RNA extraction from wild small mammals using TRIzol in BSL3 discipline.

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This protocol describes the biosafety level 3 discipline used for the extraction of RNA/DNA from wild bats & rodents using guanidinium thiocyanate-phenol-chloroform extraction (TRIzol™ or RNA_{GET}™). Use of the TRIzol trade name is for identification only and does not imply endorsement nor preference by our lab. The monophasic solution of phenol and guanidine isothiocyanate is designed to isolate separate fractions of RNA, DNA, and proteins from cell and tissue samples. As the true extent of viral burden in wild bat and rodent populations of the north central Mexican state of San Luis Potosí remains unknown, this protocol exaggerates perceived risk to imply the presence of risk group 3 and 4 agents such as Hantavirus, Arenavirus, Filovirus, Coronavirus and other related agents. Although risk group 4 viral pathogen isolation in cells (or any protocol with viable virus) should only be carried out in a level of equivalent biosafety (BSL-4) the carrying out of preliminary tests (genomic detection by PCR) should and can be conducted as long as a process of inactivation of the sample in a BSL-3 lab is used to subsequently allow the safe handling in a BSL-2 environment. After homogenizing the sample with TRIzol™, chloroform is added and the homogenate allowed to separate into a clear upper aqueous layer (containing RNA), an interphase, and a red lower organic layer (containing DNA and proteins). RNA is precipitated from the aqueous layer with isopropanol whereas DNA is precipitated from the interphase/organic layer with ethanol. Protein can then be precipitated from the phenol-ethanol supernatant by isopropanol precipitation. The precipitated RNA, DNA, or protein is purified to remove impurities and resuspended for downstream use.

Sample inactivation

IMPORTANT NOTE: All sample inactivation steps should be carried out within a class II type A2 biological safety cabinet located within the biosafety level 2 enhanced (BSL2+) lab and following biosafety level 3 (BSL3) discipline!

STRICT ADHERENCE TO BIOSAFETY LEVEL 3 DISCIPLINE IS EXPECTED! Work is to be supervised using the buddy system, after donning appropriate personal protective equipment (PPE) and in strict adherence to BSL3 work flow discipline for biological safety cabinets.

1) Unpack sample container (when sample has been referred to the lab for processing) ONLY ONCE INSIDE the BSL2+ laboratory and within the class 2 type A2 biological safety cabinet. Store samples in BSL3 -80ºC ultra-low freezer located in BSL2+ Laboratory until further use.

2) Once ready to process, retrieve biological sample (tissue or biospecimen) from BSL3 -80ºC ultra-low freezer and allow to thaw in ice for 10 minutes (inside the class 2 type A2 biological safety cabinet).

3) Open sample vial and retrieve approximately 5 mm^3 or 50–100 mg of tissue sample and place in 1.5 mL Eppendorf microcentrifuge tube and keep on ice. Immediately recap original sample vial and return to BSL3 -80ºC ultra-low freezer located in BSL2+ Laboratory.

4) Within a class 2 type A2 biological safety cabinet, add 500 µL of liquid nitrogen to previous Eppendorf tube and carefully homogenize tissue with crystal pestle taking special care to prevent splashes and aerosols. This step can be omitted for some tissue types, such as rodent lung and intestine.

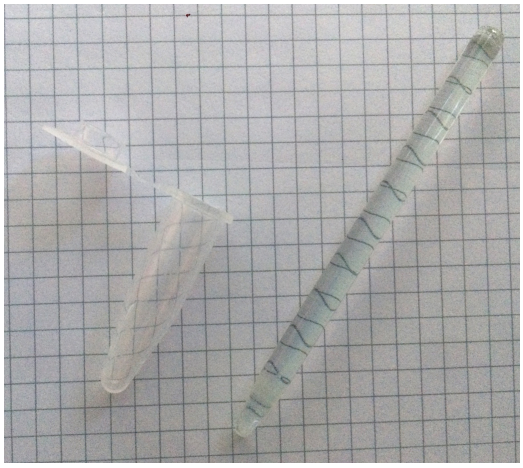


Figure 1. This pestle was improvised by our lab to allow for the processing of small samples against the inside conical end of a 1.5 mL Eppendorf tube. In our experience this method has shown to produce less aerosols than the use of tissue homogenizers (which SHOULD NOT BE USED PER BSL3 DISCIPLINE).

5) Add 25 µL of 1x DEPC-treated PBS solution to Eppendorf tube and carefully homogenize the tissue with crystal pestle without generating aerosols.

6) Add either 1mL or 750 µL or 500 µL of TRIzol and mix by inversion (see table 1). If needed, continue homogenizing tissue with glass pestle.

7) Cap the 1.5 mL Eppendorf carefully and place in ice tray. Biospecimen is now considered INACTIVATED as no viable viral particles should have survived TRIzol reagent mix.

8) Place used crystal pestle in 0.5% NaOCl or Benzalkonium chloride solution for a minimum decontamination contact time of 15 min. Clean with a plastic bristle brush and wipe dry with 70% ethanol for re-use.

Sample type	TRIzol volume	RNA Concentration (Mean ± SD)	RNA Yield (Mean ± SD)	A260/280 (Mean ± SD)	A260/230 (Mean ± SD)
Lung tissue	1 mL	373 ± 47 ng/µL	14.9 µg	1.96 ± 0.03	1.61 ± 0.25
	750 µL	269 ± 17 ng/µL	10.77 µg	1.99 ± 0.02	1.7 ± 0.03
	500 µL	111 ± 10 ng/µL	4.44 µg	1.97 ± 0.04	1.65 ± 0.22
Intestinal tissue	1 mL	1038 ± 402 ng/µL	41.5 µg	1.92 ± 0.09	1.38 ± 0.26
	750 µL	1789 ± 479 ng/µL	71.57 µg	1.96 ± 0.03	2.05 ± 0.09
	500 µL	1198 ± 243 ng/µL	47.92 µg	1.99 ± 0.01	2.03 ± 0.05

Table 1. Different volumes of TRIzol have been evaluated at our lab to produce wither high concentrations of RNA or higher yields of RNA or higher quality RNA, depending on the downstream procedures to be used. While using less TRIzol helps maintain processing costs down, RNA yields decrease when using rodent lung samples of same volume/weight. On the other hand, for some tissues such as rodent intestines, the use of lower volumes of TRIzol (between 500 and 750 µL) appears to produce higher RNA yields and quality.

9) Clean exterior of 1.5 mL Eppendorf with 70% Ethanol and re-label with specimen ID, place in clean pre-decontaminated microcentrifuge tube rack and transfer from biological safety cabinet to working buddy for him to place in previously NaOCl-decontaminated ice box with cold packs at -20ºC for transferring outside of BSL2+ laboratory.

USE COLD PACKS, NOT FLAKED ICE, AS REFRIGERANT!

10) Wipe or spray external surface of icebox with 0.5% NaOCl and place within a plastic bag. Do not rinse icebox clear of NaOCl. Tie bag shut and spray exterior with 0.1% NaOCl and let sit for 10 minutes before leaving BSL2+ Laboratory.

11) Thoroughly clean and decontaminate inside of biological safety cabinet and properly dispose of biological waste according to BSL3 work practices. Work with BSL3 discipline is now finished.

12) Transfer bag with ice-box and samples from BSL2+ Laboratory to BSL2 Laboratory.

13) Samples can now be considered risk group 1 and can be stored indefinitely in BSL2 -80ºC ultra-low freezer as a TRIzol-tissue homogenate.

Continuing RNA extraction on BSL2 setting

IMPORTANT NOTE 2: These following steps should be carried out within the static air PCR enclosure located within the RT-PCR and RNA work area, an area classed as biosafety level 2 (BSL2) and following strict BSL2 and molecular disciplines!

14) Unpack sample container from ice-box and decontaminate 1.5 mL Eppendorf's with 70% ethanol, keep these in ice-tray within the PCR enclosure.

15) Spin on microcentrifuge at 15,000 G's for 3 minutes.

16) Transfer supernatant to a new 1.5 mL Eppendorf microcentrifuge tube.

17) Add 200 µL of chloroform, securely re-cap the microcentrifuge tube, vortex briefly (10 seconds) and incubate for 3 minutes on ice.

18) Spin on microcentrifuge at 16,000 G's for 5 minutes. The mixture separates into a lower red phenol-chloroform organic phase and an interphase (containing DNA and proteins), as well as a colourless upper aqueous phase (containing RNA).

19) Transfer upper aqueous phase (containing RNA) to a new 1.5 mL Eppendorf microcentrifuge tube using P200 micropipette. Avoid disturbing interphase.

20) Save the interphase and organic phase if you want to isolate DNA or protein.

21) If the starting sample is small ($<10^6$ cells or <10 mg of tissue) add 5–10 µg of RNase-free glycogen as a carrier to the aqueous phase. The glycogen is co-precipitated with the RNA but does not interfere with subsequent applications.

22) Add 350 µL of 100% isopropanol to the aqueous phase and incubate for at least 12 hours at -20ºC.

23) Mix by inversion and incubate 5 min at room temperature.

24) Spin on microcentrifuge at 16,000 G's for 5 minutes. Discard supernatant and recover total RNA precipitate forming the white gel-like pellet at the bottom of the microcentrifuge tube.

25) Resuspend the RNA pellet in 1 mL of 70% ethanol. The extracted RNA can now be stored in this ethanol for at least 1 year at –20°C, or at least 1 week at 4°C. Use DEPC treated water to prepare this 70% Ethanol!

26) Vortex the sample briefly (10 seconds) and then spin on microcentrifuge at 16,000 G's for 5 minutes.

27) Discard the supernatant by inversion and remove excess with a P10 micropipette. Vacuum or air-dry the RNA pellet for 7 minutes.

28) Resuspend the pellet in 40 µL of RNase-free water, DEPC-treated water, 0.1 mM EDTA, or in a 0.5% SDS solution. Mix by pipetting up and down several times.

29) Check spectrophotometric concentration and quality of extracted RNA using the nanodrop (see online protocol provided in http://youtu.be/k2tZTDDyxaw).

30) Store resuspended RNA in BSL2 -80 ºC ultra-low freezer until further use.

Notes

1) Use disposable, individually wrapped, sterile plastic ware and sterile, disposable plastic ware.

2) 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.

3) In the step 3, clean and decontaminate dissection scissors and forceps by dipping in a tray with Benzalkonium Chloride or freshly prepared 0.5% NaOCl for 15 minutes and rinsing afterwards with 70% Ethanol between samples.

Warnings

1. Corresponding MSDS should be read, studied and understood before using GTPC. GTPC can lead to serious chemical burns and permanent scarring upon skin or eye contact, it is toxic if swallowed, inhaled, may cause respiratory irritation, it is suspected of causing genetic defects, may cause damage to organs through prolonged or repeated exposure
and is harmful to aquatic life and the environment.

2. Special caution should apply so as to avoid breathing dust/fume/gas/mist/vapors/spray and to avoid release to the environment. Wear protective gloves/protective clothing/eye protection/face protection and wash hands thoroughly after handling.

3. GTPC is light sensitive and should be stored in a dark-colored, glass container covered in aluminium foil and refrigerated. GTPC solution should be bright pink in color, solutions of other colors should be discarded according to health and safety and environmental regulations.

4. Contact of GTPC with acids or bleach (NaOCl) liberates toxic gases, DO NOT DECONTAMINATE with acids or bleach!

References

1. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987 Apr;162(1):156-9. PubMed PMID: 2440339.

2. Pan American Health Organization (PAHO) and World Health Organization (WHO) Regional Office for the Americas. General procedures for inactivation of potentially infectious samples with Ebola virus and other highly pathogenic viral agents. 2014

Revision history

1.0 Original document.

2.0 Centrifuge speed increased from 13 kG to16 kG, deleted some incubation steps and added additional warnings and notes. Document reformatted for new header.

3.0 Changed title of document to indicate “TRIzol”.

4.0 Modified TRIzol volumes recommended in Step 6 and wording of Step 27 to incorporate recent optimizations by Emma Felix Hernández Romano.