Lab Work Organization

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Sample prep, RNA extraction, RT-qPCR, cDNA conversion, library prep, ONT sequencing, etc.

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1 Troubleshooting: Sample Preparation

- Extract all trizol samples according to manufacturers protocol
- Add glycogen (0.5µl) for the samples after the sucrose cushion (samples 7-11).
- Determine viral RNA content through RT-qPCR.

Step	Mechanistic Details	Comments for Troubleshooting
1. Get 50 mosquitoes from insectary.	1. Obtain the necessary number of mosquitoes for the experiment.	1. Ensure mosquitoes are healthy and of the correct species/strain.
2. Make 5 pools each consisting of 10 mosquitoes in 2ml tubes containing steel balls.	2. Pooling increases sample size and reduces variability.	2. Avoid cross-contamination between pools.
3. Make 3 Medium aliquots containing 2ml medium each.	3. Medium is used as a negative control.	3. Prepare medium under sterile conditions.
4. Add 10^4/ml particles to one 2ml aliquot -> mix.	4. Create a spike-in sample with a known concentration of particles.	4. Verify particle concentration and mix thoroughly.
5. Add 10^6/ml particles to the second 2ml aliquot -> mix.	5. Create a spike-in sample with a higher concentration of particles.	5. Verify particle concentration and mix thoroughly.

Step	Mechanistic Details	Comments for Troubleshooting
6. Add 750µl of the 10^4 solution to two pools each and 250µl of the 10^4 solution to Trizol LS (Sample 1).	6. Spike-in controls help assess extraction efficiency.	6. Pipette accurately to ensure proper spike-in concentrations.
7. Add 750µl of the 10^6 solution to two pools each and 250µl of the 10^6 solution to Trizol LS (Sample 2).	7. Spike-in controls help assess extraction efficiency.	7. Pipette accurately to ensure proper spike-in concentrations.
8. Add 750µl of the control medium to the last mosquito pool and 250µl of the medium to Trizol LS (Sample 3).	8. The control medium sample serves as a baseline for comparison.	8. Ensure control medium is sterile and particle-free.
9. Store Trizol LS samples @ -80°C.	9. Trizol LS preserves RNA/DNA integrity.	9. Maintain consistent -80°C storage for all samples.
10. Homogenize the pools 2 min @ 30 Hz.	10. Disrupts mosquito tissues and releases nucleic acids.	10. Check homogenizer settings and ensure thorough disruption.
11. Centrifuge all samples for 3 min @ 20000g.	11. Separates cellular debris from the supernatant containing nucleic acids.	11. Use appropriate centrifuge settings and time.
12. Take 250µl of the supernatant of the smaller control and pools and add it to Trizol LS -> -80°C (Samples 4-6).	12. Collects a representative sample for later RNA/DNA extraction.	12. Avoid disturbing the pellet when collecting supernatant.
13. Transfer the remaining supernatant to a fresh tube (for all pools).	13. Removes debris and potential inhibitors from the supernatant.	13. Transfer supernatant carefully to avoid contamination.
14. Centrifuge for 10 min @ 20000g.	14. Further clarifies the supernatant.	14. Check centrifuge settings and time.
14_1. Take out the plunger from a filtering syringe, and put the empty cylinder on top of a new tube.	14_1. Prepare the syringe for filtration.	14_1. Ensure the filter is compatible with the sample type.
14_2. Transfer the sample into the empty cylinder.	14_2. Begin the filtration process.	14_2. Avoid spilling or losing sample during transfer.

Step	Mechanistic Details	Comments for Troubleshooting
14_3. Hold the cylinder firmly, and insert the plunger. Push it to filter the sample through the filter. Some dead volume will not be passed through.	14_3. Complete the filtration step.	14_3. Apply consistent pressure to the plunger for even filtration.
15. Transfer supernatant/filtered volume to ultracentrifuge tubes.	15. Prepare for ultracentrifugation.	15. Use appropriate ultracentrifuge tubes. These tubes are in the ultrcentrifugation room in the closet.
16. Underlay the sample with 20% Sucrose in PBS.	16. Sucrose cushion helps to concentrate particles during ultracentrifugation.	16. Prepare sucrose solution accurately. Find out the heaviest one and add PBS to other tubes to make the same volume. Add 1.2mL PBS first to the sample and then add the 20% sucrose.
17. Pellet particles 1h @ 483 750 g (or higher).	17. Ultracentrifugation pellets particles.	17. Use correct ultracentrifuge settings and time. We need 60WS to get 48375g.
18. Carefully remove supernatant (best by careful decanting).	18. Removes unwanted supernatant.	18. Avoid disturbing the pellet when removing supernatant.
19. Leave the tubes bottoms up for a few minutes to drain any fluid.	19. Ensure all supernatant is removed.	19. Complete drainage before proceeding to the next step.
20. Add 1ml Trizol to the pellets (Samples 7-11) -> store @ -80°C till processing.	20. Trizol preserves RNA/DNA in the pelleted particles.	20. Add Trizol directly to the pellet to prevent degradation.

2 Troubleshooting: RNA Extraction

2.1 Procedural Guidelines

- Perform all steps at room temperature (20–25°C) unless otherwise noted.
- Use cold TRIzol™ Reagent if the starting material contains high levels of RNase, such as spleen or pancreas samples.
- Use disposable, individually wrapped, sterile plastic ware and sterile, disposable RNase-free pipettes & pipette tips, and tubes.
- Wear disposable gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin; change gloves frequently, particularly as the protocol progresses from crude extracts to more purified materials.
- Always use proper microbiological aseptic techniques when working with RNA.
- Use RNaseZap™ RNase Decontamination Solution (Cat. no. AM9780) to remove RNase contamination from work surfaces and non-disposable items such as centrifuges and pipettes used during purification.

2.2 Lyse samples and separate phases

1. Lyse and homogenize samples in TRIzol™ Reagent according to your starting material.

- Tissues:
  Add 1 mL of TRIzol™ Reagent per 50–100 mg of tissue to the sample and homogenize using a homogenizer.
- Cell grown in monolayer:

a. Remove growth media.

b. Add 0.3–0.4 mL of TRIzol™ Reagent per 1×10^5—10^7 cells directly to the culture dish to lyse the cells.

c. Pipet the lysate up and down several times to homogenize.

- Cells grown in suspension:

a. Pellet the cells by centrifugation and discard the supernatant.

b. Add 0.75 mL of TRIzol™ Reagent per 0.25 mL of sample (5–10 × 10^6 cells from animal, plant, or yeasty origin or 1 × 10^7 cells of bacterial origin) to the pellet.
Note: Do not wash cells before addition of TRIzol™ Reagent to avoid mRNA degradation.

c. Pipette the lysate up and down several times to homogenize.
Note: The sample volume should not exceed 10% of the volume of TRIzol™ Reagent used for lysis.

STOPPING POINT Samples can be stored at 4°C overnight or at –20°C for up to a year.

2. **(Optional)** If samples have a high fat content, centrifuge the lysate for 5 minutes at 12,000 g at 4–10°C, then transfer the clear supernatant to a new tube.

Step	Mechanistic Details	Comments for Troubleshooting
3. Incubate for 5 minutes to permit complete dissociation of the nucleoproteins complex.	3. Incubation allows the denaturing agents in TRIzol to disrupt cellular structures and dissolve membranes.	3. If complete dissociation doesn't occur, increase incubation time or use a higher concentration of TRIzol.
4. Add 0.2 mL of chloroform per 1 mL of TRIzol™ Reagent used for lysis, then securely cap the tube.	4. Chloroform further denatures proteins and promotes phase separation.	4. Ensure proper mixing of chloroform and TRIzol. Vortex briefly after adding chloroform.
5. Incubate for 2–3 minutes.	5. This step enhances phase separation and ensures thorough mixing.	5. Check sample temperature to ensure it's close to room temperature.
6. Centrifuge the sample for 15 minutes at 12,000 × g at 4°C. The mixture separates into a lower red phenol-chloroform, and interphase, and a colorless upper aqueous phase.	6. Centrifugation causes the sample to separate into distinct layers based on density.	6. Ensure correct centrifuge speed and time. Incomplete separation may indicate issues with centrifugation.
7. Transfer the aqueous phase containing the RNA to a new tube by angling the tube at 45° and pipette the solution out.	7. The aqueous phase contains RNA, while DNA and proteins are in the interphase and organic phase, respectively.	7. Use a micropipette with a wide-bore tip to avoid shearing RNA. Be careful not to disturb the interphase.

IMPORTANT! Avoid transferring any of the interphase or organic layer into the pipette when removing the aqueous phase.

Proceed directly to "Isolate RNA" on page 2.

Save the interphase and organic phase if you want to isolate DNA or protein. See "Isolate DNA" on page 3 or "Isolate proteins" on page 4 for detailed procedures. The organic phase can be stored at 4°C overnight.

3 Troubleshooting: RT-qPCR

- I will make a shiny app for the calculation online for any number of `#sample`.

N.B. For the NTC, we need everything except the RNA sample (use RNase-free water instead of sample).

- Using `WNV-Ag-ID-Path.prcl` One-Step RT-PCR protocol.

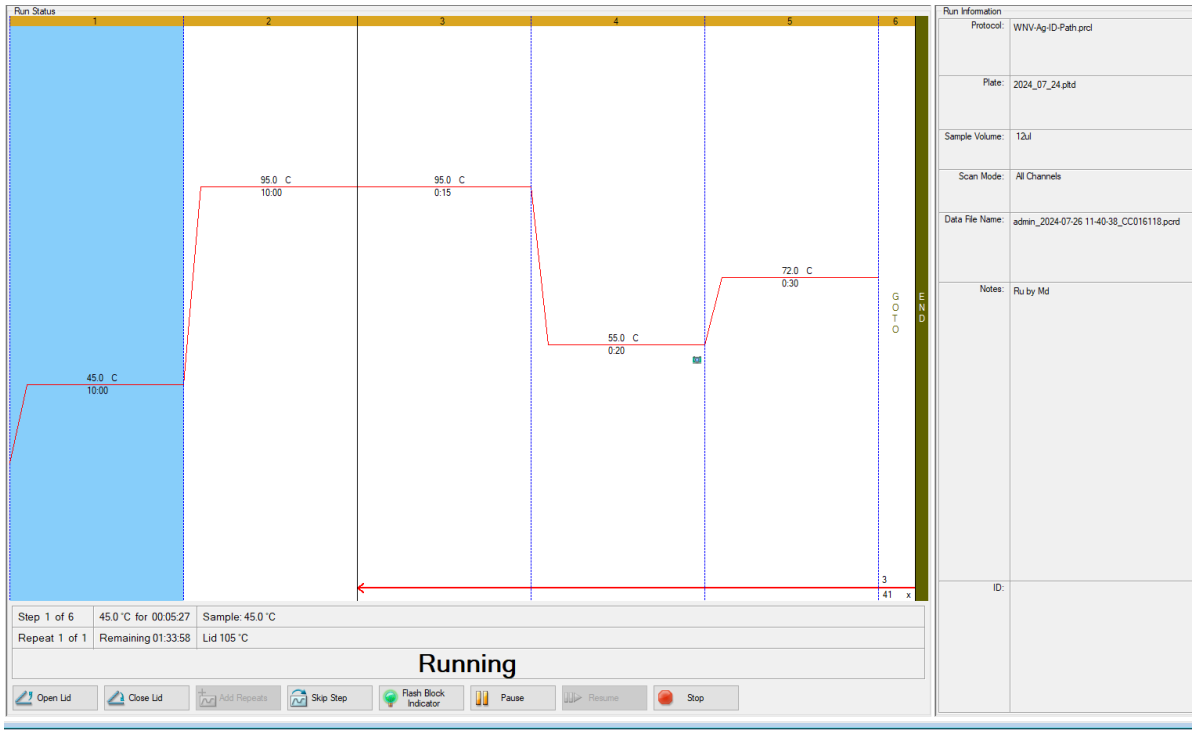

Figure 1: RT-qPCR Run Info

- There are 6 steps in the RT-qPCR. Here goes the mechanistic details of the steps:

Steps	Mechanism
Step 1: Reverse Transcription (RT)	The reverse transcriptase enzyme is active at this temperature, converting the RNA template into complementary DNA (cDNA). This is a crucial step as qPCR amplifies DNA, not RNA.
Step 2: Initial Denaturation and Enzyme Activation	The high temperature serves two purposes: Denaturation: Separates double-stranded RNA/DNA into single strands, making them accessible for primers. Hot-start activation: Activates the hot-start Taq polymerase, which prevents non-specific amplification at lower temperatures.
Step 3: Denaturation	Repeated in each cycle, this step ensures complete separation of newly synthesized DNA strands, allowing primers to bind in the next step.
Step 4: Annealing	The primers, designed to be complementary to specific regions of the target DNA, bind (anneal) to their respective targets. Lowering the temperature facilitates this binding.
Step 5: Extension	The Taq polymerase extends the primers, synthesizing new complementary DNA strands. This results in the doubling of the target DNA in each cycle.
Step 6: Data Acquisition and Analysis	The duration varies (usually a brief step at a temperature between annealing and extension) FAM Fluorescence: The machine measures the fluorescence of the FAM dye, which is typically incorporated into the amplifying DNA (either through a probe or intercalating dye like SYBR Green). The FAM signal increases proportionally with the amount of target DNA. ROX Fluorescence: The ROX dye acts as a passive reference. Its fluorescence should remain constant throughout the run. The machine uses the ROX signal to normalize the FAM signal, correcting for well-to-well variations and potential errors in pipetting.

3.1 Organizational Stuffs

- Reagents are in -20°C fridge (opposite of -80°C fridge).
- Turn on all the switches on the sample hood and rotate the key to turn on. Take the slash up (folded).
- Look if the scissor is there to cut your plate and sealing accordingly.
- Print out the design and keep there to organize the Master Mix (MM) and samples accordingly. Use FAM and ROX for this experiment.
- Keep the enzyme mix always on the cooling block till use.
- Take gloves.
- The cooling blocks change color depending on how hot they are.
- Take 10µL, 20µL, 100µL, 200µL tip boxes if needed. Pipettes are there already inside the hoods.
- Samples on ice.
- Take 1 black box and take ice in it (from downstairs, left hand side while entering). Put the ice boxes in it.
- Take another black box with plate & sealing (touch only with gloves).
- Take 2 Eppendorf tube holders, one for sample and one for H2O, Buffer.
- Touch the RNA sample and reagents only with gloves.
- Keep FAM and Enzyme inside the black box, because it is color sensitive.
- Take the small white box to keep the well plate (with sealing) on the ice box inside it to remove potential contamination issue. Take out when needed after making the MM.
- Clean the MM hood and Sample hood with alcohol first and then start working/putting things inside.
- The centrifuge machine for well plate works this way: Turn on –> Place the plate –> Run for 10-20 secs –> Take out –> Turn off

- Use the first point to pipette off the 11.5 µL MM, don't push too much. A bit will remain in the tip. That's fine. It will be the same for all the wells. No air bubble (aerosol) will be formed this way.

Consumables	Location
Cooling blocks (blue and yellow) for PCR tubes and reagents	Shelf XIV, middle room
RNase-free water	Rack A, Box 3
2x RT-PCR buffer (has things for ROX)	Rack A, Box 3
25x RT-PCR enzyme mix (Rev Transcriptase + Pol)	Rack A, Box 3
FAM mix (Primer + Probe)	Rack B2, Box 2
96-Well-Plate + sealing	Top-right drawer of RNA side
RNA Sample(s)	-80°C fridge (Rack N, Box 4)
A waste bag	You know where
Scissors	Inside MM hood or you take

- No. of sample = depends on your experiment & used in the equation to calculate the total volume of MM.
- Take the waste bag out of MM hood to the sample hood, never the other way around.
- Design the well-plate beforehand using correct fluorophores (FAM/ROX), standard samples, concentration of the standards, unknown and/or negative samples, NTC, biological/technical replicates, etc.
- Prepare the reaction mix following this exact order. **& See the points on precautions below:**

Reagent	1x (µL)	38x (µL)	Mechanism
RNase-free water (100µL aliquot)	3.75	142.5	To make the correct volume
2x RT-PCR buffer (130µL aliquot?)	6.25	237.5	Has buffering activity and ROX for internal control
FAM mix (10µL aliquot)	1.00	38	Has primer pairs and probe for amplification and fluorophore detection, respectively
25x RT-PCR enzyme mix (10µL aliquot)	0.50	19	Has the polymerase for amplification

Reagent	1x (µL)	38x (µL)	Mechanism
Total vol of MM	11.50	437	X
RNA template (20µL)	1.00	x	Inital element/RNA for RT-qPCR
Total vol of reaction mix	12.50	475	X

- Cut the plate and the sealing accordingly using the scissors. Name/date & orientation should be written.
- Turn on air circulation, & white light in the MM hood.
- Vortex everything (MM, samples) before adding (so, prep the MM in a Eppendorf tube).
- **MM handling:** Put the MM in each and every well carefully. Don't release the pipette inside the well or near the plate. Keep it held. Release only on the waste bag and discard. Otherwise, you might take something from the well and make aerosol (contaminant).
- **Sample handling:** Don't press hard (second point), pipette multiple times and take carefully. Then put in the well carefully. Again follow the same procedure of pipetting.
- Put on the sealing nicely; otherwise aerosol will form and disperse.
- Clean, turn on the UV light in th MM hood.
- Clean, turn off the switches in the sample hood. Turn on the UV. Go back after 20-30 mins to turn of the UV (for the sample hood, MM hood is automatic). Turn off using the key.
- Centrifuge the plate in the other lab (Short pulse for 20 secs). **Procedure:** Open lid –> Place on the plate –> Switch on –> Close lid –> Hold the button for "Impuls" for 20 secs –> Take out and turn off
- RT-qPCR running: Open the program –> Click on "open lid" –> place on the well-plate as designed –> Put a balance for even heat circulation (if not using the full plate) –> Click on "close lid" –> Choose the right options.
- Run the RT-qPCR using the right protocol (`WNV-Ag-ID-Path.prcl`) and set the right location for saving the result.
- Open the result using Bio-Rad CFX Maestro and explore!