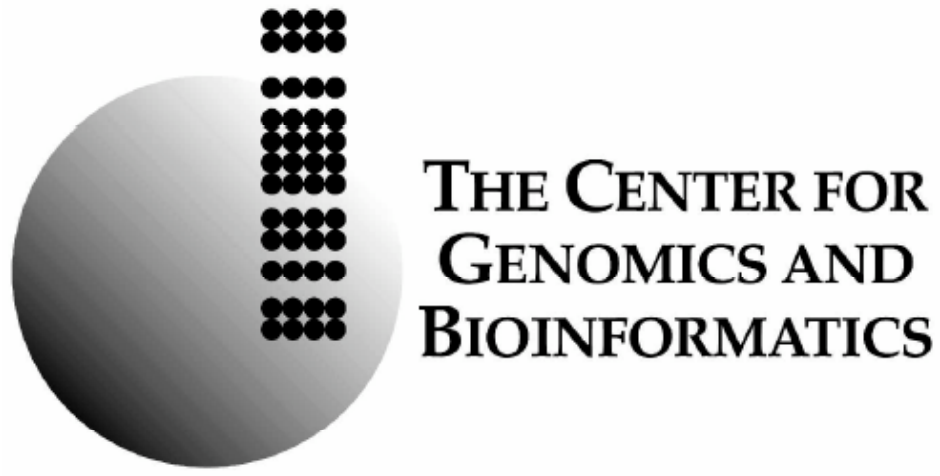

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Extraction of Total RNA from *Drosophila*

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INTRODUCTION

In this protocol, RNA is first isolated by organic extraction with the phenolic Trizol reagent and further purified by solid-phase purification using Qiagen RNeasy columns. The protocol is modified from the manufacturer's instructions. We find better results using 1.5 - 2 times more Trizol than is recommended. This is particularly the case for fatty tissues such as ovaries. In many instances it is not necessary to perform the solid-phase purification. The Trizol protocol alone will yield RNA pure enough for labeling purposes.

PRECAUTIONS

Laboratory safety. It is assumed that users have a sound knowledge of molecular biology techniques and safe laboratory practices. Before undertaking a new protocol or using unfamiliar reagents users should review relevant Material Safety Data Sheets to identify potential hazards and recommended precautions. For background in general molecular biology please see *Molecular Cloning A Laboratory Manual*, J. Sambrook and D. W. Russell. Cold Spring Harbor Laboratory Press. For background in microarray techniques please see *DNA Microarrays A Molecular Cloning Manual*. D. Bowtell and J. Sambrook. Cold Spring Harbor Laboratory Press.

Prevent particulate and fluorescent contamination. When working with microarrays it is essential to avoid fluorescent and particulate contamination. Please follow the these precautions. Filter solutions through 0.2 um filters to remove particles. Only use powder free nitrile gloves (latex gloves can cause fluorescent background).

Prevent RNase contamination. When working with RNA it is essential to avoid contamination with RNase. Please follow these precautions. Use RNase-free virgin plastic ware. Use RNase-free solutions (Note all traces of DEPC must be removed to avoid degradation of Cy dyes). Do not handle tubes or reagents with ungloved hands. Clean the inside and the outside of the barrel of micropipettes and/or use barrier tips.

MATERIALS

- TRIzol reagent; Invitrogen Life Sciences, Cat. # 155-96-011.
- Chloroform; EM Scientific, Cat. # CX1055-6.
- Ethanol.
- Disposable plastic homogenizer for microcentrifuge tubes.
- 1.5 ml and 2 ml microcentrifuge tubes.
- Temperature-regulated centrifuge; Eppendorf 5415 R.
- Ultrapure Distilled Water, Nuclease Free; Gibco-Invitrogen, Cat. # 10977-015. (*Note see Molecular Cloning A Laboratory Manual, J. Sambrook and D. W. Russell. Cold Spring Harbor Laboratory Press. for instruction on preparing DEPC treated RNase free solutions*).
- RNeasy Mini Kit; Qiagen, Cat. # 74104.

PROCEDURE

Trizol RNA isolation

Adapted from Invitrogen Life Technologies Trizol manual. The protocol is written for 50 mg of tissue that is homogenized in a microcentrifuge tube with a plastic pestle. This can be readily scaled up using a mortar and pestle and/or a Dounce homogenizer to homogenize frozen tissue. Make sure that flies/tissue is snap frozen in liquid nitrogen, stored at -80 ^{o}C and not allowed to thaw prior to being homogenized rapidly in Trizol. If you are dissecting tissue make sure to snap freeze the material as it is collected. This can be done by progressively adding dissected tissue to a microcentrifuge tube sitting in dry ice as you perform the dissections.

Please note that Trizol (phenol) and chloroform are toxic by contact, ingestion and inhalation. Additionally Trizol (phenol) is highly corrosive. Use these reagents in a fume hood and use gloves protective clothing and eye protection.

1. To 50 mg frozen flies (or frozen fly tissue) in a 1.5 ml microcentrifuge tube add 1 ml Trizol reagent and homogenize immediately with a disposable plastic pestle.
   (Note: Work quickly. RNA is protected from RNase in the frozen tissue and in the phenolic solution but is susceptible to degradation during the homogenization process)
2. Incubate at room temperature for 5 minutes.
3. Centrifuge at 12,000 rcf for 10 minutes at 4^{o}C to pellet insoluble debris such as exoskeleton.
4. Transfer the supernatant to a new microcentrifuge tube. *(Note: Take great care not to take pellet or fat layer, which can lead to phenol contamination of the aqueous layer when the phenol becomes trapped in fat micelles.)*
5. Add 200 ul of Chloroform (no isoamyl alcohol) to each tube.
6. Shake vigorously by hand (do not vortex which will lead to DNA Contamination).
7. Incubate tubes at room temperature for 3 minutes.
8. Centrifuge at 10,000 rcf for 15 minutes at 4^{o}C.

9. Transfer upper aqueous phase (~0.6 ml) to a fresh RNAse-free microcentrifuge tube. (*Note: Make sure to avoid RNase contamination from this point on.*)
10. Add 0.5 ml isopropanol
11. Incubate at room temperature for 10 minutes.
12. Centrifuge at 12,000 rcf for 10 minutes at 4°C.
13. Remove the supernatant and wash the pellet with 1ml 75% ethanol.
14. Centrifuge at 7,500 rcf for 5 minutes. at 4°C.
15. Remove the supernatant.
16. Centrifuge briefly and carefully remove the last of the supernatant with a micropipette.
17. Air dry for 10 minutes. (*Note: Do not dry under vacuum or heat.*)
18. Resuspend the pellet in 100 ul RNase-free water.
19. Quantify a 1/100 dilution of the RNA on spectrophotometer.

RNeasy cleanup

In many instances it is not necessary to perform the solid-phase purification. The Trizol protocol alone will yield RNA pure enough for labeling purposes. This protocol is identical to that outlined in detail in RNeasy Mini Handbook; RNeasy Mini Protocol for RNA Clean-up, p. 79-81 (http://www.qiagen.com/literature/rnalit.asp#mini). Please refer to the Handbook. The protocol is summarized here for convenience.

1. The binding capacity of each RNeasy Mini column is 100 ug RNA. Bring the RNA sample to a concentration of no more than 1 ug/ul in a total volume of 100 ul of RNase-free water.
2. Add 350 ul Buffer RLT and mix. (*Note: make sure to add beta-mercaptoethanol to buffer RLT before use.*)
3. Add 250 ul ethanol and mix by pipetting
4. Apply the sample to an RNeasy column in a 2 ml collection tube.
5. Centrifuge at 8,000 rcf for 15 seconds.
6. Transfer column to a new collection tube and discard the flow through.
7. Add 500 ul Buffer RPE to the column. (*Note: Make sure that ethanol has been added to buffer RPE before use.)*
8. Centrifuge at 8,000 rcf for 15 seconds.
9. Discard the flow-through and return the column to the same collection tube.
10. Add 500 ul Buffer RPE to the column.
11. Centrifuge for 2 minutes at 8,000 rcf.
12. Transfer the column to a new 2 ml microcentrifuge tube.
13. Centrifuge at full speed for 1 minute.
14. Transfer column to a new 1.5 ml collection tube.
15. Add 50 ul RNase-free water to the center of the column bed.
16. Incubate at room temperature 1-2 minutes.
17. Centrifuge at full speed for 1 minute.
18. Repeat steps 15-17.

19. Quantify 1/100 dilution of RNA on spectrophotometer.
20. Visualize appropriate aliquot (2-4 ug) RNA on a 1% agarose 0.5x TBE mini-gel (0.5 ug/ml EtBr), run in 0.5x TBE ~60 min at 100 V. (*Note: There should be a uniform smear of RNA with prominent ribosomal bands. The ribosomal bands should not be smeared and there should be no insoluble material in the wells.*)
21. Store the RNA at -20°C. (*Note: For long term storage store at -20°C or -80°C as ethanolic precipitate.*)

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