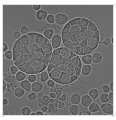

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Working

RNA isolation and cDNA synthesis from the marine ichthyosporean Sphaeroforma arctica

PLOS Genetics

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Multicellgenomelab

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ABSTRACT

This protocol describes the steps for isolation of total RNA for cDNA synthesis in the marine Ichthyosporean *Sphaeroforma arctica*. In general, Ichthysoporeans are characterized by having a thick cell wall, of uncknown composition, that is particularly hard to brake, making cell lysis difficult. Here we have introduce a simple modification on the conventional RNA extraction protocol using TRIzol reagent, by including two cycles of freezing-tawing of the cells-TRIzol suspension, in order to facilitate cell lysis. This modification improved significantly the RNA isolation yield. Total RNA is further treated with DNAse I enzyme and purified by precipitation to minimize genomic DNA contamination. Highly pure total RNA is then used to synthetize cDNA for downstream applications.

EXTERNAL LINK

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Ocaña-Pallarès E, Najle SR, Scazzocchio C, Ruiz-Trillo I (2019) Reticulate evolution in eukaryotes: Origin and evolution of the nitrate assimilation pathway. PLoS Genet 15(2): e1007986. doi: 10.1371/journal.pgen.1007986

PROTOCOL STATUS

Working

Collect cells by centrifugation

1

- Remove the cells from the culture flasks with aid of a cell scraper (Nunc).
- Transfer cells to a 15 ml centrifuge tube. Pellet cells by centrifugation at 4500 xg for 5 min at 12 °C .
- Carefully remove supernatant by pipetting. Be cautious not to perturb the pellet, to avoid loosing material.

00:08:00

Lyse cells and extract total RNA

2

- Resuspend the cell pellet with 1 ml of TRIzol (ThermoFisher Scientific) by gently pipetting up and down, until the pellet is completely dissolved. Incubate suspension for 5 min at room temperature.

Make sure that no pellet remaining is visible at the bottom of the tube after resuspension. See manufacturer's protocol for more details on using this reagent.

- Transfer the TRIzol-suspended material to a new 1.5 ml microcentrifuge tube with safe lock (Eppendorf).
- Freeze the suspension by submerging the tube in liquid nitrogen. Once it is completely freezed (nitrogen stops boiling), transfer the tube to a thermal block pre-warmed at 50 °C and taw the solution for 5 min.
- Repeat freeze-tawing cycle once.

STOPPING POINT: TRIzol suspensions can be kept at -20 °C for up to 2 weeks or at -80 °C for long-term storage.

00:25:00

Separate phases and isolate RNA

3
- Add 100 µl of 1-bromo-3-chloropropane (Sigma-Aldrich). Cap the tube and seal it with parafilm to avoid leaks.

1-Br-3-Cl-propane performs better than *chloroform* for phase separation helping to avoid gDNA contamination (interphase is more 'compact' after centrifugation).

- Shake the tube vigorously by hand for 15 seconds and incubate at room temperature for 15 min.
- Centrifuge at 12,000 xg for 15 min at 4 °C .
- Transfer the aqueous (superior) phase containing RNA (aproximately 600 µl) to a clean, new 1.5 mL tube.

Interphase contains genomic DNA. So, be careful while pipetting to avoid DNA contamination in the RNA sample. Lower phase contain proteins.

00:30:00

Precipitate RNA

4
- Add 500 µl of isopropanol. Invert 3-4 times to mix and incubate 15 min at room temperature.
- Centrifuge at 12,000 xg for 10 min at 4 °C .
- Discard supernatant and add 1 ml of 75% ethanol (freshly prepared), vortex briefly and centrifuge at 8,500 xg for 5 min at 4 °C .
- Discard supernatant completely and dry at room temperature for about 10 min.

CRITICAL!: Avoid overdrying, as it will make RNA very difficult to resuspend.

00:40:00

Resuspend RNA

5 Add 30 µl of nuclease-free water, and resuspend by gently pipetting while heating at 56 °C . Spin down to collect the whole volume at the bottom of the tube.

Water volume can be adjusted as needed. Tipically 30 to 50 uL are used.

00:05:00

Measure RNA concentration

6 Measure RNA concentration by spectrophotometry (for example, using nanodrop instrument).

00:02:00

DNAse I treatement

7 Transfer up to 10 µg of total RNA to a clean, new tube. Complete volume with nuclease-free water to reach a final volume of 40.5 µl and then add:

- 5 µl 10X DNAseI buffer (Thermofisher)
- 2 µl RNAseOUT (Thermofisher)
- 5 µl DNAseI (Thermofisher)

Incubate at room temperature for 15 min and inactivate the reaction by adding 1 µl EDTA solution and incubating for 10 min at 65 °C .

00:20:00

RNA precipitation

8 - Add 5 µl of 5M LiCl plus two volumes (110 µl) of 100% ethanol, previously cooled at -20 °C . Precipitate overnight at -80 °C .

NEXT DAY:

- Centrifuge at maximum speed for 15 min at 4 °C . Discard supernatant carefully with pipette.
- Wash once with 1 ml 70% ethanol (cold at -20 °C).
- Centrifuge at maximum speed for 5 min at 4 °C , discard supernatant and dry at room temperature for 10-15 min.

CRITICAL!: Be extremelly careful not to overdry the sample. Dry RNA is very difficult to resuspend.

- Resuspend pellet (sometimes not visible) with 20 µl of nuclease-free water, by pipetting up and down. Flick the tube, spin down, and incubate overnight at -80 °C for complete resuspension.

48:00:00 **(Includes two overnight incubations.)**

Measure RNA concentration

9 Measure RNA concentration by spectrophotometry (for example, using nanodrop instrument).

00:02:00

cDNA synthesis

10 - Calculate the appropriate volume of DNAse-treated total RNA for 0.5 to 3 µg of RNA. Volume should not exceed 8 µl. Assemble the following reaction preferably in DNA LoBind 0.5 µl tubes (Eppendorf).

If DNA LoBind tubes are not available, use normal PCR tubes.

Total RNA ---------- x µl
Nuclease-free water --------- 8 - x µl
oligo dT primer ---------- 1µl
10X dNTP mix ---------- 1µl

- On thermocycler, heat the mix for 5 min at 65 °C, then cool down immediately on ice.
- While on ice, add 10 µl of cDNA reaction mix of the following composition:

  10X Superscript III buffer ----- 2 µl
  25 mM $MgCl_2$ ---------- 4 µl
  0.1 M DTT ---------- 2 µl
  RNAseOUT ---------- 1 µl
  Superscript III ---------- 1 µl

- Homogenize the mixture, quickly spin down and immediately incubate for 75 min at 50 °C, followed by 5 min incubation at 85 °C to inactivate the enzyme.
- Treat samples with 1 µl RNAse H (provided with the SuperScript kit), for 1 hour at 37 °C (in a water bath).

Reagents used in this step are from SuperScript III First Strand Synthesis Kit (Invitrogen).

02:30:00