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RNA isolation and cDNA synthesis from the marine ichthyosporean *Sphaeroforma arctica*

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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, Ichthyosporeans are characterized by having a thick cell wall, of unknown composition, that is particularly hard to break, making cell lysis difficult. Here we have introduced a simple modification on the conventional RNA extraction protocol using TRIzol reagent, by including two cycles of freezing-thawing 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 synthesize cDNA for downstream applications.

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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 losing 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.

Note

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 frozen (nitrogen stops boiling), transfer the tube to a thermal block pre-warmed at 50 °C and thaw the solution for 5 min.
- Repeat freeze-thawing cycle once.

Note

STOPPING POINT: TRIzol suspensions can be kept at $-20\text{ }^{\circ}\text{C}$ for up to 2 weeks or at $-80\text{ }^{\circ}\text{C}$ for long-term storage.

⌚ 00:25:00

Separate phases and isolate RNA

- 3
 - Add $100\text{ }\mu\text{L}$ of 1-bromo-3-chloropropane (Sigma-Aldrich). Cap the tube and seal it with parafilm to avoid leaks.

Note

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 $12,000\text{ }xg$ for 15 min at $4\text{ }^{\circ}\text{C}$.
- Transfer the aqueous (superior) phase containing RNA (approximately $600\text{ }\mu\text{L}$) to a clean, new 1.5 mL tube.

Note

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 $500\text{ }\mu\text{L}$ of isopropanol. Invert 3-4 times to mix and incubate 15 min at room temperature.
 - Centrifuge at $12,000\text{ }xg$ for 10 min at $4\text{ }^{\circ}\text{C}$.
 - 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.

Note

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.

Note

Water volume can be adjusted as needed. Typically 30 to 50 µL are used.

⌚ 00:05:00

Measure RNA concentration

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

⌚ 00:02:00

DNase I treatment

- 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 μ l EDTA solution and incubating for 10 min at 65°C .

00:20:00

RNA precipitation

- 8
- Add 5 μ l of 5M LiCl plus two volumes (110 μ l) 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.

Note

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

- Resuspend pellet (sometimes not visible) with 20 μ l 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 μ g of RNA. Volume

should not exceed 8 µl. Assemble the following reaction preferably in DNA LoBind 0.5 µl tubes (Eppendorf).

Note

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

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

- 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 MgCl₂ ----- 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).

Note

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

02:30:00