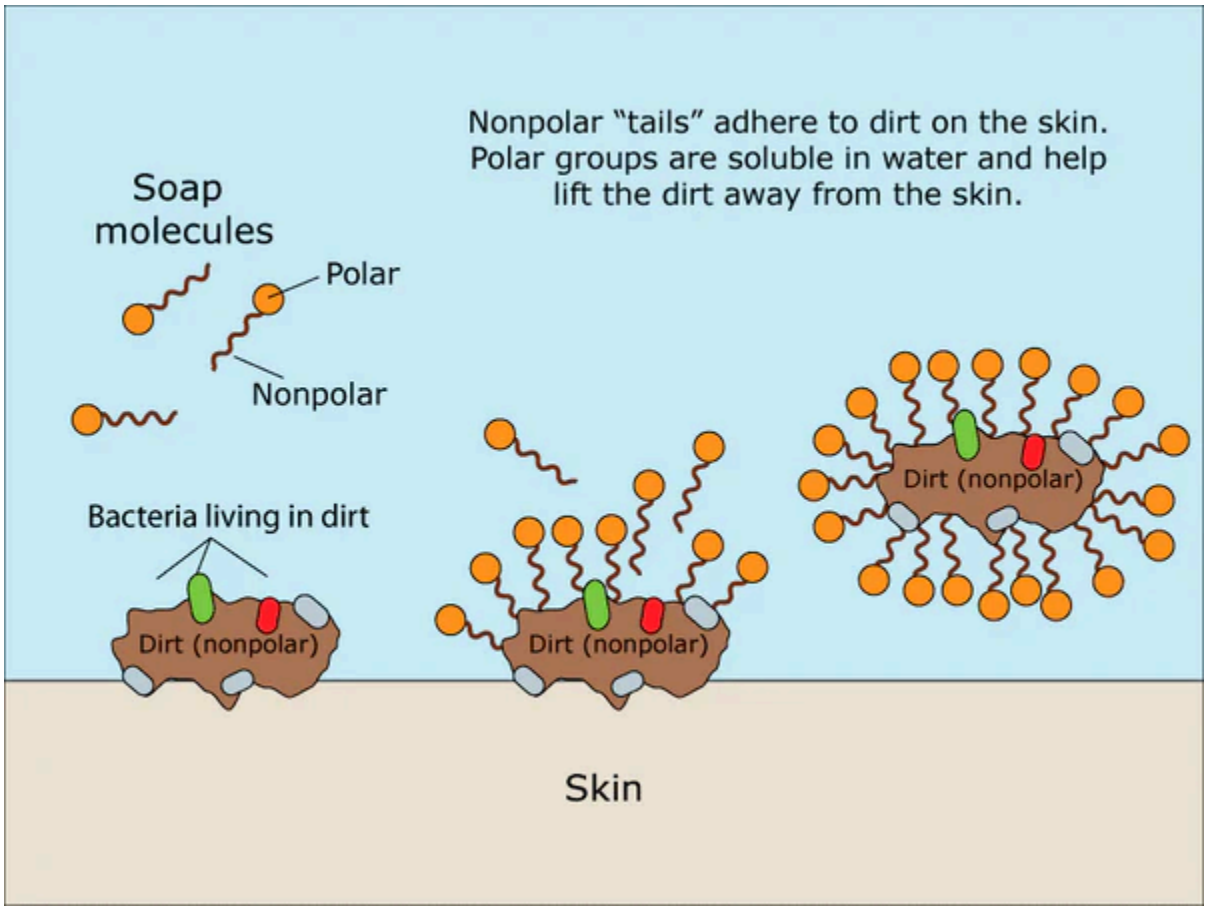
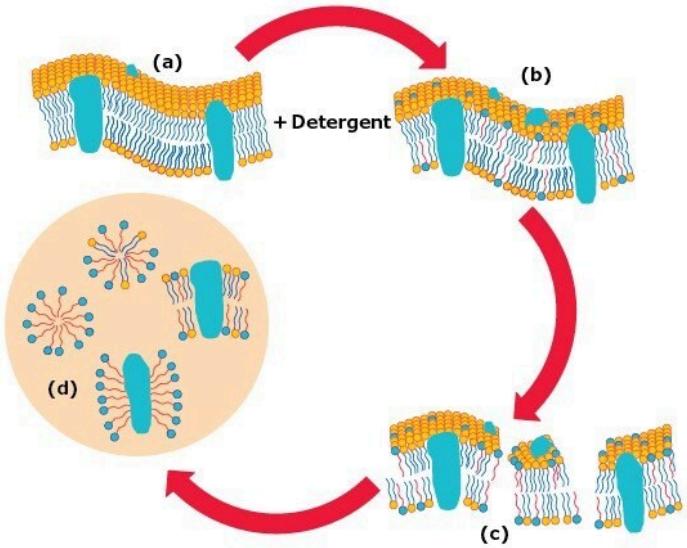
1.] How does soap clean grease?

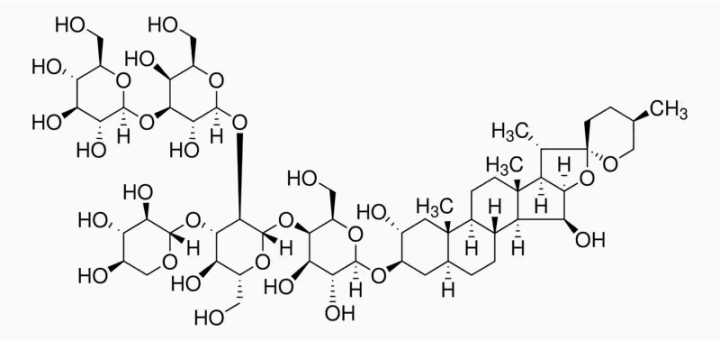


Soap forms micelles, whereby the nonpolar tails stick to the nonpolar, forming a solubilizable product, allowing one to use polar substances to wash away pieces of the dirt as it breaks apart due to intrusion of the soap molecules.

2.] How does this relate to processes occurring with plasma membranes?

The plasma membrane is a more complex lipid bilayer structure - the "dirt" piece is replaced by another layer of lipids. The membrane serves as a key barrier regulating entry and exit of solutes.





3.] Why does digitonin disrupt plasma membranes?

Like soap, digitonin has a nonpolar and polar piece, which interact with the plasma membrane.

It has been demonstrated the disruptive activity specifically requires the presence of cholesterol (10.3390/molecules201119682, 10.1016/j.molliq.2020.114598).

4.] How could we utilize digitonin to isolate mitochondria from cells? What other techniques could we potentially employ?

Digitonin can at an appropriate concentration and time duration solubilize the plasma membrane, while leaving the mitochondrial membrane intact. Other mitochondrial isolation techniques include dounce homogenization, where mechanical shearing pressures attempt to perform the same task, or needle extraction, where the size of the needle selects for plasma membrane lysis.

5.] If we were to isolate extracellular mitochondria within blood plasma, which steps can we skip from the initial protocol?

INITIAL PROTOCOL

All the steps were performed on ice.

~~1. Count and harvest cells (1 × 10⁶ to 1 × 10⁸ cells per sample) by trypsinization. Cells were counted using the InvitrogenTM CountessTM Automated Cell Counter.~~

~~2. Wash cells with 1X PBS and pellet at 215 × **g**, 5 min at 4 °C.~~

~~3. Resuspend pelleted cells in 500 µl of STM buffer with RNASE inhibitor and incubate on ice for 5 min.~~

~~4. For cell lysis, add 50 µl of 0.2 % high purity digitonin to achieve a final concentration of 0.02%. Vortex vigorously (5–10 s) every minute for 5 min.~~

5. Add 1:1 volume (500 µl) of STM buffer with RNASE inhibitor to the samples and centrifuge immediately in a benchtop microfuge at 700 × **g** for 10 min at 4 °C to remove unbroken cells and cell debris.

6. Carefully transfer the supernatant to a new tube and centrifuge at 10,000 × **g** for 15 min at 4 °C. Remove the supernatant (cytoplasmic fraction).

OPTIONAL: Add RNASE A, diluted in 50 µl STM buffer, at 100 U/µl. Incubate at room temperature for five minutes. RNASE A Prep: RNase A, DNase and protease-free (10 mg/mL), dilute 1 µl in 99 µl of STM buffer

7. For washing the mitochondria-containing pellet, add 500 µl of 1x STM buffer with RNASE inhibitor on top of the pellet. Do not resuspend; instead, invert the position of the pellet in the centrifuge and spin at 10,000 × **g** for 5 min at 4 °C.

8. Aspirate the supernatant completely and store the mitochondria-containing pellet at -80 °C until further use.

There are many considerations and protocols to keep in mind during RNA isolation.

RNAseAway purpose & function

- ★ Removes RNase contamination from labware (glassware and plasticware)
- ★ Spray like disinfectant spray then wash with distilled water!

When should you use RNAseAway during your procedure?

Right at the beginning & any-time you return to the bench from another lab space for active work.

Qiagen RNAeasy kit

- ★ Cells! Buffer RLT (350µl for dish <6cm / 600µl for 6-10cm)
- ★ Add 1 volume of 70% Ethanol (pipette to mix), then transfer up to 700µl to the RNeasy Mini Spin column. Centrifuge for ~15 seconds (speed of ≥8000 x g).
    - ○ Discard flow-through
    - ○ DNase Digestion Happens Here
- ★ Add 700 µl Buffer RW1 to the RNeasy spin column. centrifuge for 15 s at the same speed. Discard the flow-through.
- ★ Add 500 µl Buffer RPE to the RNeasy spin column. centrifuge for 15s at same speed. Discard the flow-through.
- ★ Add 500 µl Buffer RPE to the spin column. Centrifuge for 2 min at same speed.
- ★ Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Centrifuge for 1 min at same speed to elute the RNA.
- ★ If the expected RNA yield is >30 µg, repeat the previous using another 30–50 µl of RNase-free water or using the eluate from the previous step (if high RNA concentration is required). Reuse the collection tube from step 7.

DNase Digestíon

- ★ DNase I stock solution by injecting 550 µl RNase-free water into the DNase I vial using an RNase-free needle and syringe.
- ★ Mix gently by inverting the vial. NO VORTEX
- ★ Add 350 µl Buffer RW1 to the RNeasy column centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard flow-through.
- ★ Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube. Centrifuge briefly (<5s presumably)
- ★ Add DNase I incubation mix (80 µl) directly to RNeasy column membrane, and place on the benchtop (20–30°C) for 15 min.
- ★ Add 350 µl Buffer RW1 to the RNeasy column and centrifuge for 15 s at same speed. Discard flow-through. Continue with the next step of the RNAeasy kit.

Why should you not vortex the DNAse vial? Prevent protein denaturation

What is the purpose of the DNAse digestion step? Remove genomic DNA

Qubit Invitrogen RNA Concentration Protocol

* This protocol assumes that we are preparing standards for calibrating the Qubit Fluorometer (Components C and D) .

1. Set up the required number of Qubit tube for standards (C and D) and samples.
    - Use only thin-wall, clear 0.5-mL PCR tubes for the Qubit 4
    - Use only 8 x 200 - microL tube strips for Qubit flex
2. Label the tube lids
3. Prepare the Qubit working solution by diluting the Qubit RNA HS reagent 1:200 in qubit RNA HS buffer. Use clean plastic tube each time you prepare QUBIT working solution
    - For each sample we will need 199 microL of dilution buffer (Make sure to include enough for excess and for the two standards
    - Add 200x fluorometric reagent. 1 microL for each standard and the 2 standards.
4. Add Qubit solutions to each tube such that the final volume is 200 microL.

	Standard assay tubes	User sample assay tubes
Volume of working solution	190 µL	180–199 µL
Volume of standard	10 µL	—
Volume of user sample	—	1–20 µL
Total volume in each assay tube	200 µL	200 µL

5. Add 190 microL of the working solution to standards 1 and 2
6. Add between 180 and 199 microL of working solution to each sample (depends of dilution)
7. Add 10 microL of each Qubit standard to the appropriate standard tubes
8. Add 1-20 microL of each user sample to the appropriate tube (Depends on the anticipated concentration)
9. Vigorously vortex for 3-5 seconds
10. Allow tube to incubate at room temperature for 2 minutes, then proceed to read standards and samples

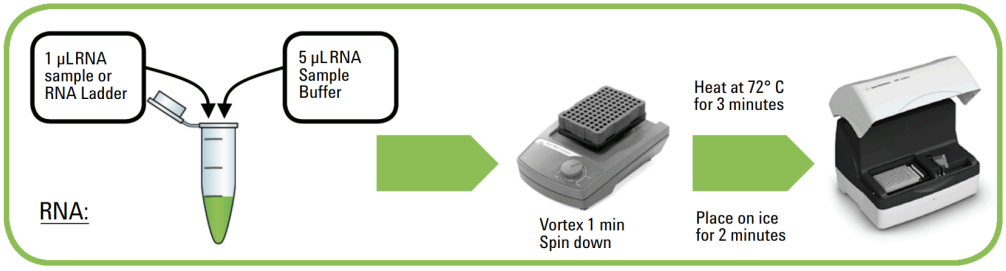
What should be the amount of sample that is added to this protocol? Between 1-20 µl ; generally select 10 µl

What is the principle behind the Fluorometer? The protocol employs fluorescent dyes that only emit wavelengths when bound to DNA / RNA. This is different from spectroscopy, which excites the DNA / RNA molecules directly.

Why do we need standards? The standards provide a date and machine-specific calibration curve, allowing for the interpolation of values between a low and high standard [see example].

Agilent Tapestation Protocol:

1. Allow reagents to equilibrate at room temp for 30 min
2. Vortex mix before use
3. Thaw total RNA samples on ice
4. Prepare sample by mixing 5uL RNA sample buffer with 1 uL RNA sample
    a. For best results, use reverse pipetting technique
5. Spin down, then vortex using IKA vortexer and adaptor at 2000 rpm for 1 min
6. Spin down to position the sample at the button of the tube
7. Sample denaturation
    a. Heat sample at 72 C (162 F) for 3 min
    b. Place samples on ice for 2 min
    c. Spin down to position the sample at the bottom of the tube

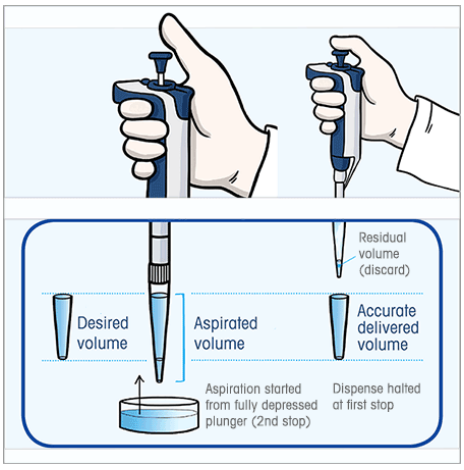


What is the purpose of vortexing the mix? Ensure that there is no sedimentation of different materials in the buffer, which could cause a difference in concentration, with downstream consequences on the effectiveness of the assay.

Why is the sample heated at 72˚C for 3 minutes before the protocol? The aim is to minimize intermolecular interactions between the RNA molecules.

What is the purpose thereafter of placing the samples on ice for 2 minutes? The purpose is to return the sample to an approximate temperature near RT.

Describe in your own words the reverse pipetting technique:
see picture



What is the function of the IKA vortexer and adaptor?
equilibrate the buffer and RNA input