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RNA purification and cDNA synthesis

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We use this protocol and it's working

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Abstract

This protocol compiles multiple methods for purifying RNA from an *S. rosetta* lysate and provides a modified reverse transcriptase protocol to robustly synthesize cDNA from transcripts with higher GC content.

Culture cells for lysate

1 **Grow enough culture for ~50x10^7 cells.** 2d

- for swimming cultures: seed 40 mL acclimated cells at ~ [M] 8*10^4 cells/ml then culture at 27 °C for 24:00:00
- for thecate cultures: seed 100 mL acclimated cells in culture plate then culture at 27 °C for 24:00:00

Number of cells doesn't necessarily matter. You just want enough cells to extract sufficient nucleic acid from taking in account the loss of yield in subsequent steps.

2 **Follow preferential lysis protocol**

Extract RNA

3 **You can extract total RNA (go to step 3.1) or mRNA (go to step 3.2)**

3.1 **Trizol LS total RNA extraction** 1h 15m 30s

1. Add 3:1 Trizol LS to lysate and incubate for 00:05:00
2. Add 200 µL chloroform to 750 µL of added Trizol LS and incubate for 00:03:00
3. Centrifuged sample at 12000 x g, 4°C, 00:15:00
4. Collect aqueous layer

RNA precipitation

1. Add 500 µL cold isopropanol per 750 µL of added Trizol LS to collected aqueous layer and incubate on ice for 00:10:00
2. Centrifuge sample at 12000 x g, 4°C, 00:30:00
3. Washed pellet with cold 75% ethanol

4. Centrifuge sample at 7500 x g, 4°C, 00:05:00
5. Resuspend with [M] 1 millimolar (mM) citrate , pH 6.4 .
6. Measure RNA concentration

or

RNeasy Cleanup

we use the ***RNeasy MinElute Cleanup Kit***

1. Adjust the sample to a volume of 100 µL
2. Add 250 µL 96–100% ethanol to the diluted RNA, and mix
3. Transfer the sample (700 µL) to an RNeasy MinElute spin column placed in a 2 ml collection tube. Centrifuge for 8000 x g, Room temperature, 00:00:15 . Discard the flow-through.
4. Place the RNeasy MinElute spin column in a new 2 ml collection tube. Add 500 µL Buffer RPE to the spin column. Close the lid gently, and centrifuge for 8000 x g, Room temperature, 00:00:15
5. Add 500 µL of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 8000 x g, Room temperature, 00:02:00
6. Place the RNeasy MinElute spin column in a new 2 ml collection tube. Open the lid of the spin column, and centrifuge at full speed for 00:05:00 .
7. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube. Add 14 µL RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA
8. For long-term storage, supplement the RNA with [M] 1 millimolar (mM) sodium citrate , pH 6.4

3.2 mRNA extraction

13m

*We use the **NEB Magnetic mRNA Isolation Kit***

1. Equilibrate 100 µL beads with 200 µL binding buffer
2. Add lysate and mix for 00:10:00
3. Pull down beads with magnet and remove supernatant
4. Add 500 µL Wash Buffer 1 and mix for 00:01:00
5. Pull down beads with magnet and remove supernatant
6. Repeat step 4-5
7. Add 500 µL Wash Buffer 2 and mix for 00:01:00
8. Pull down beads with magnet and remove supernatant
9. Repeat step 7-8
10. Add 500 µL Low Salt Buffer and mix for 00:01:00
11. Pull down beads with magnet and remove supernatant
12. Add 20 µL elution buffer *(can vary elution volume for desired concentration; can vary elution buffer, we have used Nano-pure water or nylon filtered 10 mM Tris-acetate pH 8.0)*
13. Measure mRNA concentration
14. For long-term storage, supplement the RNA with [M] 1 millimolar (mM) sodium citrate , pH 6.4

cDNA synthesis

6m

4 We use Invitrogen SuperScript™ IV Reverse Transcriptase kit

4.1 Anneal oligo $d(T)_{20}$ primer to RNA sample

6m

1. Assemble the reaction according to this table:

	A	B
	Component	**Volume**
	50 µM oligo d(T)20 primer or 2 µM gene-specific reverse primer	1 µl
	10 mM dNTP mix	1 µl
	RNA sample (10pg-5µg total RNA or 10pg-500 ng mRNA)	up to 11 µl
	DEPC-treated water or nuclease-free water	to 13 µl

2. Mix and incubate reaction at 65 °C for 00:05:00
3. Place on ice for 00:01:00

4.2 Reverse transcription to make cDNA

30m

1. Vortex 5x SSIV Buffer
2. To the annealed RNA templates from go to step #4.1, add the following components:

	A	B
	Component	**Volume**
	5x SSIV Buffer	4 µl
	100 mM DTT	1 µl
	RNaseOUT™ Recombinant RNase Inhibitor	1 µl
	SuperScript IV Reverse Transcriptase (200 U/µL)	1 µl

4. Incubate reaction at 60 °C for 10 minutes (IMPORTANT: reaction temperature is increased from kit instructions to account for higher GC content in some transcripts)
5. Inactivate reaction by incubation at 80 °C or 00:10:00
6. Remove RNA with incubation with 1 µL of RNase H at 37 °C for 00:20:00

5 Clone gene of interest with cDNA

Use generated cDNA in PCR reaction with gene specific primers. To verify that the RNA purfication and cDNA synthesis was successful, amplify a highly expressed transcript, such as cofillin (PTSG_01554).