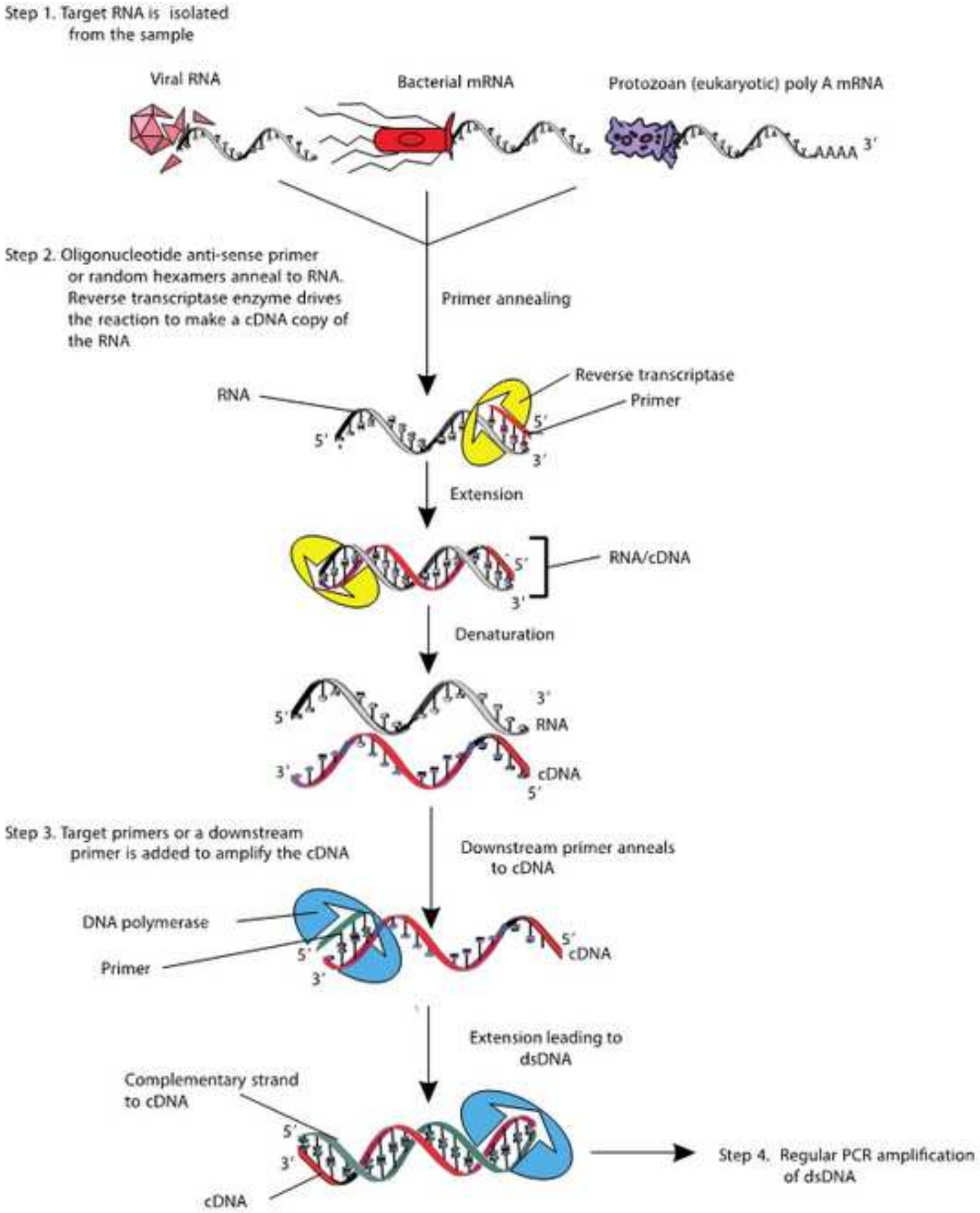
Figure 1: Step-by-step process of RNA transcribing to cDNA.

Figure 2: Capped 8-tube strip containing master mix and extract.

Table 1: RT - PCR Master Mix Ingredients.

Table 2: RT - PCR Reaction Temperature Profile.

Click here to download Photo or Graphic File: Pepper_RT_PCR_Figure 1.jpg



Click here to download Photo or Graphic File: Pepper_RT_PCR_Figure 2.jpg

RT – PCR Master Mix Ingredients

Reagent	Volume per 1 reaction (µL)	"X" factor	Volume added into Master Mix (µL)
10x RT Buffer	2.0	18.5	37
25x dNTPs	0.8	18.5	14.8
10x Random Primer	2.0	18.5	37
Multiscribe	1.0	18.5	18.5
Rnase Inhibitor	1.0	18.5	18.5
Molecular Grade H_2O	3.2	18.5	59.2
Total Volume	10		185

Click here to download Photo or Graphic File: Pepper_RT_PCR_table2.docx

Step 1	Step 2	Step 3	Step 4
25 ^{O}C , 10 minutes	37 ^{O}C , 120 minutes	85 ^{O}C , 5 minutes	4 ^{O}C , ∞

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~~———~~PCR methodologies rely on double--stranded nucleic acid templates~~,s, therefore,~~ inhibiting their capabilities to amplify RNA from organisms present in the environment. However, RT-PCR provides a means for single--stranded RNA to be converted into cDNA by specialized enzymes, known as reverse transcriptase. After the double--stranded configuration is amplified, environmental samples can be analyzed ~~using~~ by implementing the new cDNA as the starting template for further downstream assays.

Many viruses found throughout the environment house RNA as their genomic material, requiring RT-PCR as the pretreatment before further molecular assays. Several RNA--based viral pathogens, such as *Norovirus*, and indicator organisms, such as Pepper Mild Mottle Virus (PMMoV), do not have culture-based detection methods for quantification. In order to determine the presence and influences of RNA viruses across soil, water, agriculture, etc., molecular assays rely on RT-PCR as a precursor methodology. Without RT-PCR, microbiologists would not be able to assay and research numerous RNA--based viruses that pose risks to human and environmental health.

RT-PCR can also be employed as a tool to measure microbial activity in the environment, as messenger RNA (mRNA) is the single--stranded template for protein translation and gene expression. This indicates which genes and microbes are synthesizing into proteins within ~~in~~ the environment in order to perform functions necessary for metabolic pathways and survival. Analyzing gene expression gives clues to~~on~~ what functional characteristics prokaryotes and eukaryotes are using in order to survive within environmental conditions. Community gene expression can give an overall picture of the genes organisms need to adapt to variable conditions. In some cases, gene expression can be utilized to determine which organisms may survive best in harsh conditions and have capabilities for bioremediation in contaminated soil or water.

Reverse transcription can be controlled to amplify only desired products or an entire community of~~f~~ nucleic acids found within an environmental sample. This is important, as soil and water samples are often saturated with various nucleic acids that aren'~~ not~~ desired

Comment [AW1]: This is a good primer to RT-PCR, but it needs to be more explicit about the connection to environmental science.

Comment [BS2]: I really like this sentence as it ties the purpose together, however, if we need to create space, this is the sentence that could be deleted

for specific environmental analyses. Random primers are initiation sequences incorporated into RT-PCR to start amplification of most RNA present. These primers are able to attach to nucleic sequences ~~that are~~ broadly found throughout many types of microbes. This enables the reverse transcriptase enzyme to polymerize the genetic material for numerous microbes, so ~~that~~ the sample can be analyzed for multiple organisms and/or their relative abundance in the environment. However, specific primers initiate cDNA amplification for precise sequences found in only one organism -without interfering nucleic acids. This allows an environmental sample to be tested for a predetermined purpose, such as determining whether *Norovirus*, which can cause gastrointestinal illnesses to a human population, is present in water ~~that is causing gastrointestinal illnesses to a human population~~.

~~Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) involves the same process as conventional PCR, cycling temperature gradients to amplify nucleic acids. However, conventional PCR amplifies deoxyribonucleic acids (DNA), whereas RT-PCR exponentially increases the amount of ribonucleic acids (RNA) through the formation of complementary DNA (cDNA). This transforms single-stranded nucleic acids into double-stranded structures, enabling RNA-based organisms to be analyzed utilizing methods and technologies designed for DNA.~~

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~~throughout many types of microbes. This enables the reverse transcriptase enzyme to polymerize the genetic material for numerous microbes, so the sample can be analyzed for multiple organisms and/or their relative abundance in the environment.~~

~~However, specific primers initiate cDNA amplification for precise sequences found in only one or a few microbes. This allows singular analysis without interfering nucleic acids.~~

Procedure:

1. Sample Collection.

1.1. Collect a s~~S~~oil s~~S~~ample.

1.1.1. Find a ~~S~~sample location ~~is found~~ via GPS, coordinates, or sight.

1.1.2. Push and twist a ~~H~~hand auger ~~is pushed and twisted~~ into the ground soil to a predetermined depth.

1.1.3. Lift the auger. The~~Auger is lifted and~~ soil is found within the hollow stem of the auger.

1.1.4. Scrape the ~~S~~soil at the bottom of the auger ~~is hit/scraped i~~into a soil collection bag.

1.1.5. Be sure not to touch or contaminate the soil.

~~1.1.6.~~ Label the ~~B~~bag ~~is labeled~~ properly with location, name, date, and time.
1.1.6. Include any other necessary labels.

1.1.7. Transfer the ~~S~~soil ~~is transferred~~ to the laboratory.

1.1.8. Sieve the soil~~Soil is sieved~~ through 2 mm to remove gravel and rock.

1.1.9. Analyze a ~~P~~portion of the soil ~~is analyzed~~ for soil moisture content.

1.2. Collect a w~~W~~ater s~~S~~ample.

1.2.1. Find the ~~S~~sample location ~~is found~~ via GPS, coordinates, or sight.

1.2.2. Collect the ~~W~~water ~~is collected~~ in a Nalgene bottle.

1.2.3. Make sure to have the correct bottle size depending on the volume needed (either 250 ml or 1 L).~~Various bottle sizes depending on volume needed.~~
~~250 mL or 1 L.~~

Comment [BS3]: Refer/Link to other JoVE manuscript on soil moisture

Comment [JR4]: May be unnecessary.

~~1.~~ Immediately test the ~~W~~water ~~immediately tested~~ for any parameters needed/required for the experiment (temperature, pH, conductivity, salinity, nitrogen, phosphorous) using probe(s).

1.2.4. ~~Temperature, pH, conductivity, salinity, nitrogen, phosphorous.~~

1.2.5. Place the ~~B~~bottle containing the water ~~is placed~~ in a cooler with ice.

1.2.6. ~~Cooler t~~Transfer the cooler~~red~~ to the laboratory.

~~1.1. Soil Sample~~

~~2.~~

~~2.1.1. Collect soil samples from multiple depths of a soil profile using an auger or shovel.~~

~~3.~~

~~3.1.1. Sieve soil through 2 mm to remove gravel and rock.~~

~~4.~~

~~4.1. Water Sample~~

~~5.~~

~~6.~~2. Nucleic Acid Extraction

~~6.1.~~2.1. Extract RNA from viruses, as capsids are broken, which allows nucleic acid to be accessed.

~~6.1.1.~~2.1.1. Obtain a commercial extraction kit.

~~6.1.1.1.~~2.1.1.1. Soil – MoBio Power Soil RNA Isolation Kit.

~~6.1.1.2.~~2.1.1.2. Water – MoBio Power Water RNA Isolation Kit or Zymo ZR Viral DNA/RNA Extraction Kit.

~~6.2.~~2.2. Extract mRNA from eukaryotes and prokaryotes via cell lysis.

~~6.2.1.~~2.2.1. Grow colonies on a Petri dish containing proper substrate for desired microbe growth.

~~6.2.2.~~2.2.2. Add colony to sterile tube containing 500 µl~~L~~ molecular grade water.

~~6.2.3.~~2.2.3. Vortex to suspend pellet.

~~6.2.4.~~2.2.4. Add locking cap to tube and boil for 10 min using a hot plate.

~~6.2.5.~~2.2.5. Vortex for 1-2 min to shear DNA.

~~6.2.6.~~2.2.6. Centrifuge for 5 min at max speed to pellet cell debris.

~~6.2.7.~~2.2.7. Transfer the top 100 µl~~L~~ of supernatant to a clean tube.

Comment [JR5]: For Section 2, the author(s) planned on writing a detailed manuscript that addresses the extraction process. Should they still expand upon this section here? Or will there be too much overlap?

We could always insert a reference to the other video, if that solves the problem.

Comment [AW6]: These are the types of steps we'd like to thoroughly demonstrate, as an environmental video. Even if the kits come with instructions on use, we need to be able to script and shotlist the process. Please flush this out.

Comment [AW7]: What organisms will you be demonstrating? Bacteria, yeast?

6.2.8.2.2.8. Dilute DNA to 1:10 and 1:100.

7.3. Reverse Transcriptase Polymerase Chain Reaction.

7.1.3.1. Thaw reagents inside a "clean" hood at room temperature (stored at -20 °C).

7.1.1.3.1.1. 25x dNTP, 10x Reverse Transcription Buffer, 10x Random Primer, Multiscribe Reverse Transcriptase (50 U/µl), and Rnase Inhibitor.

7.2.3.2. Calculate the final cDNA volume needed to be obtained from each sample.

7.2.1.3.2.1. C = cDNA volume needed per sample.

7.2.2.3.2.2. 2 = duplicates for each organism being analyzed.

7.2.3.3.2.3. n = number of organism species, plus one extra, to be analyzed.

7.2.4.3.2.4. r = volume of cDNA per reaction utilized during future analysis.

7.2.5.3.2.5. Add an additional 10% and round up to the nearest ten.

$$C = 2\ n\ r$$

Example: 4 RNA-based viruses need to be amplified into cDNA for future qPCR analysis that utilizes 2.5 µl per reaction.

cDNA per sample = 2 duplicates x 5 virus types x 2.5 µl per reaction = 25 µl

25 µl + 2.5 µl (10%) = 27.5 µl ≈ 30 µl cDNA per sample

7.3.3.3. Calculate the master mix volume that needs to be created.

7.3.1.3.3.1. Split the previously calculated volume cDNA in half to create 1:1 ratio of master mix to sample extract.

7.3.2.3.3.2. e = number of sample extracts to be processed + one positive control.

7.3.3.3.3.3. m = master mix volume needed for each sample.

7.3.4.3.3.4. M = total master mix volume needed.

7.3.5.3.3.5. Add an additional 10% and round up to the nearest ten.

cDNA per sample = 30 µl = 15 : 15 ratio

15 µl master mix : 15 µl sample extract

m = 15 µl

Example: There are 10 sample extracts to be processed for future qPCR analysis.

M = e m

Master Mix volume = 11 extracts x 15 µl master mix per sample = 165 µl

165 µl + 17 µl (10%) = 182 µl ≈ 185 µl Total Master Mix needed

7.4.3.4. Calculate the factor by which reagents are to be multiplied by to create the master mix volume needed.

7.4.1.3.4.1. M = total master mix volume needed.

7.4.2.3.4.2. v = total component volume of master mix for one reaction.

7.4.2.1.3.4.2.1. One reaction requires 10 µl master mix.

7.4.3.3.4.3. x = factor to multiple reagents by.

M = v x

185 µl = 10 x

x = 18.5 µl

7.5.3.5. Calculate the volume of each reagent needed to create the total master mix volume required for the cDNA amplification of all samples. Multiply the reagent volume needed in one reaction by the factor calculated from the previous step (**Table 1**).

7.6.3.6. Prepare 1.5 ml LoBind Eppendorf tube. LoBind prevents binding to plastic.

7.7.3.7. When reagents thaw, add calculated volumes to LoBind Eppendorf tube.

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

7.7.2.3.7.2. Change pipette tip between adding each reagent to prevent contamination and to ensure precision when adding volumes.

7.7.3.3.7.3. Put reagents back into storage (-20 °C) after adding into master mix.

~~7.8.~~3.8. After all reagents have been added into the 1.5 ml Eppendorf tube, vortex and spin to create a heterogenous mixture.

~~7.9.~~3.9. Prepare 8-tube strip, with tube "1" positioned on the left and tube "8" on the right.

~~7.9.1.~~3.9.1. If preparing more than 8 samples/tubes, multiple strips are needed.

~~7.9.2.~~3.9.2. Designate a tube for each sample, including negative controls with only H_2O.

~~7.10.~~3.10. Aliquot calculated amount of master mix required into each tube on the strip.

~~7.10.1.~~3.10.1. Amount to be added was calculated in Step 3.4.3.

cDNA per sample = 30 µl~~L~~
15 µl~~L~~ master mix : 15 µl~~L~~ sample extract

Aliquot 15 µl~~L~~ master mix into each tube.

~~7.11.~~3.11. Thaw extracts and add calculated amount into each sample's designated tube.

cDNA per sample = 30 µl~~L~~
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Aliquot~~e~~ 15 µl~~L~~ extract into each tube.

~~7.12.~~3.12. Cap strip securely on 8-tube strip, and mini-centrifuge to ensure all liquid is collected at the bottom of each tube (**Figure 2**).

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~~7.14.~~3.14. Run thermal cycler for High Capacity Reverse Transcripase (**Table 2**).

~~7.15.~~3.15. When thermal cycler is finished, the tubes contain cDNA product.

~~7.16.~~3.16. Analyze product via gel electrophoresis to ensure cDNA was created.

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Applications:

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Legend:

Figure 1: Step-by-step process of RNA transcribing to cDNA.

Figure 2: Capped 8-tube strip containing master mix and extract.

Table 1: RT - PCR Master Mix Ingredients.

Table 2: RT - PCR Reaction Temperature Profile.