

Chapter 2

Genomic-Wide Methods to Evaluate Transcription Rates in Yeast

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Abstract

Gene transcription is a dynamic process in which the desired amount of an mRNA is obtained by the equilibrium between its transcription (TR) and degradation (DR) rates. The control mechanism at the RNA polymerase level primarily causes changes in TR. Despite their importance, TRs have been rarely measured. In the yeast *Saccharomyces cerevisiae*, we have implemented two techniques to evaluate TRs: run-on and chromatin immunoprecipitation of RNA polymerase II. These techniques allow the discrimination of the relative importance of TR and DR in gene regulation for the first time in a eukaryote.

Key words: Yeast, *Saccharomyces cerevisiae*, Transcription rate, Functional genomics, ChIP-on-chip, Run-on

1. Introduction

Transcription rate (TR) is the rate at which RNAs are produced as molecules per time unit. Measurement of TRs is not as straightforward as the measurement of mRNA amounts (RA). Even at the individual level, the TR of a given gene has been rarely measured because of the difficulty of quantifying nascent RNA molecules. One possibility of evaluating TR is by measuring the RNA polymerase densities in the transcribed regions of the genes. Since each elongating enzyme has a single nascent RNA molecule, the number of RNA polymerases on a gene reflects the number of RNAs being produced, while density reflects the TR if we assume a constant RNA polymerase speed. RNA polymerase II (Pol II) density can be counted by either the run-on (1) or the chromatin immunoprecipitation (chIP) techniques using specific antibodies (Abs).

The run-on technique can be used in many kinds of eukaryotic cells prior to nuclei isolation (2, 3). However, whole cells can be used only in yeast because sarkosyl detergent permeabilizes

cell membranes and allows labeled UTP utilization for RNA synthesis (1). This permits an instantaneous labeling of the physiologically real RNA transcription. We adapted the run-on technique to the genomic scale [genomic run-on (GRO)] using [α - ^{33}P]rUTP labeling and nylon macroarray hybridization (Figs. 1 and 2, and ref. 4). Using GRO, the nascent TRs for all the genes of an organism have been calculated for the very first time. Since the experiment includes a parallel RA determination, the mRNA stabilities can be calculated at the genomic scale if considering steady-state conditions (4, 5) or even under non-steady-state conditions (6, 7). This utility of the GRO technique will be discussed in a companion chapter of this book (8). Similar protocols have been used in other eukaryotes, but without a real determination of TRs (2, 3). The GRO technique has also been adapted to massive parallel sequencing technologies but, again, without TR calculation (9).

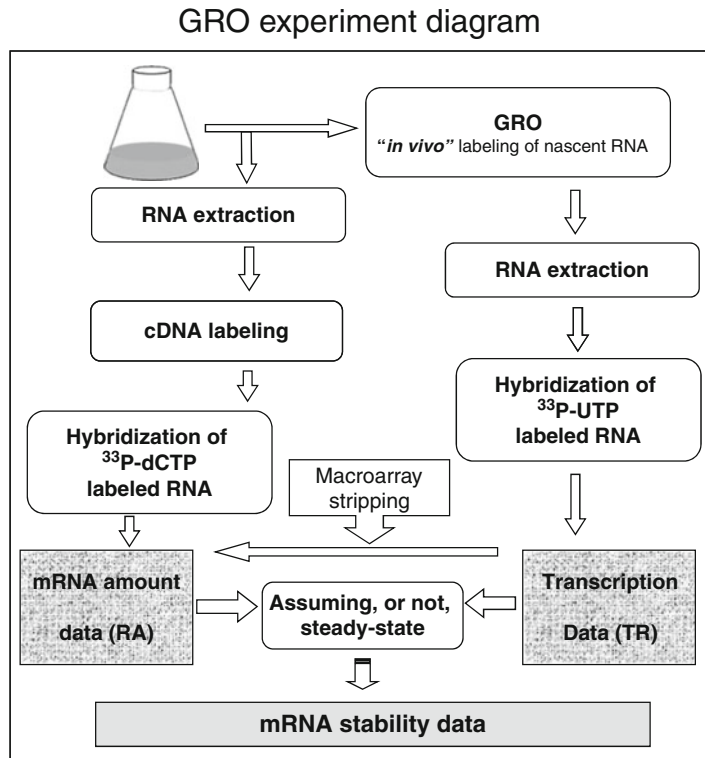


Fig. 1. Genomic run-on protocol for simultaneous TR and RA measurements. Grown cells are subjected to two independent protocols: GRO for nascent RNA labeling (*right*) and direct RNA extraction (*left*). The data from the GRO hybridized macroarrays are used to obtain transcription rates (TR) after normalization and corrections. The data from successive cDNA hybridization onto the same macroarray (after stripping it) are used to obtain mRNA amounts (RA). If one assumes steady-state conditions for mRNA amounts, it is possible to calculate mRNA stability data by dividing RA by TR. If there is no steady-state, a mathematical approximation is also possible see ref. 15.

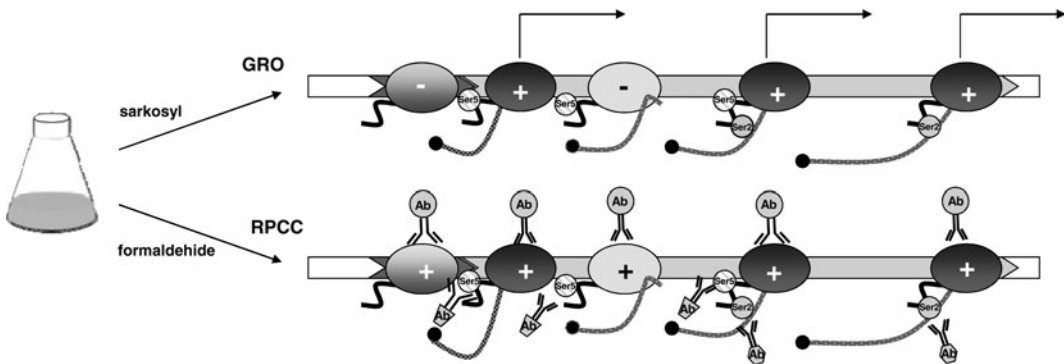


Fig. 2. Comparison of the GRO and RPCC methods. Different forms of Pol II molecules (*ovals*) are bound to a transcription unit (*horizontal rectangle*). Pol II molecules are represented with a CTD tail that can be, or not, modified in Ser5 and/or Ser2 (*dashed circles*) and with or without an mRNA molecule (long string with a *filled circle*, 5' cap). All of them are cross-linkable to the adjacent DNA sequences. If a “general” Ab is used in the RPCC method (such as 8WG16, which recognizes hypophosphorylated molecules, but also others (21) or Ab against tags is added to a Pol II subunit, different forms represented), all the cross-linked Pol II (all kinds of *ovals*) are immunoprecipitated. If specific Abs against the post-translational modifications are used, only those molecules will be precipitated. Run-on, however, only labels true elongating Pol II molecules (*dark ovals*), as well as the other nuclear RNA polymerases (I and III, not shown).

On the other hand, RNA polymerase molecules have been shown to be cross-linked to transiently bound DNA sequences (10, 11). The scaling of this method at the genomic level using DNA chips has been demonstrated for human cells (12) and yeast cells (13–15) using tiling arrays. These studies proved very powerful in terms of the description of the RNA polymerase distribution within the genome and the genes, but they were not used to calculate TRs. However, the use of DNA arrays containing whole ORF probes enables the calculation of an average distribution of Pol II density over the genes. We call this method RNA Polymerase II ChIP-on-chip (RPCC) (Fig. 2). Although the RPCC technique may be used to calculate the TRs in yeast, it is technically more complex than the GRO technique and, moreover, is affected by a higher background due to the unavoidable amplification of co-precipitated nonbound DNA, which is typical of ChIP. This results in a narrower dynamic range than that seen in the GRO technique.

Interestingly, the comparison of RPCC and GRO methods allows the detection and correction of technique-specific biases (V. Pelechano et al., in press). Moreover, the comparison between the presence of Pol II and the elongation activity measured by GRO allows the discovery of biological differences in the way in which the genes are transcribed (16). The RPCC can be done using any antibody that recognizes Pol II. However, the quality of the results depends on the antibody's affinity. We have successfully used Abs against either a tagged Pol II or the different phosphorylation forms of the carboxy terminal domain (CTD) of its largest subunit. Abs against other Pol II subunits may also be used (13, 15).

2. Materials

2.1. Run-On and Macroarray Hybridization

1. YDP medium: 1% w/v, yeast extract, 2% w/v, peptone, 2% glucose. Store at room temperature (see Note 1).
2. 10 and 0.5% w/v, L-laurylsarcosine (sarkosyl, Sigma–Aldrich Inc., St. Louis, MO)/in H₂O. Store at room temperature.
3. 2.5× Transcription buffer: 50 mM Tris–HCl, pH 7.7, 50 mM KCl, 80 mM MgCl₂. Store at room temperature (see Note 2).
4. ACG mix (10 mM each ATP, CTP, GTP, Roche, Mannheim, Germany). Store frozen.
5. 0.1 M DTT (Invitrogen, Carlsbad, CA). Store frozen.
6. [α -³³P]rUTP (~3,000 Ci/mmol, 10 μ Ci/ μ L, PerkinElmer, Waltham, MA). Store at 4°C (see Note 3).
7. Transcription mix: 120 μ L of 2.5× Transcription buffer, 16 μ L AGC mix, 6 μ L 0.1 M DTT, and 16 μ L of [α -³³P]rUTP. Prepare fresh (see Note 4).
8. LETS buffer: 100 mM LiCl, 10 mM EDTA, 10 mM Tris–HCl, pH 7.5, 0.2% w/v, SDS. Store at room temperature.
9. Acid phenol:chloroform:isoamyl alcohol (125:24:1), equilibrated with water, not buffered. Store at 4°C.
10. 5 M Lithium chloride. Store at room temperature.
11. Hybridization solution: 0.5 M sodium phosphate buffer, 1 mM EDTA, 7% w/v, SDS, pH 7.2, 100 μ g/mL sonicated salmon sperm DNA. Do not autoclave. Store at room temperature. Add the DNA (stored frozen in 10 mg/mL solution in small aliquots) just before use (see Note 5).
12. Wash buffer I 1× SSC, 0.1% w/v, SDS and wash buffer II 0.5× SSC, 0.1% w/v, SDS. 20× SSC is 300 mM Na citrate, 3 M NaCl, pH 7.0 adjusted with HCl. Store at room temperature (see Note 5).
13. 1 M and 50 mM NaOH. Store at room temperature.
14. Neutralizing buffer: 50 mM Tris–HCl, pH 7.5, 0.1× SSC, 0.1% w/v, SDS. Store at room temperature.
15. Stripping solution: 5 mM sodium phosphate buffer, pH 7.0, 0.1% w/v, SDS. Store at room temperature.
16. Yeast nylon macroarrays. Described in (17).

2.2. cDNA Labeling

1. 5× First Strand Buffer (Invitrogen). Store frozen.
2. 0.1 M DTT (Invitrogen). Store frozen.
3. RNase OUT (Invitrogen). Store frozen.
4. DNase I (RNase free, 10/ μ L) (Roche). Store frozen.

5. Chloroform (Panreac, Barcelona). Store at room temperature.
6. 3 M Sodium acetate, pH 4.5. Store at room temperature.
7. Random Hexamers (3 $\mu\text{g}/\mu\text{L}$) (Invitrogen). Store frozen.
8. Oligo dT ($T_{15}\text{VN}$) (500 $\text{ng}/\mu\text{L}$). Store frozen.
9. dNTP's mix: 16 mM each of dATP, dGTP, dTTP, and 1 mM dCTP. Divide into small aliquots and store frozen.
10. [α - ^{32}P]dCTP (~3,000 Ci/mmol, 10 $\mu\text{Ci}/\mu\text{L}$) (PerkinElmer). Store at 4°C.
11. SuperScript II Reverse Transcriptase (200 U/ μL) (Invitrogen). Store frozen.
12. 0.5 M EDTA, pH 8.0 buffered with NaOH. Store at room temperature.
13. ProbeQuant G-50 or SR-H300 columns (GE, Niskayuna, NY). G-50 columns at room temperature and SR-H300 columns at 4°C, according to the suppliers.

2.3. Chromatin Immunoprecipitation

1. 37% w/v, formaldehyde solution in H_2O (Sigma–Aldrich). Store at room temperature.
2. 2.5 M Glycine. Store in small autoclaved aliquots at room temperature.
3. TBS buffer: 20 mM Tris–HCl, 140 mM NaCl, pH 7.5.
4. Glass beads, acid-washed and autoclaved (425–600 μm , Sigma–Aldrich). Store at room temperature.
5. 8GW16 antibody (Covance Inc., Berkeley, CA). Store frozen; once thawed, keep at 4°C.
6. Dynabeads[®] Protein G for immunoprecipitation (Invitrogen). Store at 4°C.
7. 5 mg/mL bovine serum albumin (BSA) in PBS buffer: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.4. Divide into small aliquots and store frozen.
8. 10 mg/mL yeast tRNA (Applied Biosystems, Austin, TX). Store frozen.
9. Lysis buffer: 50 mM HEPES–KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% v/v, Triton X-100, 0.1% w/v, sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine and one pill of complete protease inhibitor cocktail (Roche) for 50 mL of buffer. Prepare fresh (see Note 6).
10. Wash buffer: 10 mM Tris–HCl, pH 8.0, 250 mM LiCl, 0.5% w/v, Nonidet P-40, 0.5% w/v, sodium deoxycholate, 1 mM EDTA, pH 8.0. Prepare fresh.
11. TE: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA. Store at room temperature.

12. Elution buffer: 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% w/v, SDS. Store at room temperature.
13. Proteinase K (Roche) stock solution: 1 mg/mL in water. Store frozen divided into aliquots.
14. QIAquick PCR purification columns (Qiagen, Valencia, CA). Store at room temperature.
15. Neutral phenol:chloroform: Phenol:chloroform:isoamyl alcohol (25:24:1, saturated with 50 mM Tris-HCl, pH 7.5 buffer). Store at 4°C.

2.4. Ligation-Mediated PCR (LM-PCR) DNA Amplification

1. T4 DNA polymerase. Store frozen.
2. T4 DNA ligase. Store frozen.
3. Linkers oJW102 (5'-GCGGTGACCCGGGAGATCTGA ATTC) and oJW103 (5'-GAATTCAGATC) (18). The linker oligonucleotides are mixed to a final concentration of 15 μ M in the presence of 250 mM Tris-HCl (pH 7.9). The mixture is distributed into 50 μ L aliquots and denatured for 5 min at 95°C. Then they are transferred to a 70°C heated block and allowed to cool down slowly to room temperature. Afterward, the block with the tubes is placed at 4°C and allowed to cool down again. The linkers are then stored frozen, and should always be thawed and kept on ice.
4. Glycogen 20 mg/mL (Roche). Store frozen.

2.5. Macroarray Hybridization

1. Hybridization, washing, and stripping solutions are identical to those described for GRO (see Subheading 1).

3. Methods

3.1. Genomic Run-On

3.1.1. Run-On

1. Allow cells to grow to the desired OD₆₀₀ (we normally use 0.4–0.6).
2. Two aliquots of the culture are needed: 50 and 20 mL (corresponding to about 6×10^8 and 2.5×10^8 cells, respectively). Other volumes may be required if using different cell densities for the transcription rate (TR) and the mRNA amount (RA, see Subheading 3.1.5) measurements, respectively (see Note 4).
3. Cells are pelleted in a 50-mL falcon tube by centrifugation at $2,500 \times g$ -force for 3 min.
4. Eliminate the supernatant and resuspend the cells in 5 mL of 0.5% sarkosyl at room temperature (see Note 7).

5. Pellet the cells as before. The aliquot for RA is directly frozen in dry ice (see Note 8) and the TR aliquot is resuspended in 1 mL 0.5% sarkosyl.
6. Transfer resuspended cells into a 1.5-mL tube, and pellet the cells in a microcentrifuge by centrifuging at $3,300 \times g$ -force for 30 s. Discard the supernatant and centrifuge again, if necessary, to eliminate any remains of sarkosyl.
7. Resuspend the cells in 120 μ L (see Note 4) of RNase-free water. Pre-warm both cells and mix separately at 30°C for 5 min. Add 158 μ L of the transcription mix: the final reaction volume should be \sim 300 μ L (see Note 9).
8. Incubate the mix at 30°C for 5 min in a Thermomixer (Eppendorf, Hamburg, Germany), or similar, with 600 rpm agitation (see Note 10).
9. Stop the run-on reaction by adding 1 mL of ice-cold RNase-free water. Recover cells by centrifuging at $3,300 \times g$ -force for 1 min and discard the supernatant (which contains the non-incorporated radioactive nucleotide).
10. Start the RNA extraction by resuspending cells in 500 μ L of LETS buffer.
11. Transfer the cells resuspended in LETS to a fresh tube containing 500 μ L of glass beads and 500 μ L of acid phenol:chloroform.
12. Break cells by vortexing tubes three times for 30 s at 5.5 intensity in a Fast-Prep 24 (MP Biomedicals, Solon, OH) (see Note 11).
13. Centrifuge tubes for 5 min at $13,400 \times g$ -force to separate the phases, and transfer the upper water phase to a fresh tube. Add one volume of acid phenol:chloroform, mix well by vortexing, and centrifuge as before.
14. Transfer the new upper aqueous phase to a fresh tube and add 0.1 volume of 5 M LiCl and two volumes of cold 96% ethanol. Mix and incubate at -20°C for at least 3 h (see Note 12).
15. Recover the total RNA by centrifugation at $13,400 \times g$ -force in a microcentrifuge for 15 min. Discard the supernatant and wash the pellet with 0.7 mL of 70% ethanol. Dry the pellet in a Speed-vac (Thermo Savant, Waltham, MA) for 2–3 min, and dissolve the RNA in 300 μ L of RNase-free water (see Note 13).
16. Prepare a 1:100 dilution of the dissolved RNA in H_2O . Quantify the extracted RNA by measuring at A_{260} . A spectrophotometer that is capable of measuring low volumes (as 50 μ L) will avoid losses of the valuable material. Use 5 μ L of each one from the same dilutions to measure the

radioactivity incorporated into a scintillation counter. The radioactivity obtained ranges of between 0.8 and 3.5×10^7 dpm (see Note 14). All the labeled RNA is used in hybridization.

3.1.2. Hybridization of Run-On Samples

1. Prehybridize the yeast nylon macroarray (17) for a minimum of 30 min at 65°C with 5 mL of hybridization solution in a hybridization tube on a roller oven (see Note 15).
2. Hybridization is performed with fresh hybridization solution by adding the labeled RNA. The volume of fresh hybridization solution may be adjusted to obtain in a hybridization solution of between 1 and 7×10^6 dpm/mL. Allow to hybridize for 20–24 h at 65°C in a roller oven (see Note 15).
3. After hybridization, wash the macroarray once with washing buffer I at 65°C for 10 min, and twice with washing buffer II at 65°C for 10 min (see Note 5).
4. After washing, the membranes are saran-wrap sealed and exposed between 1 and 7 days to an Imaging Plate (Fujifilm BAS IP or similar), depending on the intensity of the signal measured with a Geiger counter (see Note 16).

3.1.3. Analysis of Run-On Hybridized Macroarrays

1. Scan the macroarrays in a suitable phosphorimager (such as a Fujifilm FLA, Fujifilm BAS, GE Storm, or GE Typhoon), with a resolution of at least 50 μm .
2. The macroarray image data are analyzed by using ArrayVision 7.0 (Imaging Research Inc., Ontario, Canada) or by other array analysis softwares. Biological replicates of the experiment should be done. We recommend at least three.
3. Before manipulating the raw data, we use genomic hybridizations to eliminate any differences due to the filter (see Note 17). Thus, each run-on hybridization dataset was divided by the corresponding genomic hybridization dataset done on the same nylon membrane. This procedure also serves to normalize the signals of the different probes, which enables comparable TR results for all the genes.
4. Values for each replicate are corrected by the number of cells used (see Note 18).
5. Hybridization values for each gene probe in each replicate are normalized and averaged by using ArrayStat 1.0 (Imaging Research Inc.), or other statistical array analysis softwares, in order to obtain a sure transcription value per cell for each gene (TR values).
6. Average values from step 5 are corrected for each gene by the percentage of U in each probe-coding strand.

7. RNA polymerase densities reflect transcription rates if we consider they have a constant elongation speed (4). The TR values obtained are, however, in arbitrary units (radioactive intensities). In order to convert them into real rates (i.e., molecules/min) it is necessary to use a reference. We have used the known TR for *HIS3* gene, 0.43 mRNAs/min (19). In this way, knowing the ratio of the radioactive intensities between *HIS3* and a given gene, the real TR can be calculated for that gene. Another possibility is to use the whole set of absolute values for mRNA concentrations (called m or RA) and mRNA half-lives $t_{1/2}$, e.g., that described in ref. 20 to determine a set of indirect TR using the Eqs. 2 and 3 described in the companion chapter (8) and plot it against the arbitrary units set to obtain a conversion factor (V. Pelechano et al., in press). This last method is more robust than the one previously described.

3.1.4. Stripping Run-On Hybridizations

Nylon macroarrays can be used several times (up to ten times in our hands). Therefore, it is necessary to strip them of the radioactive sample before they are reused. They should be stripped even if they are not to be used immediately (see Note 16).

1. Incubate the membrane inside the hybridization tube with 25 mL of 50 mM NaOH at 45°C for 45 min.
2. Wash once with the same volume of neutralizing buffer at 45°C for 15 min.
3. Transfer the filter to a plastic box and perform an additional washing step with boiling stripping solution for 5–10 min with agitation.
4. Membranes can be reused directly or stored after air-drying.

3.1.5. cDNA Labeling: RNA Extraction

A cDNA labeling experiment requires a series of independent protocols that we describe independently (from Subheadings 3.1.5–3.1.10).

Two different procedures can be followed depending on the primer used in the cDNA synthesis: random primers (RP labeling) or oligo d(T) (dT labeling). If RP labeling is used, it is necessary to perform a DNase I digestion of the RNA in order to eliminate any remains of contaminant DNA that co-extracted with the RNA. This is not necessary with dT labeling because it only primes at poly(A)-mRNAs (see Notes 19 and 20).

1. Total RNA is extracted from the 20-mL frozen culture aliquot for mRNA measurements as in an in vivo run-on protocol. The RNA extraction yield is evaluated by A_{260} (see Subheading 3.1.1, steps 2 and 10–16, but also see Note 12).

3.1.6. DNase I Digestion

1. Use a total of around 100 µg of total RNA (to prevent loss after the phenolization and precipitation steps). Dissolve it in 17 µL of H₂O.
2. Add 2 µL of 5× first strand buffer (Invitrogen), 1 µL of RNase OUT (Invitrogen) and 0.6 µL of RNase free-DNase I.
3. Incubate at 37°C for 30 min. Once again, add 0.4 µL of RNase free-DNase I, and incubate under the same conditions for 30 min more.
4. Remove the RNase free-DNase I by extracting once with acid phenol:chloroform and once with chloroform.
5. Precipitate the RNA with 0.1 volume of 3 M sodium acetate, pH 4.8, and 2.5 volumes of 96% ethanol, incubating at −20°C for a minimum of 1 h.
6. Recover the RNA by centrifugation in a microcentrifuge at 13,400 × *g*-force for 15 min. Remove the supernatant and wash with 0.7 mL of 70% ethanol, and centrifuge again at 13,400 × *g*-force for 5 min.
7. Dry the RNA for 1–2 min in a Speed-vac (see Note 13).

3.1.7. Labeling Reaction

1. Take 50 µg of total RNA (DNase I-digested or not, see Note 12) in a volume of 12.3 µL, add 1 µL of RNase OUT and, alternatively, 1.2 µL of random hexamers (3 µg/µL) or 1.2 µL of Oligo d(T) (500 ng/µL), depending on the labeling option. The final volume of that mix must be 14.5 µL.
2. Incubate the mix at 70°C for 10 min and leave at room temperature for 5–10 min. Then place it on ice.
3. To the previous sample, add 6 µL of the 5× first strand buffer, 3 µL of 0.1 M DTT, 1.5 µL of dNTP's mix, 4 µL of [α -³²P]-dCTP, and 1 µL of SuperScript II Reverse Transcriptase. The final reaction volume must be 30 µL (see Note 9).
4. Incubate at 42°C for 1 h and stop the reaction by adding 1 µL of 0.5 M EDTA, pH 8.0.
5. Add water to the reaction to a final volume of 50 µL, and eliminate the nonincorporated nucleotides by using Probe-Quant G-50 or SH-300R columns according to the manufacturer's instructions.
6. Estimate the radioactive incorporation by measuring 1 µL in the scintillation counter to calculate the total dpm.

3.1.8. Hybridization of cDNA Samples

1. Perform a prehybridization of the macroarray as for the run-on samples (Subheading 3.1.2, step 1).
2. Denature the labeled sample at 95°C for 5 min and transfer to an ice bath.

3. Add the denatured labeled cDNA sample to the corresponding volume of hybridization solution to obtain a radioactivity concentration ranging between 5 and 10×10^6 dpm/mL.
4. Hybridization, washing, and scanning are performed as previously described (Subheading 3.1.2, steps 2–5).

3.1.9. Stripping cDNA Hybridizations

1. Perform three washes in a dish with boiling stripping solution for 5–10 min in agitation.
2. Membranes can be reused directly or kept air-dried.

3.1.10. Analysis of the cDNA Hybridized Macroarrays

1. The hybridized macroarrays are scanned and the images are analyzed as before with the run-on samples. Biological replicates of the experiment should be done. Again, we recommend at least three.
2. As in Subheading 3.1.3, genomic hybridizations are used for eliminating any differences due to the filter; again, ArrayStat or a similar software was used to normalize and average the cDNA hybridization values (see Note 17).
3. When different conditions are analyzed, normalized, and averaged, the cDNA values are corrected by the combined factor of total RNA per cell (see Note 18) and the proportion of mRNA in the total RNA (see Note 21) in order to obtain the mRNA values per cell (RA values).
4. Average values from step 3 are corrected for each gene by the percentage of G in each probe-coding strand.
5. As for TR values, the RA values obtained are in arbitrary units (radioactive intensities). In order to convert them into real units (molecules/cell) it is necessary to use a reference. We have used the whole set of absolute values for mRNA concentrations described in ref. 20, and plot it against the arbitrary units to obtain a conversion factor, and transform the arbitrary units into real ones.

3.2. RNA Polymerase-ChIP-on-Chip

The first step of this protocol, and the most critical one, is chromatin immunoprecipitation (IP). To obtain reliable and reproducible results, it is important to ensure that the Pol II IP is successful. It is advisable to perform a control PCR to check IP efficiency using a gene that is known to be expressed as a positive control before proceeding to the array hybridization (11, 21).

The genomic RPCC data should be obtained using the IP data that have been normalized by a positive control of the total chromatin (whole cell extract, WCE). A negative control (such as an IP without a specific antibody) is highly variable between different technical replicates due to the low amount of contaminant DNA. Therefore, although it is advisable to perform negative

control replicates to discard any nonspecific IP, they are not used to normalize the final IP data.

3.2.1. Chromatin Immunoprecipitation

1. For each IP reaction or for the negative control, 50 mL cells of yeast culture ($OD_{600} \sim 0.5$) are cross-linked by adding formaldehyde at a final concentration of 1% for 15 min at room temperature. Then the reaction is quenched by the addition of glycine at a final concentration of 125 mM (see Note 22). Cells are washed four times with 30 mL ice-cold TBS buffer, frozen in liquid N_2 , and stored at $-20^\circ C$ until use. Samples can be kept several weeks in this stage.
2. Thaw cells on ice and resuspend them in 300 μL lysis buffer. Then, transfer cells to an ice-cold 1.5 mL screw-capped tube with 0.2 mL of glass beads and break them by vortexing at the maximum power for 12 min at $4^\circ C$ in a Genie 2 vortex (Scientific Industries Inc., Bohemia, NY) or similar.
3. Add 300 μL lysis buffer to the tubes and transfer the lysed cells to a new tube. Sonicate the chromatin at $4^\circ C$ (see Note 23).
4. Remove the cell debris by centrifugation at $14,000 \times g$ at $4^\circ C$ for 5 min. A 10 μL aliquot of this WCE is kept as a positive control.
5. The magnetic beads with the Ab should be prepared 1 day prior to their use. Beads (50 μL /sample) are washed twice with 600 μL PBS/BSA using a magnet (DynaMag™-2, Invitrogen). Then they are resuspended with 15 μL 8WG16 Ab (2 $\mu g/\mu L$) and 1 μL yeast tRNA as a blocking agent. For a no-Ab negative control, the volume of Ab is changed by an equal volume of PBS/BSA. Beads are kept in a tube rotator overnight at $4^\circ C$ (Roto-Torque, Cole-Parmer, Vernon Hills, IL). The next day, beads are washed four times with 600 μL PBS/BSA. Afterward, they are resuspended in 30 μL of PBS/BSA and the sonicated chromatin obtained from 50 mL cells (step 4) is added. The samples with the beads are incubated in a rotator for 1.5 h at $4^\circ C$ (see Note 24). Wash beads twice with 1 mL lysis buffer, twice with 1 mL lysis buffer supplemented with 360 mM NaCl, twice with 1 mL wash buffer, and once with 1 mL TE. In order to elute the samples, beads are resuspended in 50 μL of elution buffer and incubated for 10 min at $65^\circ C$ under agitation (600 rpm in a Thermomixer). Then 30 μL of eluted sample is recovered and an additional amount of 30 μL of elution buffer is added. Repeat this incubation and recover an additional amount of 30 μL of the eluted sample. It is important in this step to be careful not to touch beads excessively with the tip to avoid contamination or any bead carryover. Raise the final volume of the samples to 300 μL with TE and incubate overnight at $65^\circ C$

with agitation (600 rpm in a Thermomixer) to reverse the cross-linking.

6. To digest the proteins, 142.5 μL TE and 7.5 μL proteinase K (to 20 $\mu\text{g}/\text{mL}$) are added to each sample. Incubation is kept at 37°C with agitation (600 rpm) for 1.5 h. Samples are purified using QIAquick PCR purification columns (or similar) with two binding steps and the same column for each sample. The sample is eluted in 50 μL . Up to 5 μL sample should be used in this step to check IP efficiency by performing a standard PCR analysis for an expressed control gene (11, 21). These DNA samples are only stable for a few days at -20°C . For this reason, the rest of the sample should be used as soon as possible for the DNA amplification step (next paragraph).

3.2.2. DNA Amplification by LM-PCR

1. The ends of the DNA molecules are blunted. The entire IP sample is used for this, but only 2 μL of the sample is used for the WCE (4% of the total). The reaction is allowed to proceed for 20 min at 12°C in the presence of 0.6 U of T4 DNA polymerase in its buffer supplemented with 80 μM dNTPs. Then, the sample is extracted twice with neutral phenol:chloroform and precipitated with two volumes of ethanol in the presence of 0.1 volume of sodium acetate and 12 μg of glycogen.
2. Ligate the blunt-ended sample overnight at 16°C using 0.5 U of T4 DNA ligase in a final volume of 50 μL in the presence of the annealed linkers oJW102 and oJW103 (1.5 μM) (18). Precipitate the ligated sample with ethanol and resuspend it in 25 μL of milliQ sterile water.
3. Amplify the sample in a 50- μL PCR mix using 1 μM of oJW102 primer. The PCR program is 2 min at 95°C, 30 (or less) cycles (30 s at 95°C, 30 s at 55°C, and 2 min at 72°C), with a final cycle of 4 min at 72°C. The number of PCR cycles should be tested and kept as low as possible. Precipitate the DNA with ethanol and resuspend it in 50 μL of milliQ water (see Note 25). In this state, the sample can be kept at -20°C for months.

3.2.3. Sample Labeling and Macroarray Hybridization

1. Label the sample by one additional cycle of PCR in the presence of α -[^{33}P]-dCTP. 15 μL of sample containing 1–2 μg of DNA from LM-PCR in 50 μL final volume, including: 1 \times Taq DNA pol buffer, 2 mM MgCl_2 , 0.2 mM dATP, dTTP, and dGTP, 25 μM dCTP, 1 μM oJW102, 0.8 μCi α -[^{33}P]-dCTP, and 5 U Taq DNA pol. Denature the mix for 5 min at 95°C, anneal for 5 min at 50°C, and amplify for 30 min at 72°C (see Note 26). Purify the reaction product with a ProbeQuant G-50 column following the manufacturer's

instructions to remove unincorporated [^{33}P]-dCTP and the remaining oligonucleotides.

2. Yeast nylon macroarrays (17) should be used. The conditions for the hybridization, washing scanning, and stripping of the macroarrays are identical to those described for the cDNA experiments (see Subheadings 3.1.8 and 3.1.9).

3.2.4. Analysis of the Hybridized Macroarrays

The macroarray image data are analyzed by ArrayVision 7.0 (Imaging Research Inc.) or other array analysis softwares. Relative immunoprecipitation is computed as the ratio between the IP and WCE samples. Biological replicates of the experiment should be done. We recommend at least three. To compare the RPCC data under the different conditions, the median binding ratio for spots with negligible Pol II binding (e.g., probes for the rRNA) can be taken and arbitrarily set to 0 to normalize the RPCC ratio under these conditions.

4. Notes

1. Although YPD is the most common culture medium, other complete or synthetic media may also be used. We have experienced that the total labeling obtained depends on the culture growth rate. This is mainly due to the high dependence of Pol I + Pol III TR ($\geq 60\%$ of the total TR, our unpublished observations using an inhibitor of Pol II, α -amanitin) on the cells growth rate.
2. All home-made buffers and most solutions are autoclaved at 2 kg/cm^2 for 1 h to inactivate DNases and RNases.
3. We have found that there are important differences in the quality of the [α - ^{33}P]rUTP depending on the supplier. We recommend testing the efficiency of incorporation if a different supplier has been used.
4. We have checked different amounts of [α - ^{33}P]rUTP. Depending on the cells' run-on efficiency (see Note 1), between 13 and 25 μL may be used, and the water volume needs to be checked to resuspend cells. We also have checked different amounts of cells in the assay affect the total incorporation of ^{33}P . We recommend to use always the same number of cells.
5. This solution allows a faster hybridization and higher signals than that originally described (4). However, it has a greater tendency to cause radioactive stains on the macroarray. Alternatively, you may use: $5\times$ SSC, $5\times$ Denhart's, 0.5% SDS, 100 $\mu\text{g/mL}$ salmon sperm DNA, and hybridize for 40–48 h.

- In this case, washing is done once for 20 min with $2\times$ SSC 0.1% SDS, and twice for 30 min with $0.2\times$ SSC 0.1% SDS (4).
6. In order to avoid precipitation when preparing the buffer, all the compounds, except for benzamidine, the PMSF and the protease inhibitor are dissolved first at room temperature. Then benzamidine is added. The solution is mixed and cooled on ice. Finally, all the remaining compounds are added.
 7. We have checked that centrifuging cells at 4°C and/or washing them with cold sarkosyl, or cold water (as in ref. 4), induces a cold stress response that affects TR and RA of some genes. By resuspending cells in room temperature sarkosyl this stress is avoided because cells are quickly killed under nonstressing conditions. Alternatively, 10% w/v, sarkosyl stock solution can be added directly to the cells' culture medium to obtain a concentration of 0.5% w/v, then proceed with cell recovery under the same conditions as before.
 8. We have observed that the slow freezing of sarkosyl-treated cells causes some RNA degradation. It is recommended that the cells for total RNA extraction are to be frozen in liquid nitrogen or on dry ice.
 9. For multiple reactions, prepare a master mix with an excess of 5–10% to compensate minor pipetting inaccuracy.
 10. We have checked that longer incubation times do not improve labeling. This coincides with previously described run-on protocols (1). Probably, the run-on reaction is completed in only a few minutes. Temperature, however, has a clear effect on run-on labeling. We have checked that it increases up to 50% from 30°C to 37°C . Traditionally, run-on in *Saccharomyces cerevisiae* is done at 30°C (1) because this is the standard growth temperature for this yeast. Nevertheless, because run-on is an in vitro assay in which yeast cells are dead, there is no obstacle to perform it at a different temperature. Different temperatures could, however, affect the length of the elongation in nascent mRNA which may cause differential effects on genes. Therefore, we recommend using the same temperature for all the experiments.
 11. Other RNA extraction methods are also possible, such as hot-phenol or commercial RNA extraction kits. It is important to verify that the selected method is highly efficient because the amount of in vivo labeled RNA can be limiting for sensitive detection.
 12. Instead of ethanol, one volume of 5 M LiCl may also be used, followed by incubation as before. This precipitation procedure is more selective for RNA and avoids contaminant DNA. This is not a major problem, however, because DNA is not

labeled. Li^+ ions could inhibit an enzymatic processing of the RNA, such as cDNA synthesis. Therefore, it is not a problem for the RNA sample obtained with the GRO protocol (Subheading 3.1.1), but may be a problem in RNA precipitation in the sample for cDNA labeling (Subheading 3.1.5). For this reason, we recommend a new RNA precipitation using 0.1 volume of 3 M sodium acetate, pH 4.8, and two volumes of 96% ethanol.

13. Over-drying the pellet results in a difficulty to dissolve RNA. For a complete dissolution keep the RNA pellet with water in a Thermomixer at 40°C for about 30 min. Lower temperatures and longer times may also be used. Check the dissolution by carefully inspecting while pipetting.
14. A precise quantification of the incorporated radioactivity is only needed in certain experiments. In such cases, we recommend taking several independent measures of RNA amount and scintillation counting of each one. In other cases, a single measurement or, even a simple Geiger estimation, of the incorporated radioactivity may be more convenient.
15. In order to compare hybridizations between samples it is necessary to use the same volume for all samples although the dpm/mL concentration was different (4). We use cylindrical plastic tubes with a slightly longer diameter circumference than the macroarray width and a slightly longer length than the macroarray length. In this way, the required volume for hybridization is kept at a minimum and the concentration of the radioactive sample is kept at a maximum.
16. We use both a thick plastic base and a saran-wrap cover and heat seal them to avoid macroarray drying which could irreversibly link the radioactivity to the nylon. The thin saran-wrap facing the exposure side of the filter reduces the shielding caused by the plastic on the ^{33}P radioactive emission.
17. Genomic DNA is labeled by random-priming using standard protocols (17). A single sample of labeled genomic DNA is added to the hybridization solution to give between 4 and 7×10^6 dpm/mL, and is divided into aliquots to hybridize all the macroarrays to be used in a given experiment. Intensity values are then corrected for each gene by the percentage of C + G in the probe. In this way, the differences in hybridization will be due only to the differences in the particular macroarrays, and will serve to correct and normalize the GRO and cDNA results.
18. The cell number in each GRO experiment should be very similar to avoid differences in labeling during the run-on.

We estimate the real number of cells used from the amount of RNA obtained after purification (Subheading 3.1.1, step 16). If the amount of RNA per cell is known (this can be obtained from a series of independent RNA purifications from the known amount of cells), the number of cells is derived from it.

19. Depending on the primer used in cDNA labeling, the radioactive label will either be more uniformly distributed along the target ORF (RP labeling) or more concentrated at the 3'-end of the ORF (dT labeling). This should be considered when analyzing the results. We originally used RP labeling (4) because the uniform labeling along the ORF was similar to the distribution of Pol II along the transcribed region. RP-labeled cDNA is, however, less efficient for hybridization than dT labeling. We currently recommend dT labeling for this reason and because of the similar bias toward 3' as the GRO.
20. The TR calculated by GRO in our macroarrays (17) has a bias with regard to gene length which is due to the 3'-oriented movement of the RNA polymerases during run-on elongation (V. Pelechano et al., in press). This effect can also be seen while doing cDNA labeling using oligo d(T) instead of random primers (Fig. 3). It is possible to use RPCC data, which do not show this effect, to correct the GRO dataset (Fig. 3). For most instances, however, such a correction is not necessary because the GRO-determined TR values are to be compared between themselves.
21. mRNA per total RNA can be obtained by doing a dot-blot hybridization of the total RNA samples (maybe those used in the experiment or others obtained from similar cells) with a 5'-labeled oligo-d(T) probe (see refs. 4, 22 for details). This procedure assumes that all the mRNA is polyadenylated.
22. Both the cross-linking and sonication times can affect the final size of the chromatin obtained. It is advisable, therefore, to check that 15 min is the optimum time for cross-linking your samples.
23. The sonication time should be optimized for each condition and the chromatin size checked by running an agarose gel after reversing the cross-linking. We routinely use five pulses of 30 s at a high output (200 W) in a Bioruptor (Diagenode SA, Liège, Belgium), and obtain DNA fragments whose average size is 350 ± 150 bp.
24. For less efficient antibodies, this incubation time could be extended by up to 4 h.
25. A 5- μ L aliquot of DNA of the LM-PCR amplified sample should be analyzed in 1.2% w/v, agarose gel to check both

size and PCR efficiency. A similar sized smear of the original chromatin fragments should be seen. Smears reaching a longer size do not represent a problem, but discrete bands in the gel are indicative of very low IP efficiency, thus the IP step should be repeated.

26. For this, we recommend the use of a set of three independent tube incubators, each at a different temperature. That at 72°C may be a Thermomixer at 600 rpm agitation.

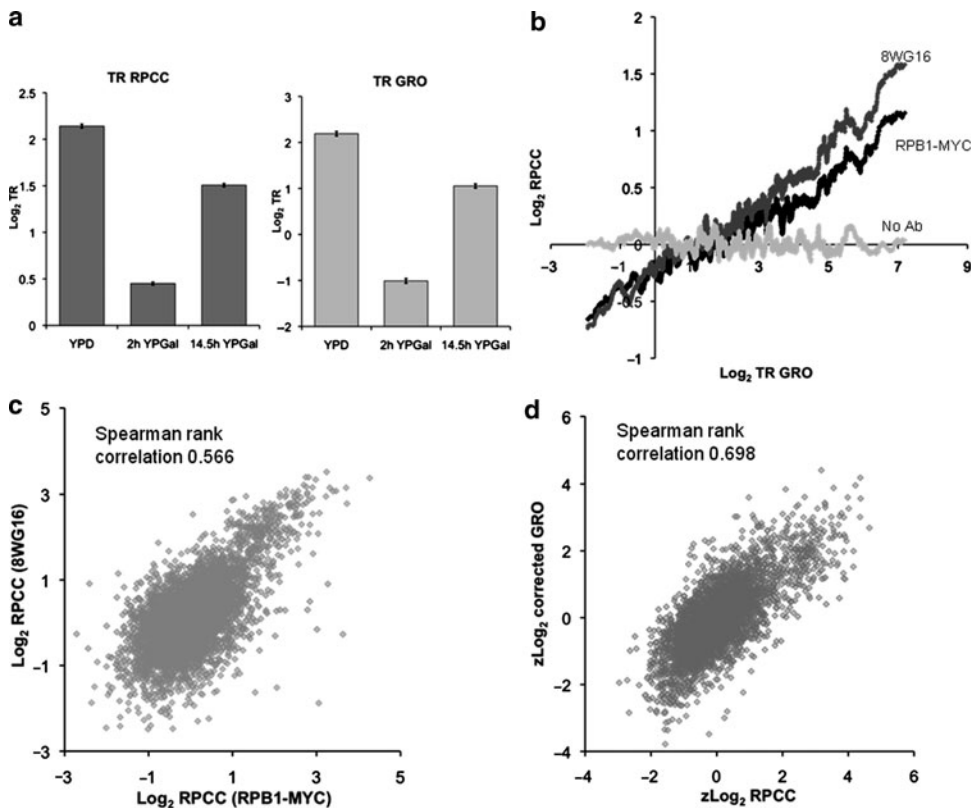


Fig. 3. Correlation between the RPCC and GRO measurements (16). There is a general correlation between the amount of Pol II present in the genes detected by RPCC and the transcriptional density measured by GRO. (a) Average transcription rate for all the genome using either RPCC or GRO under three different conditions (exponentially grown in YPD, nongrowing cells after 2 h of changing them to YPGal, and exponentially growing cells in YPGal 14.5 h after a change of medium; refs. 4, 16). The 95% confidence interval of the median is presented. (b) General correlation between the RPCC datasets using two different antibodies (Rpb1-Myc in black, and 8WG16 which recognizes the hypophosphorylated CTD in dark gray) toward the GRO TR. A negative control using a mock IP (NA, No-antibody IP in light gray) is also shown. All the curves represent the smoothness of the data using the average values for a sliding window of 100 genes. All the values are presented in arbitrary units. (c) A comparison is made between RPCC using two different antibodies: one against the total Rpb1-tagged RNA pol II (Rpb1-MYC) and another against hypophosphorylated CTD (8WG16). Spearman's rank correlation coefficient is shown. (d) Comparison between RPCC (8WG16) and GRO using standardized values (Z-scores) Spearman's rank correlation coefficient is shown.

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