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Global Run-On sequencing (GRO-seq)

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Abstract

Transcription occurring at gene loci results in accumulation of mature RNA molecules (i.e. mRNAs) that can be easily assayed by RT-PCR or RNA-sequencing. However, the steady-state level of RNA does not accurately mirror transcriptional activity *per se*. In fact, RNA stability plays a major role in determining the relative abundance of any given RNA molecule. Here, I describe a protocol of Nuclear Run-On assay coupled to deep sequencing to assess real-time transcription from engaged RNA polymerase. Mapping nascent transcripts at the genome-wide scale provides a reliable measure of transcriptional activity in mammalian cells and delivers a high-resolution map of coding and noncoding transcripts that is especially useful for annotation and quantification of short-lived RNA molecules.

Keywords

Nuclear Run-On; Nascent RNA; RNA polymerases; transcription initiation; transcription elongation; non-coding RNA; enhancer RNA; deep sequencing

1. Introduction

The Nuclear Run-On Assay was introduced over 40 years ago as a method to assess RNA polymerase that is transcriptionally engaged [1,2]. Nuclei from mammalian cells are isolated, washed to remove free nucleotides and kept at ice-cold temperature to arrest ongoing transcription. Transcription is resumed *in vitro* when nuclei are incubated at 30°C in the presence of radiolabeled nucleotides and the anionic detergent sarkosyl, which prevents *de novo* assembly of the pre-initiation complex and avoids re-initiation. Transcripts that were initiated at the time of nuclei isolation (commonly referred to as *nascent RNA*) will be further elongated by engaged RNA polymerase, to allow incorporation of radioactive nucleotides. Traditionally, radiolabeled RNA is hybridized to an array of specific DNA probes (representing different genes or different portions of a given gene) that are cross-linked to a nylon membrane using a dot blot system. The extent of nascent transcription is ultimately revealed by autoradiography. Nuclear Run-On has been successfully performed on a variety of cell types and organisms, including plants [3], *D. Melanogaster* [4] and fission yeast [5].

Recently, Lis et al. developed a modified Run-On protocol to isolate nascent RNA that can be ultimately converted into a DNA library suitable for deep sequencing [6]. Such high-

throughput evolution of the Run-On assay has been named GRO-seq (Global Run-On sequencing) and allows unbiased mapping of nascent transcripts genome-wide. The main alteration to the original Run-On assay lies in the use of brominated nucleotides instead of radioactive analogs. RNA molecules that have incorporated BrUTP can be affinity purified by means of commonly used antibodies against bromodeoxyuridine (anti-BrdU). Such immunoprecipitation step is fundamental to ensure proper enrichment of nascent RNA before initiating library preparation. Illumina-compatible DNA libraries are prepared similar to conventional protocols for directional sequencing of total RNA.

Major limitations of GRO-seq are the laboriousness of the technique and the amount of starting material (the number of cells that are required lies in the 10^7 range). Nonetheless, GRO-seq is an exceptionally sensitive method to estimate transcriptional activity throughout the entire genome and has generated crucial information on RNA polymerase II (RNAPII) density at different classes of protein coding genes [6]. GRO-seq has shown unprecedented accuracy to ascertain defects in RNAPII elongation and pause-release [7,8,9] as well as termination [10]. Additionally, GRO-seq has revealed that RNAPII fires bi-directionally at most mammalian promoters [6], initiating noncoding RNAs that are transcribed antisense with respect to the messenger RNA. Owing to their instability, these transcripts do not accumulate in the nucleus and elude most RNA detection protocols. Due to its sensitivity, GRO-seq is suitable to assess low-abundant long noncoding RNAs, such as the recently characterized enhancer-associated RNAs (eRNAs). Bi-directional eRNAs are hard to detect by conventional sequencing methods that gauge steady-state transcription. GRO-seq has been employed to reveal the full extent of eRNA transcription in response to stimuli such as estrogen, LPS and Epidermal Growth Factor [10,11,12].

2. Materials

2.1 Nuclei isolation

1. Swelling Buffer (500mL): 492.5 ml MilliQ water, 5 ml 1M Tris-HCL pH 7.5 (final 10mM), 1 ml 1M MgCl₂ (final 2mM), 1.5 ml 1M CaCl₂ (final 3mM).
2. Swelling buffer with glycerol: 90 ml of swelling buffer, 10 ml of pure glycerol.
3. Lysis buffer: 99 ml of swelling buffer with glycerol, 1ml of Igepal (NP-40).
4. Freezing buffer: 27.5 ml MilliQ water, 20 ml glycerol, 2.5 ml Tris-HCl pH 8, 250 ul of 1M MgCl₂, 10 ul of 0.5M EDTA.
5. Ice-cold PBS.
6. SUPERase In RNase Inhibitor (20 U/ μ L), ThermoFisher Scientific (AM2696).

2.2 Nuclear Run-On

1. 2 $\times$ Nuclear Run-On (NRO) buffer: 10mM Tris-HCl pH 8, 5mM MgCl₂, 300mM KCl, 1mM DTT, 500 uM ATP, 500 uM GTP, 500 uM Br-UTP, 2uM CTP, 200 U/ml Superase In, 1% N-Laurylsarcosine (sodium salt solution), nuclease free water. Prepare 100 ul per sample.
2. TRIzol LS reagent (ThermoFisher Scientific).

3. Chloroform, molecular biology grade.
4. 100% ethanol and 75% ethanol in nuclease free water.
5. Sodium Chloride solution, 5M.
6. GlycoBlue coprecipitant (ThermoFisher Scientific).

2.3 RNA immunoprecipitation

1. RNase-free DNase (Turbo DNA-free kit, ThermoFisher Scientific).
2. RNA fragmentation reagents (Ambion, ThermoFisher Scientific).
3. Micro Bio-Spin P-30 Gel Columns (BioRad).
4. T4 Polynucleotide Kinase (PNK), 10× PNK buffer (New England Biolabs).
5. Binding buffer: 0.25× SSPE, 0.05% Tween 20, 37.5 mM NaCl, 1mM EDTA in nuclease free water.
6. Blocking buffer: 1× binding buffer with the addition of 0.1% polyvinylpyrrolidone and 0.1% BSA (use Ultrapure BSA in solution).
7. Low salt wash buffer: 0.2× SSPE, 0.05% Tween 20 (50ul 10% tween), 1mM EDTA in nuclease free water.
8. High salt wash buffer: 0.2× SSPE, 137.5 mM NaCl, 0.05% Tween 20, 1mM EDTA in nuclease free buffer.
9. Elution buffer: 50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 20 mM DTT, 1 mM EDTA in nuclease free water.
1. TE+Tween buffer: TE (0.01 M Tris, 0.001 M EDTA, pH 7.4) + 0.05% Tween 20.
10. Anti-BrdU-conjugated agarose beads (Santa Cruz Biotechnologies).
11. 100% ethanol and 75% ethanol in nuclease free water.
12. Glycogen solution (20 mg/ml), molecular biology grade.

2.4 Library preparation

2. *E. Coli* Poly(A) Polymerase (New England Biolabs), which contains the enzyme, 10× reaction buffer and 10 mM Adenosine-5'-Triphosphate (ATP). Prepare a 2.5× dilution of ATP in nuclease free water.
3. Superscript III Reverse Transcriptase kit (ThermoFisher Scientific).
4. Oligonucleotides: NTI223 - /5Phos/GAT CGT CGG ACT GTA GAA CTC T/ idSp/CA AGC AGA AGA CGG CAT ACG ATT TTT TTT TTT TTT TTT TTT VN where 5Phos indicates 5' phosphorylation, idSp indicates the 1',2'-Dideoxyribose modification that introduces a stable abasic site, and VN indicates degenerate nucleotides.

NTI200 - CAA GCA GAA GAC GGC ATA

NTI201 - AAT GAT ACG GCG ACC ACC GAC AGG TTC AGA GTT
CTA CAG TCC GAC G

NTI202 – CGACAGGTTTCAGAGTTCTACAGTCCGACGATC.

- 5 Exonuclease I.
- 6 NaOH 1M and HCl 2M.
- 7 10 % polyacrilamide pre-cast gel (TBE-Urea for denaturing ssDNA PAGE and TBE for size selection of dsDNA library).
- 8 Non-denaturing DNA sample buffer (2× concentrated).
- 9 Non-mutagenic DNA stain (i.e. SYBR Gold, ThermoFisher Scientific).
- 10 Blue-light transilluminator.
- 11 Elution Buffer I: TE (0.01 M Tris, 0.001 M EDTA, pH 7.4) with 0.1% Tween 20.
- 12 100% ethanol and 75% ethanol in nuclease free water.
- 13 Ultrafree MC-HV columns (Millipore).
- 14 Betaine 5 M.
- 15 Circular Ligase kit (CircLigase, EpiBio. Includes enzyme, buffer, ATP, MnCl₂).
- 16 Human apurinic/apyrimidinic (AP) endonuclease, APE 1.
- 17 4× relinearization mix: 100mM KCl, 2mM DTT in nuclease free water.
- 18 Proofreading DNA polymerase (i.e. Phusion Polymerase with High-Fidelity 5× buffer).
- 19 dNTP mix (dATP, dCTG, dTTP, dGTP, 10 mM each).
- 20 6× Gel Loading buffer for DNA.
- 21 PCR purification columns for microcentrifuge.
- 22 Corning Costar Spin-X centrifuge tube filters (Sigma-Aldrich).
- 23 Elution Buffer II: TE (0.01 M Tris, 0.001 M EDTA, pH 7.4) with 0.1% Tween 20 and 150 mM NaCl.
- 24 100% ethanol and 75% ethanol in nuclease free water.
- 25 Glycogen solution (20 mg/ml), molecular biology grade.

3. Methods

3.1 Nuclei isolation

1. Collect between 10⁷–10⁸ cells (accurate cell count to be performed during step 8) in a 50 ml polypropylene tube and immediately put on ice (see **Note** ¹). Centrifuge at 4°C at 1,000 RPM.

2. Wash cell pellet twice with ice cold PBS.
3. Resuspend in 10 ml of ice cols swelling buffer, incubate on ice for 5 min.
4. Centrifuge at 400g for 10 min.
5. Discard supernatant and gently resuspend cells in 10 ml of swelling buffer with glycerol. Add Suprase In to the buffer (2U/ml).
6. Slowly add 10 ml of lysis buffer while agitating the tube (see **Note** ²), incubate on ice for 5 min. Add Suprase In to the buffer (2U/ml).
7. Add 25 ml of lysis buffer and centrifuge at 600g for 5 min.
8. Discard supernatant and resuspend nuclei in 10 ml of freezing buffer (add Suprase In), transfer to a 15 ml tube. Use 5–10 ul of cell suspension to count nuclei with a Neubauer chamber (no intact cells should be visible at this stage).
9. Centrifuge at 900g for 6 min, discard supernatant and resuspend in an appropriate amount of freezing buffer (10 ul per 1×10^6 of nuclei). Proceed to the Nuclear Run-On reaction or store nuclei at -80°C . If properly stored, frozen nuclei can be used several months after collection.

3.2 Nuclear Run-On

1. Use 100 ul of frozen nuclei preparation that correspond to approximately 1×10^7 nuclei (see **Note** ³).
2. Prepare individual 1.5 ml tubes with 100 ul of $2 \times$ NRO buffer, add 100 ul of nuclei with immediate mixing. Pipette gently several times and incubate at 30°C for 7 min. During this step brominated UTP is incorporated in the nascent RNA.
3. Block the reaction by adding 600 ul of TRIzol LS reagent. Vortex thoroughly until nuclei dissolve. Incubate at room temperature for 5 min.
4. Add 160 ul of chloroform, shake vigorously for 20 sec. Incubate at room temperature for 3 min. Centrifuge at 4°C (12,000g) for 30 min.
5. Transfer the aqueous phase (upper layer) to a clean 1.5 ml tube. Add NaCl (up to 300 mM), 1 ul of GlycoBlue and 1 ml of cold 100% ethanol. Incubate at -20°C for at least 2 hours (can be done overnight).
6. Precipitate RNA at 4°C in a microcentrifuge (maximum speed) for 30 min.
7. Discard supernatant and wash with 75% ethanol, centrifuge for 5 min at maximum speed.

¹Adherent cells should be trypsinized as fast as possible and collected using ice-cold medium to ensure that transcription is arrested. Alternatively, culture dishes can be placed over an ice bed to collect cells with a disposable scraper.

²Gentle agitation is needed while the detergent solution (lysis buffer) is added to the swollen cell preparation to disrupt the cytoplasmic membrane. Avoid excessive foaming that would cause the nuclear membrane to break. This step can be performed with the aid of a vortex benchtop mixer using mild rotation settings.

³Place frozen nuclei on ice for approximately 5 minutes before use. Unused nuclei can be frozen again and stored at -80°C .

8. Carefully remove supernatant and air dry the RNA pellet (do not let over-dry, otherwise the pellet may become difficult to resuspend).
9. Resuspend RNA in 20 μ l of DNase and RNase free water supplemented with Suprase In (1U/ μ l).

3.3 RNA immunoprecipitation

Part A. DNase treatment

- 1 Incubate resuspended RNA at 60°C for 10 min.
- 2 Add 2.8 μ l 10 $\times$ DNase buffer and 3 μ l of DNase (from Turbo DNA-free kit). Incubate at 37°C for 30 min.
- 3 Mix well the DNase inactivation reagent (from Turbo DNA-free kit) and add 2 μ l to the reaction. Incubate at room temperature for 5 min. Mix 2-3 times during incubation by flicking the tube.
- 4 Centrifuge at 10,000g for 2 min, carefully transfer the supernatant to a clean tube. Repeat DNase treatment: add 3 μ l of DNase and incubate 20–30 min at 37°C.
- 5 Clean-up with 2 μ l of DNase inactivation reagent, perform centrifugation as before and transfer the supernatant to a clean 1.5 ml tube. Proceed to fragmentation.

Part B. RNA fragmentation and PNK treatment

- 6 For 30 μ l of DNase-free RNA solution: add 24 μ l of nuclease free water.
- 7 Add 6 μ l fragmentation reagent (from Ambion RNA fragmentation kit) to each sample. Split the reaction into 0.2 ml PCR tubes (10 μ l per tube).
- 8 Incubate at 70°C for 8 min. Add 1 μ l of Stop solution (from Ambion RNA fragmentation kit) to each 10 μ l reaction and keep on ice.
- 9 Pool reactions (approximate total volume: 60 μ l) and purify using Micro Bio-Spin P-30 Gel Columns. Prepare columns as per manufacturer's instruction, apply sample on top of the resin and centrifuge at 4,000g for 4 min, and then proceed to the polynucleotide kinase reaction.
- 10 Add fresh Suprase In to the sample (2 μ l of inhibitor for 50–55 μ l of sample). Set up a reaction with 30 U of T4 Polynucleotide Kinase (PNK) and 1 $\times$ PNK buffer. Incubate at 37°C for 60 min.
- 11 Add 20 U of PNK and EDTA (final 10mM) and incubate for additional 60 min at 37°C. Inactivate the enzyme at 75°C for 5 min.

Part C. Immunoprecipitation

- 12 Bring samples to 200 μ l with binding buffer (see **Note 4**).

- 13 Prepare beads for the RNA immunoprecipitation: use 50 ul of bead slurry per sample. During the washing and blocking procedure beads can be pooled (up to 5 samples or 250 ul of bead slurry). Precipitate beads by centrifugation at 900g for 3 min, wash beads twice with blocking buffer and resuspend them in 5 volumes of blocking buffer (1 volume refers to the dry bead pellet). Rotate at room temperature for 60 min.
- 14 Wash beads twice with 5 volumes of binding buffer, aliquot beads in individual tubes for immunoprecipitation. Beads are finally resuspended in 500 ul of binding buffer (for each 50 ul of original bead slurry).
- 15 Add 200 ul of RNA sample to 500 ul of resuspended beads and incubate at room temperature for 60 min in a rotisserie-style tube rotator.
- 16 Precipitate beads at 900g for 3 min, discard the supernatant and perform the following washes: 2× with 500 ul of binding buffer, 2× with 500 ul of low salt buffer, 1× with 500 ul of high salt buffer, 2× with 500 ul of TE+tween buffer. All washes are performed at room temperature, with rotation, for 2–3 min followed by centrifugation at 900g for 2 min.
- 17 Elute brominated nascent RNA from beads using 100 ul of elution buffer during 10 min incubation (see **Note** ⁵). Repeat the elution 3 additional times and collect the resulting 400 ul eluate in a clean 1.5 ml tube.
- 18 Purified nascent RNA by ethanol precipitation: add 1 ul glycogen, 300 mM NaCl and 1ml of cold 100% ethanol. Incubate at –20°C for 2 hours or overnight. Centrifuge tubes at maximum speed for 30 min wash once with 75% ethanol, air dry and resuspend in 5 ul of nuclease free water (containing 0,05% Tween 20 and 1 U/ul of Suprase In).

3.4 Library preparation

1. Before cDNA synthesis and adapter ligation, immunopurified RNA fragments are subjected to a poly-adenosine tailing reaction. Addition of a 3' poly-A stretch allows first strand cDNA synthesis to be performed with the NTI223 library adaptor, which contains a poly-dT stretch for priming. To each RNA sample add the following: 0.7ul of poly(A) polymerase buffer, 0.25 ul of diluted ATP, 0.7 ul of poly(A) polymerase (3.75 U). Incubate reactions at 37°C for 30 min.
2. Prepare the reverse transcriptase reaction by adding 0.9 ul of dNTP mix (10 mM each, included in the RT kit) and 0.9 ul of NTI223 oligo. Heat the sample at 75°C for 3 min, chill on ice. Add 1.7 ul of 10× RT buffer (provided with the kit), 3 ul of 25 mM MgCl₂, 1.7 ul of 0.1M DTT, 0.5 ul of Suprase In, 1 ul of SuperScript III. Incubate at 48°C for 40 min in a thermal cycler.

⁴All buffers used during immunoprecipitation should be supplemented with RNase inhibitor right before use (add Suprase In to a final concentration of 4 U/ml). Buffers should be kept cold to preserve the inhibitor, however, all binding and washing steps can be performed at room temperature.

⁵Elution are best performed at 37°C with continuous shaking to keep beads in suspension. Better results are achieved using a tube mixer and incubator such as the Thermomixer (Eppendorf).

3. The excess of NTI223 oligonucleotide is removed by digestion with 3 ul of exonuclease I (20 U/ul). Samples are incubated at 37°C for 15 min.
4. Add 2 ul of NaOH 1M and incubate at 98°C for 20 min to allow enzyme inactivation and degradation of the RNA strand. Neutralize with 1 ul of HCl 2M.
5. Run samples on a denaturing 10% TBE-Urea polyacrylamide gel. Prior to electrophoresis, samples should be denatured at 70°C for 3 min, chilled on ice, and then resuspended in an equal volume of non-denaturing sample buffer. Load samples along with an appropriate ladder (see **Note** ⁶) and run at 180 volts for approximately 1 hour. Stain gel with a non-mutagenic dye and visualize using a blue-light transilluminator. Excise DNA fragments from 100 to 400 bp and elute in Elution buffer I (TE with 0.1% Tween 20). Perform elution at room temperature in a rotisserie-style tube rotator (see **Note** ⁷). Use a Millipore Ultrafree MC-HV column (as per manufacturer's instructions) to discard gel debris and recover the eluate, which contains single stranded cDNA.
6. Precipitate DNA by adding 1 ul glycogen solution, 30 ul of NaCl 5M and 1 ml of cold 100% ethanol. Incubate at -20°C for 2 hours or overnight. Centrifuge at maximum speed for 30 min, discard the supernatant and wash pellet once with cold 75% ethanol. Air dry pellet and resuspend in 7.5 ul of nuclease free water.
7. The NTI223 adaptor bears both 5' and 3' library adaptors, separated by a cleavable spacer. The adaptor is ligated to the free 3' end of ssDNA with a circularization reaction, followed by cleavage of the abasic spacer in NTI223 oligo to re-linearize cDNA. Circularization is performed by adding the following reagents to the 7.5 ul sample: 1ul of CircLigase 10× reaction buffer, 0.5 ul of 1mM ATP, 0.5 ul of 50mM MnCl₂, 0.4 ul of CircLigase (100 U/ul). Incubate at 60°C for 60 min, followed by 10 min at 80°C to inactivate the enzyme.
8. Re-linearization of cDNA is achieved by adding 3.3 ul of 4× re-linearization mix and 1 ul of APE1 phosphodiesterase (15 U) followed by incubation at 37°C for 45 min. Repeat the reaction by adding 1 ul of fresh APE1 and incubate at 37°C for additional 45 min. Inactivate the enzyme at 65°C for 20 min. During this reaction, the spacer in NTI223 is cleaved to generate linear cDNA inserts with 5' and 3' adapters that can be used to amplify the library by conventional PCR.
9. Dilute samples 2× with nuclease free water (approximate final volume of cDNA: 30 ul) and perform amplification in a thermal cycler using proofreading DNA polymerase and betaine to prevent secondary structures. Add the following reagents to the PCR reaction: 500 nm NTI200, 500 nm NTI201, 10 ul of 5× High-Fidelity buffer, 200 uM of dNTPs, 5 ul of betaine, 1 U of Phusion DNA polymerase. Perform reactions in a final volume of 50 ul using no more than 5 ul of cDNA as template (set up multiple reactions per sample). Use the following

⁶An appropriate ladder for single stranded nucleic acids should be loaded on the denaturing gel. Use of single stranded RNA marker with a low molecular range (i.e. 1000-100 bp) is recommended. Denature RNA ladder as per manufacturer's instructions.

⁷To maximize recovery of ssDNA from the elution, cut gel bands into small pieces using a sharp blade.

PCR routine: 30 sec at 98°C for initial denaturation, 20 cycles of (10 sec at 98°C, 30 sec at 57°C, 15 sec at 72°C), 5 min at 72°C as final elongation step.

10. Pool PCR reactions from the same sample and concentrate using a PCR purification column (alternatively, perform ethanol precipitation). Elute in 25–30 ul of nuclease free water (or a proprietary elution buffer), mix with 6× Gel Loading Buffer.
11. Pre-run a 10% polyacrylamide TBE gel for 15 min. Run samples for 2 hours at 120 volts, along with an appropriate low molecular-weight DNA ladder. Excise fragments comprised between 150 and 300 bp.
12. Elute the library using 400 ul of Elution buffer II (TE with 0.1% Tween 20 and 150 mM NaCl), rotate at room temperature for 4 hours. Transfer eluate and gel debris to a Spin X filter column and centrifuge at 14,000g for 2 min. Add 300 mM NaCl, 1 ul of glycogen and ethanol to precipitate DNA. Incubate overnight at –20°C. Centrifuge at maximum speed for 30 min, wash once with 75% ethanol and resuspend in 10–20 ul of TE (see **Note** ⁸).
13. Libraries can be clustered using Illumina platforms and sequenced with oligo NTI202. Before sequencing, samples should be evaluated on a BioAnalyzer (Agilent) using the High Sensitivity DNA analysis kit. Optimal size range for GRO-seq libraries is 200–250 bp, which is shorter than conventional RNA-seq libraries. Use KAPA library quantification kit to measure the exact concentration (expected range: 100–200 nM).

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⁸Library yield after precipitation can be evaluated by NanoDrop (Thermo Scientific). Concentration should lie between 30 and 100 ng/ul.

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