COGEME Transcriptome facility Protocols and Methods

ISOLATION OF TOTAL RNA FROM YEAST CELLS

A. PREPARATION OF YEAST CELLS:

Grow yeast cells

1. Harvest the cells by centrifugation at 4000 rpm for 3 min.
2. Resuspend in a very small volume of growth medium.
3. The suspension is sucked into a pipette and released as individual drops directly into liquid nitrogen.
4. Frozen cell drops can be stored at –80 ^{deg} C.
5. Pre – cool a 5ml Teflon vessel in liquid nitrogen.
6. Add a 7 mm bead made of tungsten carbide and frozen cells.
7. Close the flask with the pre – cooled cap and place it into the holder of the Micro – Dismembrator.
8. Set the shaking frequency to 1200 rpm and shake for 2 min.
9. Take up the still frozen powder in 2 ml TRIZOL and split into 2 eppendorfs.

B. ISOLATION OF TOTAL RNA FROM FILAMENTOUS FUNGUS

1. Soak large pestle and mortar o/n in Decon. Rinse in DEPC water. Wipe with Rnase away using paper towel. Wash with DEPC water again and dry with paper towel. Cool down with liquid Nitrogen.
2. Filter 50ml o/n filamentous culture through miracloth or muslin and immediately put biomass into liquid Nitrogen and grind to a fine powder adding more liquid Nitrogen as necessary.
3. Put ground mycelium into Falcon tube and add 2ml Trizol.

C. ISOLATION OF TOTAL RNA

1. Homogenize by vortexing for 1 min.
2. Keep samples at RT for 5 min to allow for dissociation of the nucleoproteins complexes.
3. This is an optional step to precipitate polysacchs, ECM and high Mr DNA. Centrifuge 12000 x g for 10 min, transfer the clear supernatant to a fresh tube.

4. After addition of 0.4 vol chloroform shake the samples vigorously for 15 sec by hand and leave 3 – 10 min at RT.

5. Centrifuge 12000 x g for 5 min. The mixture separates into the lower RED (phenol – chloroform phase), the interphase and the colourless upper aqueous phase. RNA is forced exclusively into the aqueous phase whereas the DNA and the proteins partition into the interphase and the lower phenol phase.

6. Transfer the aqueous phase to a fresh eppendorf (the volume of the RNA containing phase is @ 60% of the vol. used for homogenisation).

7. Precipitate the RNA with 0.5ml of IPA / ml of reagent and keep all samples at RT for 5 – 15 min. **(Can be left o/n at this stage)**

8. Centrifuge 12000 x g for 10 min. The RNA pellet should form a gel like precipitate on the bottom and the side of the tube.

9. Remove the IPA S/N carefully.

10. Wash the pellet with 1 ml 70% EtOH by vortexing and centrifuge as in step 8.

11. Briefly air dry the RNA pellet.

12. Dissolve the RNA in 500 ul DEPC treated dH_2O.

13. Add 1 vol. LiCl buffer and precipitate for at least 1 hr at –20 deg C. Centrifuge at max speed for 30 min. **(Can be left o/n at this stage)**

14. Wash the pellet twice with 70% EtOH (to remove any salt as these will act as inhibitors of Reverse Transcriptase).

15. Dry pellet and dissolve in a small volume of DEPC treated dH_2O.

16. Quantify by taking OD260 and run on 1% agarose gel (TBE) to visualise and check for quality of RNA.

TARGET SYNTHESIS / LABELLING AND PURIFICATION:

1. Prepare a labelling mix containing 500mM dCTP, 500mM dATP, 500mM dGTP, 100mM dTTP, Cy 3 - dUTP / Cy 5 d UTP, 400 U Superscript II RT, 1mM DTT and 1x RT buffer. Can make a ‘master mix’ for 20 reactions and store the unused at – 20 deg C.

   5x RT buffer 120ul

   DTT (5mM) 60ul

   dATP (100 mM) 3ul

   dCTP (100 mM) 3ul

   dGTP (100 mM) 3ul

   dTTP (100 mM) 0.6ul

DEPC Water 110.4ul

TOTAL 300ul

2. To 50ug of total **RNA** in a microfuge tube, add 2ug (4ul) of oligo dT(VN) and DEPC treated water to a total volume of 10ul.
3. Incubate the reaction mixture at 70 deg C for 10 min. and chill on ice for one min.
4. To the RNA add

   RT labelling mix (from 1) 15ul

   Cy 3 or Cy 5 dUTP (1mM) 3ul

   Superscript II RT (200 U/ml) 2ul
5. Mix thoroughly and incubate at 42 ^{deg} C for 2 hours.
6. Briefly centrifuge the reaction and add 1.5ul of 20mM EDTA to stop the reaction
7. Add 1.5ul of 500mM NaOH and heat at 70 deg C for 10 min. to degrade the RNA.
8. Neutralise the reaction by adding 1.5ml of 500mM HCl.
9. Unincorporated fluorescent nucleotides are removed by purification using GFX columns using the following protocol.
10. Place a GFX column in a collection tube for each purification to be performed.
11. Add 500ul of capture buffer to each GFX columns.
12. Transfer the labelled DNA product (up to 100ul) to the GFX column.
13. Mix thoroughly by pipetting the sample up and down 4 – 6 times
14. Centrifuge in a microfuge at full speed for 30 sec.
15. Discard the flow through and add 500ul wash buffer to the column. Centrifuge at full speed for 30 sec.
16. Discard the collection tube and transfer the GFX column to a fresh eppendorf and apply 50ul of elution buffer or ddH_2O. Allow to stand at RT for 1 min.
17. Centrifuge at full speed for 1 min.
18. Dry the probe in speedvac either to completion or to 9ul.
19. Run on gel and scan gel on Molecular Dynamics Storm 860 to check labelling efficiency.

PROTOCOL FOR HYBRIDIZATION

PREHYBRIDISATION MIX:

25 ml 20x SSC

1 ml 10% SDS

1 g BSA

To 100 ml with ddH_2O

PREHYBRIDISATION PROCEDURE:

Prehyb. in coplin jar at 42 deg C for 45 min

Wash slide by dipping 5 X in ddH_2O at RT

Dip in isopropanol at RT

Air dry.

HYBRIDISATION PROCEDURE

2x HYB. MIX

50% FORMAMIDE 5.0 ml

10x SSC 5.0 ml 20x Stock

0.2% SDS 0.2 ml 10% Stock

9.0ul CY3 + 9.0ul CY5 + 18ul hyb. mix.

Boil 3 min. to denature and spin briefly.

Apply labelled probe to slide.

Hybridise o/n 42^{deg}C if using cDNA.

WASH: With agitation.

2X SSC, 1% SDS RT 15 min (5ml 20x SSC + 5ml 10% SDS: 50ml dH_2O)

1X SSC, 0.2% SDS RT 8 min (2.5ml 20x SSC + 1.0 ml 10% SDS: 50ml dH_2O)

0.1X SSC, 0.2% SDS RT 5 min (0.25ml 20x SSC + 1ml 10% SDS: 50ml dH_2O)

Spin slide to dry – scan.

BUFFERS AND SOLUTIONS:

Chloroform

IPA – Isopropanol.

70% EtOH – with DEPC treated dH_2O.

DEPC treated dH_2O. Prepare by adding 0.1% DEPC in fume hood. Leave overnight and autoclave following day.

LiCl Buffer: 4 M LiCl, 20 mM Tris/HCl pH 7.5, 10 mM EDTA.

TRIzol® Reagent: Invitrogen product ref. 15596-026

(25 nmol) Cy 3 dUTP – Amersham. Cat # PA 53022.

(25 nmol) Cy 5 dUTP– Amersham. Cat # PA 55022.

Or Value pack of 5x Cy 3 and Cy 5 dUTP Cat # 53022

Superscript II - Invitrogen Life Technologies. Cat # 18064 – 014.

dNTP's – Roche.