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RNA purification and cDNA synthesis

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Vicki Deng¹, Fredrick leon², David Booth³

¹UT Austin; ²UCSF; ³University of California, San Francisco



Vicki Deng

UT Austin

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Protocol status: Working

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 $\sim 50 \times 10^7$ cells.

2d

- for swimming cultures: seed  40 mL acclimated cells at $\sim$ [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 μ L chloroform to  750 μ L 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 μ L cold isopropanol per  750 μ L 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  1 millimolar (mM) citrate ,  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  1 millimolar (mM) sodium citrate ,  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  1 millimolar (mM) sodium citrate ,  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 μ M oligo d(T) ₂₀ primer or 2 μ M gene-specific reverse primer	1 μ l
10 mM dNTP mix	1 μ l
RNA sample (10pg-5 μ g total RNA or 10pg-500 ng mRNA)	up to 11 μ l
DEPC-treated water or nuclease-free water	to 13 μ l

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 μ l
100 mM DTT	1 μ l
RNaseOUT™ Recombinant RNase Inhibitor	1 μ l



A	B
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 purification and cDNA synthesis was successful, amplify a highly expressed transcript, such as cofillin (PTSG_01554).