Supplementary Material 3
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PROTOCOLS FOR RNA EXTRACTION AND COPY NUMBER ESTIMATION BY RT-qPCR

Contents:

1. Overview

In order to obtain an estimate of the number of copies of a particular RNA species, several stages in the isolation and assay of the RNA need to be quantified. These are:-

1) Efficiency of cell lysis.
2) Efficiency of extraction of RNA from the cell lysate.
3) Efficiency of cDNA production by reverse transcriptase.
4) Efficiency of each PCR reaction.

1) The DNA content of cells is tightly controlled at between one and two haploid genomes (double if diploid), so if the number of cells is known then the maximum amount of DNA that can be recovered is known. An estimate of the efficiency of cell lysis is obtained by determining the amount of genomic DNA recovered compared with the expected amount. The difference between the observed and the theoretical yield of DNA will be largely due to the proportion of cells lysed and provides an estimate of the efficiency of cell lysis.

2) An estimate of the efficiency of RNA recovery from the cell lysate is obtained by adding a known amount of non-yeast in vitro transcribed RNA (we use *Arabidopsis thaliana* LSM1, accession number At3g14080), and determining by RT-qPCR how much is recovered at the end of the RNA purification.

3) and 4) The reverse transcription and qPCR reactions are quantified by comparison with known amounts of synthetic RNA standards. These are produced by in vitro transcription (IVT) and have the same sequences as the RNAs to be quantified. The IVT standards are mixed with total RNA extracted from cells lacking the reporter. When assaying RNAs with lariat structure, the primer used for lariat cDNA production and for PCR from the branch site contains an A instead of a T opposite the branch site A (Vogel et al. 1997). This is compared with a linear IVT RNA that contains a T instead of an A at the branch point to make it more like a branch structure.

2. Culture Sampling

1. Cultures are grown in appropriate conditions to the required OD (for doxycyline induction or repression OD_{600} is 0.4 to 0.7 in SD medium).
2. Doxycycline is added to a final concentration of 4 µg/ml.
3. 10 ml samples are removed at set time points and pipetted directly into 5ml 100% methanol in a 15 ml falcon tube on dry ice (final concentration 33% methanol – cools sample to approximately -10^{o}C).
4. Mix samples gently by inversion of the tubes a couple of times, then

   allow to warm up to ~4 ^{O}C on ice for several minutes.
6. Centrifuge the cells at 3000g for 5 min at 4 ^{O}C
7. Resuspend cells in 1ml H_2O at 4^{o}C, transfer to a 2 ml safe lock Eppendorf tube and spin
8. Gently decant the liquid, snap freeze cell pellet on dry ice and store at -80^{o}C until required

3. *In Vitro* Transcription (IVT)

Two kinds of RNAs are produced by IVT: 1) non-yeast RNA to estimate RNA recovery in section 4. 2) synthetic RNA copies of the transcripts to be quantified, for use as standards in section 6). Templates for T7 transcription of the RiboSys RNAs can be obtained from David Barrass (david.barrass@ed.ac.uk).

A. PCR IVT Template

1. Set up PCR Reaction:-
   - 1.1. 5µl Expand High Fidelity Buffer (with 15mM Mg^{2+})(Roche)
   - 1.2. 1µl T7 forward primer (50mM) (see Table S3; Lar-T7 for the Lariat prem-T7 for mRNA and pre-mRNA IVT templates)
   - 1.3. 1µl Reverse primer (50mM)
   - 1.4. 1µl Template DNA
   - 1.5. dNTPs to a final concentration of 200µM each
   - 1.6. 0.5µl Expand High Fidelity Enzyme mix
   - 1.7. H_2O to 50µl
2. Mix and PCR using the cycle below:-
   - 2.1. 94ºC 30sec
   - 2.2. 60ºC 30sec
   - 2.3. 72ºC 30sec
   - 2.4. Repeat 2.1 to 2.3 for a total of 30 times
   - 2.5. 72ºC 10min
3. Run about 2µl on an agarose gel to check size and that a single product is produced.

B. IVT

1. Perform the IVT reaction using Ambion MEGAshortscript kit as the manufacturer's instructions using 5µl of the PCR reaction
2. DNase (Roche) as per the manufacturer's instructions
3. Phenol chloroform extract and ethanol precipitate as the instructions in the MEGAshortscript kit
4. Run 2µl of the RNA on an agarose gel
5. The RNA should be a single band of the predicted size, if not repeat, with regard to the troubleshooting guide in the MEGAshortscript kit instructions.
6. Quantify the RNA at A_{260} using a spectrophotometer as the manufacturer's instructions

4. RNA Extraction to Estimate RNA Recovery

(Modified from Tollervey and Mattaj. (1987). EMBO J. 6/2:469-76.)

GTC mix:

Guanidine Thiocyanate (Sigma G9277)	100g
H_2O	100 ml
Tris-HCl pH 8.0 1M	10.6 ml
EDTA pH 8.0 0.5M	4.24 ml
Dissolve 10 min at 60 – 70 °C	
β-mercaptoethanol	2.1 ml
sarkosyl 20%	21.2 ml

1. To 100 µl of a 1:1 mix of GTC and phenol add 100 pg of non-yeast IVT and 100 µl glass or zirconia beads.
2. Resuspend the cell pellet directly in the GTC/phenol/beads mix
3. Vortex for 5 minutes on a multitube vortexer at $4^{O}C$
4. Add 700 µl GTC/phenol mix
   Vortex and incubate at $65^{O}C$ for 5 - 15 min
5. Add 350 µl chloroform and 120 µl 3M NaOAc (pH 5.2)
   Vortex for 20 s
   Spin 5 min, room temperature

6. Transfer 550 µl of the upper phase into a new tube, add 500 µl phenol/chloroform
   Vortex 20 s and spin 5 min, RT

7. Transfer 500 µl upper phase into a new tube and extract with 500 ml chloroform.

8. Transfer 450 µl of the upper phase
   add 1 ml 100% ethanol (-20^{O}C)
   Incubate on ice for 10 minutes.

9. Spin 10 min, 20000g, 4^{O}C.

10. Wash pellet with 70% ethanol

11. Resuspend in 50 µl water.

12. Quantify and check RNA quality using a spectrophotometer as the manufacturer's instructions.

5. DNA Extraction

Sodium Citrate Buffer: 10% EtOH, 0.1M NaCitrate

1. Take the phenol and interface layer (the lower layer) left after the aqueous layer has been removed in step 6 of the RNA extraction procedure (section 4 above).
2. Remove and discard as much of the remaining aqueous layer as possible without disturbing the interface
3. Transfer the interface and organic layer to a new tube, leaving most of the beads behind
4. Add 240µl of 100% Ethanol incubate for 2-5 min at room temperature
5. Spin 2,000g for 5min
6. Remove and discard the liquid, re-spin and remove final dregs of liquid
7. Wash the pellet by adding 800µl of 0.1 M trisodium citrate in 10% ethanol and leave for at least 30min at RT, agitating gently
8. Spin as 5
9. Repeat 6 to 8 at least once more, twice more to give a cleaner pellet
10. Wash with 1.5ml 75% Ethanol, leave for at least 20min at RT, agitating gently
11. Spin as 5
12. Gently dry the pellet and resuspend in about 5µl of 0.8M NaOH for every 1OD of yeast culture used in the sample

13. Not all the components (such as membranes, contaminating proteins and remaining beads) will redissolve. To remove these spin at >13krpm for 5min and transfer the supernatant to a new tube
14. Quantify the DNA at A_{260} using a spectrophotometer according to the manufacturer's instructions

6. RT-qPCR (Reverse Transcription quantitative PCR)

A. Reverse Transcription

1. Prior to the conversion to cDNA, 10 μg of total RNA is treated with DNase1 (0.9U RQ1, Promega) as per the manufacturers protocol. Then incubate at 65 °C for 10 min to inactivate the DNase I.
2. To 5μg of DNase-treated RNA and primer, incubate at 70 ^{o}C for 5 min then place on ice. To this, then add a 10 μl reaction mixture containing 5x First strand synthesis buffer , 0.1M DTT, 10U RNase inhibitor (Roche) 10mM each dNTP , 250nm Ribo1 specific primers and 7.5U Transcriptor RNase H- (Roche). Followed by incubation at 55 ^{o}C for 1 -2 hours.
3. Treat the remaining 5μg of RNA as 2, except substitute H_2O for the Transcriptor to provide a no RT control to assess contamination.
4. After cDNA synthesis remaining RNA is hydrolysed by the addition of 15 μl of 0.1mg/ml RNaseA and incubate at 37^{o}C for 1 hour.
5. The cDNA is then diluted 1 / 20 with dH_2O.

B. Quantitative PCR (qPCR)

1. Set up a PCR reaction (in triplicate)
   1.1. . 5 μl 2x SYBR green qPCR mix (Invitrogen) containing 1/1000th volume of Rox
   1.2. 1μl primers (300nm each primer, for primers used see supplementary table S3
   1.3. 4 μl of cDNA template
2. Mix and PCR using the cycle below:-
   2.1. 94^{o}C for 2min
   2.2. 94°C for 10ssec
   2.3. 63°C for 10 sec
   2.4. 72°C 20 sec (acquire fluorescence)
   2.5. repeat 2.2 to 2.4 for a total of 50 times

   QPCR is also performed using the A.t. Lsm1 primer pair for estimating RNA recovery

C. Quantitation against IVT Standards

IVT RNAs are used as standards to quantify the RNA. If different RNA species are to be detected, the IVT RNA has to match the detected RNA species. For example, one is needed as a standard for the Lariat, a separate one for the mRNA and Exon, and the A.t Lsm1, and a single IVT RNA is suitable for the pre-mRNA, 3'SS and exon.

To take into account the efficiency of the reverse transcription reaction and the qPCR, the IVT RNA is spiked into the same amount (5µg) of total yeast RNA as in the assay samples. This total RNA should be from a strain without the Ribo reporter to avoid high background signal. The precise amount of RNA added depends on the species to be quantified and the linearity of the PCR reaction. A serial dilution of the added IVT RNA should be used that will exceed the expected range of the RNA to be quantified. As a guide, a 10-fold serial dilution covering the range 1µg to 1pg will generally suffice. The standards are treated like the samples for reverse transcription and qPCR.

D. Reproducibility

All RT-qPCR assays are performed in triplicate. An additional indication of the reproducibility and reliability of this approach can be obtained by measuring an endogenous transcript, e.g. *ACT1,* the level of which is unaffected by doxycyclin (Fig. S4). This shows that indeed the level of *ACT1* transcripts is constant during Ribo1 derepression and that the RT-qPCR assays are highly reproducible.

7. Quantitation of RNA in Copies per Cell

A. Estimate of Cell Number

Cell number per ml of culture can be determined using a CASY® 1 Cell Counter (Scharfe System, Germany) or a Nucleocounter (New Brunswick Scientific) according to the manufacturers' instructions or using a Chemacytometer (Thomm's chamber). For convenience, a standard curve can be generated, relating cell OD_{600} to cell number for each strain under the specific conditions used. The following formula is validated to convert OD_{600} to cell numbers per ml in the case of the Ribosys tetON and tetOFF strains grown in SD medium at OD_{600} 0.22 to 2.29.

Number of cells/ml (A) = $(OD_{600} \times M) - C$

Where M = slope of the OD versus cell no curve; C = intercept on X-axis

$$= OD_{600} \times 1x10^{7} - 4.75x10^{5}$$

Multiply by the culture sample volume (in ml) to get the total number of cells in the sample.

B. Estimate of Efficiency of Cell Lysis

The estimated cell lysis is given by the formula

$$\frac{DNAAmount(\mu g)}{CellNumber(A) \times genomicDNA}$$

DNA amount is total DNA extracted from the sample. Cell number is from above (A.) The *S. cerevisiae* genome is approximately 0.012 pg DNA (Kullman, B., Tamm, H. & Kullman, K. 2005. Fungal Genome Size Database. http://www.zbi.ee/fungal-genomesize). If the exact DNA content of the cell is known (such as by PI staining and FAC analysis) it should be used. For many haploid budding yeast strains, the DNA content averaged over the whole cell cycle will be approximately 1.4 genomes, because of the time between DNA synthesis and cell division but this will vary with growth conditions. Otherwise, twice the haploid (4x if diploid) figure provides a maximum estimate of DNA content, and therefore the minimum cell lysis efficiency.

For *S. cerevisiae* = $\frac{DNAAmount(\mu g)}{CellNumber(A) \times 1.2 \times 10^{-8} \times 1.4}$

C. Estimate of RNA recovery and RT Efficiency

This is simply given by the amount of RNA recovered (taking into account the proportion of total sample used in the RT step) as measured by QPCR against the standard curve / the amount spiked in

D. Estimate of RNA Amount

This is done by reading the Ct value of the sample on the standard curve produced with the dilution series of IVT RNA. This gives the number of ng (generally, but µg or pg are possible) of that species in 5 µg of the test RNA (as used in the RT step). From this, the amount of that species of RNA in the total sample can be calculated.

E. Estimate of Copy Number

To calculate the molecular weight of the IVT RNA the following formula is used:

(ΣA x 329.2) + (ΣU x 306.2) + (ΣC x 305.2) + (ΣG x 345.2) + 177

Where ΣA is the total number of adenosine residues in the sequence
ΣU is the total number of uracil residues in the sequence
ΣC is the total number of cytosine residues in the sequence
ΣG is the total number of guanine residues in the sequence
177 is for the 5' tri PO_4 group and the 3' OH.

The number of molecules of RNA per g is

R = $\frac{AvogadroNumber}{MolWt}$

Copy number is therefore:-

Cn = $R \times RNAamount(D)$

Copy number per cell can be estimated by:-

$$\frac{Cn}{NumberofCells(A) \times RNA\,\mathrm{Recovery}Efficiency(C) \times MinimumCellLysis(B)}$$