The Fleischman Lab

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Title	**RNA Extraction and cDNA Preparation**	
Introduction	This protocol utilizes the Roche TriPure isolation reagent along with a traditional chloroform extraction to isolate RNA from DNA and proteins. In the first step, cells are lysed with TriPure and chloroform is used to separate the sample into three phases. RNA is recovered from the colorless aqueous phase via isopropanol precipitation. DNA and proteins may be isolated from the white interphase and red organic layers using separate protocols. Once the purified RNA is obtained, it is reverse-transcribed using the Invitrogen SuperScript VILO cDNA synthesis kit.	
Materials	1. Roche TriPure isolation reagent 2. Chloroform 3. Isopropanol 4. 75% Ethanol (molecular grade) 5. DEPC-treated RNase-free water 6. 1.5 ml Eppendorf tubes 7. Cold microcentrifuge 8. Nanodrop 9. Invitrogen SuperScript VILO 5x reaction buffer 10. Invitrogen SuperScript VILO 10x enzyme mix 11. Thermal cycler 12. PCR tubes	
Protocol	**A. Initial Extraction and Phase Separation**	*Notes*
1.	Count and pellet freshly-isolated MNCs. Add 1 ml TriPure reagent per 5-10 x 10^6 cells. Lyse cells by repetitive pipetting. Keep track of the amount of TriPure reagent used for each sample.	*Samples can be frozen for 1 month at -80°C after homogenizing in TriPure.* *This same protocol may be used for other cell types (e.g. cell lines, etc.).*
2.	Incubate each sample for 5 minutes at room temperature.	
3.	Add chloroform to each sample. Use 200 µl chloroform for each 1 ml TriPure reagent used.	
4.	Cap tube and shake vigorously for 15 seconds.	
5.	Incubate tube at room temperature for 10 minutes.	
6.	Centrifuge at 4°C (cold room) for 15 minutes at 12,000g. The RNA will be present in the colorless aqueous phase.	*Do not exceed 12,000g!*
	B. RNA Extraction	
1.	Transfer the colorless upper aqueous phase to a new 1.5 ml centrifuge tube. Be careful not to disturb the lower phases!	*Yield ≈ 550-600 µl per 1 ml TriPure reagent used in step 1.*

2.	Add isopropanol to the aqueous phase. Use 500 µl isopropanol for each 1 ml TriPure reagent used in step 1.	
3.	Cap tube and invert several times to mix thoroughly. Incubate for 5-10 minutes at room temperature to allow the RNA precipitate to form.	
4.	Centrifuge at 4℃ (cold room) for 10 minutes at 12,000g.	
5.	Discard the supernatant and add 75% ethanol to each tube. Use at least 1 ml of 75% ethanol for each 1 ml TriPure reagent used in step 1.	*The RNA precipitate can be stored in 75% ethanol for 1 week at 4°C or 1 year at -20°C.*
6.	Wash the RNA pellet in the ethanol by vortexing. Centrifuge at 4℃ (cold room) for 5 minutes at 7,500g.	
7.	Discard the supernatant and repeat step 6.	
8.	Discard the supernatant and remove excess ethanol by air-drying: a. Gently tap the top of the tube on paper towels. b. Leave lids open and invert tubes. Rest on paper towels for 5-10 minutes, then re-cap tubes.	*Do not leave tubes open for more than 10 minutes to prevent over-drying!*
9.	Resuspend RNA pellet in DEPC-treated RNase-free water (≈50 µl final volume). *Optional:* Incubate the solution for 10 to 15 minutes at 55-60°C to help dissolve the RNA pellet.	
10.	Measure RNA concentration on the Nanodrop and proceed to cDNA synthesis. Store RNA at -80°C.	*It is highly recommended to synthesize cDNA immediately to avoid RNA degradation!*
	C. cDNA Synthesis	
1.	Prepare the master mix as follows: a. 5x VILO Reaction Mix — 4 µl b. 10x SuperScript Enzyme Mix — 2 µl c. RNA (up to 2.5 µg) — x µl d. DEPC-treated water — up to 20 µl	*Reaction volume may be scaled up to 100 µl.*
2.	Gently mix tube and place in the thermal cycler. Run on the program "cDNA", or as follows: a. 25°C for 10 minutes b. 42°C for 60 minutes c. 85°C for 5 minutes	*Use the green support block for tube strips.*
3.	Proceed to use cDNA in qPCR or store at -20°C until use. a. For SYBR Green: if you started with >100 ng RNA, dilute the cDNA to achieve the concentration equivalent of starting with 100 ng of RNA. Then use up to 2 µl of the diluted cDNA in a 20 µl qPCR reaction (≤10% qPCR volume).	