Analysis of rRNA synthesis using quantitative transcription run-on (qTRO) in yeast

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Comparative transcriptional analyses require appropriate and precise normalization. Here we describe a modified transcription run-on (TRO) method, named quantitative TRO (qTRO), that allows quantification of nascent transcription activity. The most critical improvement it introduces is a new standardization method for RNA isolation and hybridization steps, enabling transcription activity quantification and comparative biological analysis. We used this technique with chromatin immunoprecipitation to investigate RNA polymerase I (RNAPI) transcription activity and its rDNA gene profiles in *Saccharomyces cerevisiae*. We designed a set of new oligonucleotide probes complementary to nascent ribosomal RNA (rRNA) transcripts and standardized their hybridization strength. The qTRO method could be successfully implemented to study RNAPI transcription in response to oxidative stress and in two mutant strains with impaired rRNA synthesis.

One of the most widely used techniques to investigate nascent transcription activity is RNA polymerase chromatin immunoprecipitation (ChIP) or ChIP sequencing, which allows the mapping of polymerase densities along the targeted genes but may not directly reflect transcription activity in regions where the enzyme pauses. Subsequently, the transcription run-on (TRO) method allows the measurement of nascent transcript synthesis.

The classical TRO technique relies on nascent transcript labeling during transcriptional pulse, followed by total RNA isolation and labeled target detection. TRO approaches in yeast are based on sarkosyl detergent treatment that permeabilizes cell and nuclear membranes to efficiently deliver ³²P-labeled UTP into the nucleus [1–4]. After transcriptional pulse, total RNA is isolated, partially fragmented, hybridized to gene-specific probes (M13 ssDNA, oligonucleotides, denatured plasmids, or *in vitro* transcribed RNA) immobilized on a nylon membrane.

After hybridization, quantitative signal analysis demonstrates the transcription activity of individual genes and identifies transcription termination defects, such as read through [5–7].

Total transcription rates are measured by techniques that utilize radioactive labeling of nucleic acids *in vivo* during culture, but they do not allow the analysis of polymerase activity on specific transcription units [8]. Conversely, recent developments in high-throughput sequencing methods and nucleotide chemistry, including global run-on, native elongating transcript sequencing, and precision nuclear run-on sequencing, have enabled several genome-wide applications of TRO in yeast and the quantification of RNA polymerase transcription rates [9–12]. Although these approaches have revolutionized research on transcription, their use is still limited because of their labor intensiveness and high cost.

Despite the many advantages of the TRO method, additional controls are required to increase its analytical and

comparative potential. This method typically does not directly compare the transcription levels obtained in different experimental conditions or strains because of technical limitations. Therefore, we developed a modified quantitative TRO (qTRO) technique to ensure repeatability and reliability of obtained measurements of nascent transcription activity and validated it on RNA polymerase I (RNAPI) transcription.

rRNA-encoding genes in yeast are clustered in an 1–2 Mb region comprising ≤200 tandem copies of the rDNA locus (9.1 kb each) located on the right arm of chromosome XII. The region is transcribed by RNAPI as 35S pre-rRNA precursor molecules, which are subsequently exo- and endo-nucleolytically processed into mature 18S, 5.8S and 25S rRNA (Figure 1A; reviewed in [13,14]). rRNA synthesis constitutes approximately 60% of all transcription activity in the nucleus, making it an excellent model to validate our modified qTRO approach [15].

METHOD SUMMARY

Our quantitative transcription run-on (qTRO) protocol involved: i) the collection of equal number of cells for each condition; ii) addition of the exogenous, *in vitro* transcribed and radiolabeled spike-in RNA to each sample during cell homogenization; iii) utilization of the oligonucleotide probe complementary to the spike-in RNA for monitoring RNA recovery by comparing hybridization signals between blots; and iv) correction of the hybridization signal strength for 75–80-nt probes complementary to different regions of the rRNA by hybridization to *in vitro* transcribed and radiolabeled 35S rRNA precursors.

Figure 1. Principles of qTRO and RNAPI activity profile. (A) The rDNA gene structure diagram with marked positions of probes designed for qTRO. The individual rDNA unit is composed of the RNAPI transcribed gene encoding 35S rRNA, which is the precursor to the mature 18S, 5.8S and 25S rRNA, separated by internal transcribed spacers (ITS1 and ITS2). 35S rRNA is flanked by 5' and 3' external transcribed spacers (ETSes) and the intergenic spacers IGS1 and IGS2. Each unit is separated by RNAPIII-transcribed 5S rRNA gene. Arrows indicate the direction of RNAPI and RNAPIII transcription. (B) The outline of qTRO approach principles and two exemplary hybridization results. Signals for *AtLSM1* probe used as RNA spike-in controls are marked with red frames. (C, D) Oligonucleotide probes standardization and wild-type RNAPI transcription profile. (C) Representative blot used for correcting the hybridization strength of oligonucleotide probes (hybridization with *in vitro* transcribed, [α^{32} P] UTP-labeled 35S pre-rRNA) and their *in vivo* signals in Rpa34-13myc-tagged W303 strain. (D) Comparison of the *in vitro* probe signals with uncorrected *in vivo* and corrected RNAPI transcription profile. Data are presented as signals normalized to the 18S1 probe and represent mean values of three biological replicates with standard deviations. Yeasts were grown in liquid YPD medium (yeast extract, peptone, 2% glucose) at 30°C, 45 ml of 0.35-OD₆₀₀ cultures were collected. qTRO: Quantitative transcription run-on; RNAPI: RNA polymerase I; RNAPIII: RNA polymerase III.

A summary of the qTRO protocol and representative blots are shown in Figure 1B. Collecting equal number of cells is crucial for this technique. We strongly

recommend checking the correlation of the actual cell number with optical density of cultures measured at 600 nm wavelength (OD₆₀₀) using a Thoma cell counting chamber. The optimal OD₆₀₀ range for 50-ml cultures is 0.2–0.4. To simplify the procedure for multiple analyses, we decided to use standard curves of cell numbers at the same OD₆₀₀ range that were generated before the qTRO experiments and collect different volumes of culture to maintain consistent cell numbers. In cases where a single strain needs to be analyzed after a brief change in growth conditions, collection of cultures with equal OD is acceptable. In qTRO, we decided to not include in-cell control to monitor cell lysis efficiency, which may depend on many factors, including the cell shape and cell wall thickness. However, the protocol can be expanded to include in-cell controls for monitoring homogenization,

including genomic DNA detection using qPCR of interphase material obtained during the phenol/GTC extraction of RNA [16]. However, omitting this control results in a simpler protocol that is much easier to perform due to the high radioactivity of the preparations obtained.

To standardize RNA isolation and subsequent hybridizations, synthetic 32 P-labeled spike-in control transcript of the *LSM1* fragment from *Arabidopsis thaliana* was used, namely *AtLSM1* [16,17]. Equal amounts of *in vitro* transcribed *AtLSM1* (500 counts per second [cps]) were added to each single qTRO reaction before RNA extraction from cells. Thus, comparing the hybridization signal for the *AtLSM1* probe between blots in one experimental set allowed us to measure the correction factors of RNA isolation efficiency for individual blots and calculate TRO hybridization values for all probes used (Figure 1B).

Next, we designed a set of 16 oligonucleotide probes (Figure 1A, Supplementary Tables S1 and S2). To limit the possibility of secondary structure formation, we used sequences with a GC content of <60% and relatively high A content as they may preferentially pair with 32 P-labeled U in the nascent transcript. 12 of the 16 probes were complementary to

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mature rRNA species derived from the primary RNAPI transcript (five for 18S, one for 5.8S and six for 25S rRNA). The probe set also included additional oligonucleotides complementary to the *SCR1* and 5S rRNA, RNAPII and RNAPIII transcripts respectively, and the standardization *AtLSM1* probe. Moreover, the *agaA* transcript from *Aspergillus nidulans* served as a negative control [18]. BLAST searches of the *S. cerevisiae* genome revealed no target sequences for the *agaA* probe; thus, it was used for background correction. The value of the *agaA* hybridization signal was subtracted from the obtained signals of other probes in the set.

Importantly, we standardized the signals of 18S, 5.8S and 25S rRNA probes. When calibrating using classical TRO, the obtained signals are divided by the number of ^{32}P -labeled U in the target sequence of the nascent transcript. However, this did not reveal the hybridization strength of individual probes. In our qTRO protocol, probe signals were standardized against *in vitro* transcribed [$\alpha^{32}\text{P}$] UTP-labeled 35S rRNA. First, we optimized the *in vitro* transcription reaction conditions to increase T7 RNA polymerase processivity as we observed several preterminated transcripts under the standard assay conditions applied to *AtLSM1*. Performing the reaction at 16°C and altering the proportion of the limiting nucleotide (UTP) to other nucleotides, resulted in a significant increase in T7 RNA polymerase processivity (Supplementary Figure S1A). Although not all polymerases reach the run-off position in the template, two major ~6.5-kb RNAs are detected using both the most proximal (18S1) and distal (25S6) probes for 35S rRNA, as verified by northern blotting (Supplementary Figure S1B). Next, *in vitro* transcribed 35S rRNA (1500 cps) was processed similarly to RNA isolated from cells after ^{32}P -labelling. To reflect the conditions during the RNA fragmentation and hybridization in qTRO, hot 35S rRNA was mixed with approximately 500 µg of carrier-unlabeled total RNA, corresponding to the amount isolated from 10–20 OD₆₀₀ units of cells using the phenol/GTC method. Then it was treated with NaOH and hybridized with probes. Hybridization strength factors for each 35S rRNA probe (Supplementary Table S2) were calculated using the obtained signals (Figure 1C); the values were used

to correct the final *in vivo* TRO signals (Figure 1D). The obtained data were used to model 35S RNA transcription profiles, which revealed two distinct transcription peaks of similar amplitude in 18S and 25S rRNA (Figure 1D). A substantial decrease in RNAPI activity within the 5.8S rRNA-coding region could be explained by co-transcriptional cleavage of the pre-rRNA at the A2 site in ITS1 [19]. Another possible explanation is an increase in R-loop (RNA/DNA hybrids) formation, demonstrated by ChIP-qPCR and reflected in the decreased RNAPI occupancy over ITS1-ITS2, as observed by electron microscopy [20]. Subsequently, we investigated RNAPI occupancy over rDNA using a c-myc-tagged Rpa34 subunit and ChIP-qPCR, similar to that in previous studies [7,20]. The density distribution profile of RNAPI over the rDNA correlated with the transcription profile obtained by the qTRO approach (Figures 1D and 2A).

We investigated the possibility of conducting an efficient qTRO using flash-frozen cells, which would introduce a safe stopping point for the experiment, similar to that in biotin-genomic run-on [21]. We found that hybridization signals reduced >30% for qTRO performed using flash-frozen cells compared with qTRO performed using fresh harvested cells (Supplementary Figure S2). Therefore, we recommend avoiding freezing when working with strains or growth conditions that result in low RNAPI transcription levels and proceeding immediately with qTRO.

Next, to assess the suitability of the qTRO method in comparative biological experiments, we applied it to analyze 35S rRNA transcription under oxidative stress conditions, wherein rDNA and ribosomal protein-coding genes transcription inhibition is expected [22]. To compare the results obtained using qTRO and ChIP-qPCR methods, experiments were performed using a c-myc-tagged Rpa34 RNAPI subunit in a W303 background. As expected, the qTRO method demonstrated a significant (≤60%) decrease in RNAPI transcription activity within 5 min of oxidative stress (Figure 2B). Similarly, the observed qTRO rRNA transcription profile reflected RNAPI occupancy over the rDNA, as mapped by ChIP-qPCR (Figure 2A). Thus, the decrease in transcription under oxidative stress conditions results from polymerase complex dissociation

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Figure 2. Decrease in rRNA transcription correlates with RNAPI dissociation from the template upon oxidative stress. (A) ChIP assay revealing loss of RNAPI association on rDNA in yeast cells after induction of oxidative stress. Relative RNAPI occupancy over rDNA gene in cells treated with 1 mM H₂O₂ for 5, 15 and 30 min. Graph shows mean values of three biological replicates with standard deviations, which were corrected for background. qPCR primer positions are denoted on the rDNA scheme. The Rpa34–13myc-tagged strain was grown in YPD medium at 30°C and treated with 1 mM H₂O₂ up to 30 min. (B) Oxidative stress response involves strong RNAPI inhibition, as revealed by qTRO. Representative qTRO data obtained for control and cells treated with 1 mM H₂O₂ for 5 and 15 min. Absolute hybridization signals (corrected vs *AtLSM1* control signal and probe hybridization strength) in photo-stimulated luminescence units and standardized data presented as signal normalized to the 18S1 probe are shown. Bars represent mean values of three biological replicates with standard deviations. Strain and growth conditions as on panel A, 45 ml of 0.35-OD₆₀₀ cultures were collected. (C) Western blot analysis of Rpa34–13myc protein level with anti-myc tag antibodies after inducing oxidative stress. ChIP: Chromatin immunoprecipitation; qTRO: Quantitative transcription run-on; RNAPI: RNA polymerase I.

from the template rather than a decrease in the steady state levels of Rpa34, as verified by western blotting (Figure 2C).

Finally, to fully validate our method, we used our qTRO approach to evaluate the RNAPI transcription activity in a *rpa12Δ* strain with elevated RNAPI processivity and in *spt4Δ* with decreased rRNA transcription. Rpa12, a subunit of RNAPI, has intrinsic RNA cleavage activity and shares homology to the small subunit Rpb9 of RNAPII and Rpb11 of RNAPIII, all of which are involved in transcription termination. Deletion of the *RPA12* gene leads to minor growth effects but no pre-rRNA processing defects; however, a visible increase in RNAPI processivity is manifested by elevated transcription levels and altered RNAPI termination [5]. While measuring the RNAPI transcription activity in qTRO, we observed an average of an approximately 6.1-fold increase in 35S rRNA synthesis in the *rpa12Δ* strain compared with that in the WT strain, with a maximal signal change of approximately 12-fold for the 5.8S rRNA probe (Supple-

mentary Figure S3A). Except for changes in the 5.8S rRNA-coding region, the overall RNAPI transcription profile in the mutant resembles that in the WT. Our data are consistent with previous observations suggesting a role of Rpa12 in polymerase fidelity control and demonstrating that its absence results in increased RNAPI velocity [5].

The DRB sensitivity-inducing factor (DSIF) complex is composed of Spt4 and Spt5 and serves as a transcription elongation factor for both RNAPI and RNAPII. DSIF binds ssRNA, has diverse functions in transcription regulation (elongation, silencing, RNA processing and quality control), and is engaged in transcription-coupled DNA repair. Deletion of the *SPT4* gene results in minor growth effects, temperature sensitivity at 37°C, and pre-rRNA processing defects. A previous study has demonstrated that the total RNA synthesis rate in *spt4Δ* is approximately 70% of that in the WT strain [23]. Consistent with these findings, our qTRO data showed that in

spt4Δ mutants, RNAPI transcription is reduced to approximately 61% of that in the WT strain (Supplementary Figure S3B), but the overall RNAPI transcription profile remains unchanged.

Here we reported a new method for calibrating and quantifying yeast TRO that we successfully validated by using it to compare RNAPI transcription levels under different biological conditions and in different mutants. This modification of the TRO protocol allows rapid and direct comparison of transcription levels between mutants or conditions of interest. The results obtained by qTRO will serve as adequate preliminary results for further verification by complementary methods. The detailed qTRO protocol is available with this article.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/BTN-2018-0073

Author contributions

MK conceived the project. MK designed and performed the majority of experiments including all qTRO analyses. SM performed ChIP-qPCR experiments and optimized 35S rRNA transcription *in vitro*. MK and SM wrote the article.

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