

PROTOCOL FOR RNA ISOLATION & cDNA SYNTHESIS FROM WHOLE TISSUE

Necessary Reagents

2ml phase lock tubes
 1.5 ml eppendorf tubes
 1.5 ml teflon tissue homogenizer
 TRIzol RNA extraction reagent
 20mg/ml glycogen *optional
 Chloroform/isoamyl alcohol (24:1)
 Isopropyl Alcohol
 75% EtOH
 Rnase free water

SUPERSCRIPT II
 5X First-Strand Buffer
 0.1 M DTT
 Oligo (dT) primer
 10mM dNTP mix
 1 λ Rnase Inhibitor (40units/ λ)
 Rnase H

1. Pre-spin 2ml Phase lock Gel-heavy tubes briefly (ie- 3,000rpm for 2 min) @ RT.
2. **HOMOGENIZATION:** Obtain two rat eyes from -80°C freezer and place on ice in a clean uncontaminated 1.5 ml eppendorf tube. To homogenize add 1ml TRIZOL reagent and 10 μl of a 20 mg/ml concentration of glycogen* and homogenize.
3. Add cell lysate to the tubes containing the pre-spun Phase lock tubes and incubate for 5-10 minutes @ 15 – 30°C to allow complete dissociation of nucleoprotein complexes.
4. **PHASE SEPARATION:** Add 0.2ml of chloroform-IAA per 1 ml of TRIzol Reagent. Shake vigorously by hand for 15 seconds and incubate them @ $15-30^{\circ}\text{C}$ for 2-3 minutes.
5. Centrifuge the samples at no more than 12,000 x g for 15 minutes @ 4°C . After centrifugation the mixture should separate into a lower red , phenol chloroform phase, an intermediate phase, and a colorless upper aqueous phase (RNA will be in the upper aqueous phase). The volume is typically about 60% of the aqueous phase.
6. **RNA PRECIPITATION:** Transfer the aqueous phase to a fresh tube, and save the the organic phase if isolation of protein or DNA is desired. Precipitate the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl per 1 ml of TRIzol reagent. Mix samples by repeated inversion. Allow samples to incubate at -20°C for 10 minutes, then spin at 12,000 x g for 10 minutes @ 4°C .
7. Decant the supernatant by dumping the liquid into a waste basket (don't worry the RNA will remain attached to the inside of the tube).
8. **RNA WASH:** Add 1 ml of 75% EtOH per 1 ml TRIzol Reagent used. Mix samples to dislodge the pellet and wash salts.
9. Centrifuge samples at 7,500 x g for 5 minutes @ 4°C .
10. Carefully decant the alcohol and remove all the residual alcohol. Dissolve the pellet in 100 λ RNase free ddH₂O. Incubation @ $55-60^{\circ}\text{C}$ for 10 minutes may be helpful in dissolving this solution. RNA should be stored at -80°C after this step.

Note* typical yields that I have observed are about 1 µg/µl or 100 µg for two whole rat eyes.

Sample calculation:

Concentration = $OD^{260} \times \text{dilution factor} \times 40\mu\text{g}/\mu\text{l}$

Concentration = $.226 \times 300/3 \times 40 = 904\mu\text{g}/\text{ml}$ or $0.9\mu\text{g}/\mu\text{l}$

11. Obtain the $OD^{260/280}$ readings by spectrophotometry to assess the purity and concentration of this extract.
12. **DNase Treatment:** Mix together
 - 1µg RNA sample
 - 1 µl 10x Dnase Rxn Buffer
 - 1µl Dnase I, amp grade, 1U/µl
 - DEPC-treated H₂O to 10µl
13. Incubate for 15 minutes @ RT
14. Inactivate Dnase 1 with µl 25mM EDTA solution @ 65°C for 10 min. Store at -80°C until needed.

FIRST STRAND SYNTHESIS USING SUPERSRIPT II^{aa} FOR RT-PCR

1. A 20λ reaction volume can be used for 1 ng-5µg of total RNA or 1ng – 500 ng of mRNA. Add the following components to a nuclease free microfuge tube..
2. mix together:
 - 1µl oligo d(T)12-18 (500µg/ml)
 - 1ng to 5 µg total RNA* (ie -2λ of a 0.9µg/λ sample)
 - 1µl 10mM dNTP Mix (10mM each dATP, dGTP, dCTP, and dTTP at neutral pH.
 - Sterile, ddH₂O to 12λ
3. heat mixture to 65° C for 5 minutes and quick chill on ice. Collect the contents by quick centrifugation and add:
 - 4λ 5 X First-Strand Buffer
 - 2λ 0.1 M DTT
 - 1λ Rnase Inhibitor (40units/λ)
4. Mix contents gently and incubate at 42°C for 2 minutes.
5. Add 1λ (200 units) of SuperScript II^{aa}, mix by pipetting up and down. Incubate this for 50 minutes at 42°C.
6. Inactivate the reaction by heating at 70° C for 15 minutes. The cDNA can now be used for PCR amplification although its best to remove the RNA first (RNA complementary to DNA may interfere with some reactions.)

7. To remove RNA, add 1 λ (2 units) of E.Coli Rnase H and incubate @37° C for 20 minutes.

ORDERING INFO

Catalog #	Company	Description	Size	Contact
18064-014	Gibco - Life Tech	Superscript II	10,000U	Gibco LifeTech (800) 826-6686; http://www.lifetech.com
18427-013	Gibco - Life Tech	dNTP mix 10mM	100 μ l	Gibco LifeTech (800) 826-6686; http://www.lifetech.com
15596-026	Gibco - Life Tech	Trizol Reagent	100ml	Gibco LifeTech (800) 826-6686; http://www.lifetech.com
0032005.152	ISC BIOEXPRESS (EPPENDORF)	Phase lock Gel 2.0 ml Heavy; yellow	200tubes/unit	ISC BIOEXPRESS (800) 999-2901
27785801	Pharmacia	PdT12-18		

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