Published in final edited form as:

Methods Mol Biol. 2018 ; 1721: 63–72. doi:10.1007/978-1-4939-7546-4_6.

Total RNA Isolation and Quantification of Specific RNAs in Fission Yeast

Robert Roth¹, Hiten D. Madhani², Jennifer F. Garcia¹

¹Department of Molecular Biology, Colorado College, Colorado Springs, USA

²Department of Biochemistry and Biophysics, University of California, San Francisco, USA

Abstract

The fission yeast, *Schizosaccharomyces pombe*, is an important model organism for investigations of gene regulation. Essential to such studies is the ability to quantify the levels of a specific RNA. We describe a protocol for the isolation and quantification of RNA in *S. pombe* using reverse-transcription followed by quantitative PCR. In this procedure, the cells are lysed using zirconia beads, then total RNA is selectively isolated away from proteins and DNA using the Trizol reagent. Contaminating DNA is then removed from the RNA by using TURBO DNase, which is easily inactivated and requires no subsequent clean-up step. The RNA is then reverse transcribed into cDNA using random nine-mers and oligo dT primers. Quantitative PCR using SYBR green is then performed to quantify RNA levels. This protocol has been tested on several *S. pombe* genotypes and generates highly reproducible results.

Keywords

RNA extraction,; Reverse-transcription; qPCR; cDNA synthesis; DNase; RNase; RNA quantification; SYBR green

1 Introduction

The fission yeast, *Schizosaccharomyces pombe*, is a genetically tractable model organism used to investigate a range of questions in cell biology, including cell cycle regulation [1], cell morphogenesis [2], checkpoint regulation [3], and chromatin regulation [4], as many of these mechanisms are conserved. In particular, mechanisms that control gene expression in *S. pombe* form a particularly important area of investigation, because of its differences with the yeast, *S. cerevisiae*. Two notable and distinguishing features of *S. pombe* when compared to *S. cerevisiae* are its relative intron-richness [5] and the presence of a nuclear RNAi pathway coupled to repressive histone methylation [6].

An accurate description of the state of a cell requires knowledge of the expression levels of each gene. There are many laboratory techniques that offer a way to quantify and analyze gene expression, including western blotting to measure protein levels as well as northern blotting and reverse transcription (RT) followed by quantitative PCR (qPCR) to quantify

3. Trizol Reagent (Ambion).
4. 2 ml microcentrifuge tubes (locking or screw cap).
5. 1.5 ml microcentrifuge tubes.
6. Vortexer.
7. Disruptor Genie (or multitube/platform vortexer, or bead-beater).
8. Chloroform.
9. Isopropanol.
10. 75% ethanol prepared with RNase-free water.
11. Speed-Vac Instrument.
12. TURBO DNA-free kit (Ambion).
13. dT20N oligonucleotide (5'-TTT TTT TTT TTT TTT TTT TT[CAG]-3')
14. random nine-mer oligonucleotide (5'-NNN NNN NNN-3').
15. 0.2 ml PCR tubes with caps.
16. 10 mM dNTPs prepared in RNase-free water.
17. Superscript III Reverse Transcriptase (Invitrogen).
18. 10× PCR buffer (100 mM Tris, pH 8.3; 500 mM KCl).
19. 20 mM MgCl₂.
20. 2× SYBR green I (Invitrogen) (*see* Note 2).
21. 2.5 mM dNTPs.
22. Taq Polymerase (such as Amplitaq, Applied Biosystems).
23. Gene specific primers.
24. White 0.2 ml 96-well PCR plate (Biorad MLL9651).
25. Optically clear PCR plate sealing film (such as Biorad MSB1001).
26. Real-time PCR machine with the ability to read SYBR green fluorescence (Biorad CFX96 Touch).

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¹·RNase-free water can be made by treating water with Diethylpyrocarbonate (DEPC) to deactivate RNases. DEPC is added to a final concentration of 0.1% (v/v) to water and letting it stir overnight in a fume hood. The DEPC-treated water is then autoclaved to destroy the DEPC. DEPC is toxic and must be handled appropriately.

²·2× SYBR green I is diluted in ddH₂O from either a 100× or 10,000× stock made in Dimethyl sulfoxide (DMSO). 2× SYBR green I made with water must be used immediately.

3 Methods

3.1 Total RNA Extraction and Purification

1. Thaw frozen cell pellet containing 50–80 OD₆₀₀ of cells on ice and resuspend in 1 ml of Trizol Reagent into a 2 ml microcentrifuge tube. Briefly vortex to mix (*see Note 3*).
2. Add ~250 µl volume of zirconia beads and homogenized in a Disruptor Genie for 2.5 min. Let the samples rest on ice for 2.5 min and then homogenize for another 2.5 min (*see Notes 4 and 5*).
3. Spin the tubes at $20,000 \times g$ at 4 °C for 10 min to separate the organic and aqueous phases.
4. Transfer the aqueous (top) phase containing the RNA using a micropipette into a 1.5 ml microcentrifuge tube and add 200 µl of RNase-free chloroform (*see Note 6*).
5. Vortex for 15 s and then let it sit at room temperature for 10 min.
6. Spin at $20,000 \times g$ at 4 °C for 10 min.
7. Transfer the aqueous phase to a new tube and add 500 µl of RNase-free chloroform. Briefly vortex to mix and spin at $20,000 \times g$ at 4 °C for 10 min.
8. Transfer the aqueous phase to a new tube and add 500 µl of RNase-free isopropanol. Briefly vortex to mix and spin at $20,000 \times g$ at 4 °C for 10 min (*see Note 7*).
9. Remove the supernatant and wash pellet with 1 ml of 75% ethanol (prepared with RNase-free H₂O). Vortex to mix and spin at $20,000 \times g$ for 5 min at room temperature.
10. Remove the supernatant and dry pellets in a Speed-Vac instrument briefly and without heat for approximately 5–10 min. Alternatively, RNA pellets can be dried at room temperature by leaving tubes uncapped for 5–10 min (*see Note 8*).
11. Resuspend pellet in 50–200 µl RNase-free H₂O (*see Note 9*).

³.To prepare cell pellets, culture containing 50–80 OD of cells (i.e., 100 ml culture with an OD₆₀₀ = 0.5–0.8) was pelleted at $3000 \times g$ for 2–5 min. The media was aspirated off and the pellets were then immediately flash frozen in liquid nitrogen and stored at –80 °C. Cell pellet can also be washed prior to flash freezing. Trizol reagent contains phenol, a chemical that is hazardous and can cause chemical burns. Wear appropriate personal protective equipment such as phenol resistant gloves, protective eyewear, and a lab coat.

⁴.A PCR tube can be utilized as a good 250 µl volume equivalent for scooping the zirconia beads.

⁵.The homogenization can be done using a Disruptor Genie, multi-tube/platform vortexer, or a bead-beater. This protocol is optimized for the Disruptor Genie and the length of homogenization should be adjusted accordingly. Additionally, if using a different method, check that the samples do not warm up significantly during homogenization.

⁶.When transferring the aqueous phase be careful not to transfer any of the white substances found between the organic and aqueous phases. Exclusion of this material will improve the quantity of the RNA isolated.

⁷.After adding the isopropanol and pelleting the RNA, carefully pipet off the supernatant as the RNA pellet can easily slide around and be pipetted up.

⁸.If a Speed-Vac instrument is not available, removing as much supernatant as possible and leaving the microcentrifuge tubes uncapped with a Kimwipe draped over the top for approximately 10 min should be sufficient to let the residual alcohol evaporate from the RNA pellet.

⁹.Quantify the amount of RNA that was isolated RNA and determine the yield and purity using a spectrophotometer such as a Nano-drop.

3.2 DNase Treatment

1. Dilute 30 µg RNA in 45 µl RNase-free H₂O (*see* Note 9).
2. Add 5 µl of TURBO DNA-free 10× buffer and 1 µl TURBO DNA-free DNase to the sample and incubate at 37 °C. After 30 min, add another 1 µl TURBO DNA-free DNase to the sample and incubate for another 30 min (*see* Note 10).
3. Add 10 µl of TURBO DNase inactivation reagent and mix well. Incubate at 25 °C while occasionally mixing by hand for 5 min.
4. Spin at 10,000 × *g* for 90 s and transfer approximately 50 µl of supernatant to a 1.5 ml microcentrifuge tube.

3.3 cDNA Synthesis

1. Mix ~5 µg of DNase-treated RNA in a 0.2 ml PCR tube and add the following: (*see* Note 11):

~5 µg	DNase treated RNA
1 µl	0.5 µg/µl dT ₂₀ N
1 µl	0.5 µg/µl random 9-mer
1 µl	10 mM dNTPs (prepared in RNase-free H ₂ O)
Add RNase-free H ₂ O to 13 µl	

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2. Incubate at 65 °C for 5 min and then let it sit on ice for 5 min.
3. Add the following to reaction mix as follows:

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4 µl	5× FS buffer
1 µl	0.1 M DTT
40 units 200 U/µl Superscript III reverse transcriptase	
1.8 µl	RNase-Free H ₂ O

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4. Incubate at 25 °C for 5 min.
5. Incubate at 50 °C for 60 min.
6. Incubate at 70 °C for 15 min to inactivate the RT.
7. Optional: 5 units of RNase H can be added to each reaction to remove the RNA. Incubate at 37 °C for 30 min.

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¹⁰DNase treatments can be performed with less than 30 µg of RNA. For DNase treatments consisting of less than 2 µg of RNA, use only 1 µl of TURBO DNA-free DNase. Allow the treatment to incubate for an hour at 37 °C.

¹¹For each RNA sample, cDNA synthesis should be performed with and without RT. The “no RT” will serve as a control for contaminating DNA present in the RNA isolation.

3.4 Quantitative PCR

1. Dilute cDNA appropriately and set up DNA for standard curves (*see* Note 12–13).
2. For each RNA sample you which to quantitate use the following 1× recipe to set up a Master Mix based on the number of samples and standards that will be analyzed (*see* Note 14):

2.5 µl	10× PCR buffer
2.5 µl	20 mM MgCl ₂
2.0 µl	2.5 mM dNTP
4.0 µl	2× SYBR Green
2.5 units	Taq polymerase
0.5 µl	10 µM primer 1
0.5 µl	10 µM primer 2
Add ddH ₂ O to 20 µl	

3. Add 20 µl of master mix to 5 µl of diluted cDNA sample or standard. Run the following program (*see* Note 14):

Step 1	95 °C	3:00
Step 2	95 °C	0:20
Step 3	56 °C	0:20
Step 4	72 °C	0:40, Plate Read
Step 5	Repeat Steps 2–5 39×	
Step 6	Melt Curve 65–95 °C: Increment 0.5 °C 0:02	
Plate read		

3.5 Analysis of qPCR for Spurious Products

1. Generate melt peaks from the melt curve data. Check that the melt peaks for the DNA standards contain single peaks that have similar profiles. Two or more distinct peaks are indicative of two or more PCR products. Remove from analysis any standards that do not meet these criteria. See the examples of melting curves in Fig. 2 (*see* Note 15).

¹².Typically, our standard curves use a few microliters of cDNA from each “plus RT” reaction mixed together. This cDNA mixture is then diluted successively to create a standard curve to compare our samples. The standard curve differs for each primer set used, but it typically starts out with the following dilutions: 1:10, 1:50, 1:250, 1:1250, and 1:6250.

¹³.The cDNA is diluted so that it will fall within the range of the standard curve. Usually for abundant transcripts like Actin, the cDNA is diluted with H₂O 1:100. For low abundant transcripts such as RNAs expressed from heterochromatin, 1:10 dilutions are prepared.

¹⁴.When setting up your master mix, consider running each “plus RT” sample in triplicate, the DNA standards and “no RT” samples in duplicate, and a “no template control” (dH₂O only) in duplicate.

¹⁵.qPCR reaction volumes will vary based on the machine and be adjusted accordingly.

2. The melt peaks of the “no RT” and “no template” controls should be distinctly different from the PCRs that contain cDNA as there should be no DNA amplified within these samples. The melt curves from these controls represent spurious PCR products (such as primer dimers) or contaminating DNA present in the RT-qPCR reaction or the RNA sample. Remove from analysis any standard or sample that display similar peaks to these controls. *See example in Fig. 2 (see Note 16).*

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¹⁶Primers are typically designed and tested to generate small PCR products (80–200 bp), generally amplify a region near the 3′ end of the RNA of interest, have a melting temperature equal to or greater than 60 °C, and produce a single melt curve peak with various concentrations of cDNA template.

Fig. 1.
Flowchart of RNA extraction and RT-qPCR in *S. pombe*

Fig. 2.
Melt curves representing the various PCR products after qPCR