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Nuclear Run On Transcription Assay

PLOS Pathogens

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ABSTRACT

Adapted from Core LJ, Waterfall JJ, Lis JT. Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters. Science. 2008;322(5909):1845-8. PubMed PMID: 19056941.

This method can be used to detect nascent transcripts. The conditions given are suitable for HeLa cells and should be adapted if other cell types are used.

GUIDELINES

This protocol is for 1 confluent 15 cm² dish of HeLa cells. Parameters should be adapted for other cell types.

MATERIALS

MATERIALS

⊗ brdU/BrU conjugated beads **Santa Cruz Biotechnology Catalog #SCBT-32323Ac**

⊗ ATP **Contributed by users Catalog #035RA02825**

⊗ CTP **Contributed by users Catalog #035RC02825**

⊗ GTP **Contributed by users Catalog #035RG02825**

⊗ RNasin **Promega**

⊗ sarkosyl **Sigma Aldrich Catalog #L5777**

⊗ sspe 20x **Sigma**

BEFORE START INSTRUCTIONS

Prepare the following reagents in advance and store at +4°C:

Swelling buffer:	Stock	Final Conc	Vol (for 50 ml)
Tris-HCl, pH 7.5	1 M	10 mM	500 µl
MgCl ₂	1 M	2 mM	100 µl
CaCl ₂	1 M	3 mM	150 µl
Water			to 50 ml

Protocol status: Working
We use this protocol and it's working

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8471

Keywords: transcription, run on, nascent transcript

Lysis Buffer: Swelling buffer + Igepal Glycerol RNasin Water	Stock 50 % 100 % 40 U/μl	Final Conc 0.5 % 10 % 2 U/ml	Vol (for 50 ml) 500 μl 5 ml 100 U to 50 ml
Freezing Buffer: Tris-HCl pH 8.3 Glycerol MgCl ₂ EDTA Water	Stock 1 M 100 % 1 M 0.5 M	Final Conc 50 mM 40 % 5 mM 0.1 mM	Vol (for 50 ml) 2.5 ml 20 ml 250 μl 10 μl to 50 ml
Assay Buffer: Tris-HCl pH 8.0 MgCl ₂ DTT KCl RNasin *Sarkosyl ATP, GTP, CTP and UTP/Br-UTP Water *Add sarkosyl just before starting incubation	Stock 1 M 1 M 0.1 M 3 M 40 U/μl 10 %	Final Conc 10 mM 5 mM 1 mM 300 mM 20 U/ml 1 % 500 μM	Vol (for 5 ml) 50 μl 25 μl 50 μl 0.5 μl 2.5 μl 500 μl to 5 ml
Binding Buffer for anti BrdU: SSPE EDTA Tween20 NaCl Water	Stock 20 x 0.5 M 10 % 5 M	Final Conc 0.25 x 1 mM 0.05 % 37.5 mM	Vol (for 14 ml) 175 μl 28 μl 70 μl 105 μl to 14 ml
Blocking Buffer: Binding Buffer + polyvinylpyrrolidone (PVP) BSA Water	Stock 10 % 50 μg/ml	Final Conc 0.1 % 1 μg/ml	Vol (for 2 ml) 20 μl 40 μl to 2 ml
Low Salt Wash Buffer: SSPE EDTA Tween20 Water	Stock 20 x 0.5 M 10 %	Final Conc 0.2 x 1 mM 0.05 %	Vol (for 10 ml) 100 μl 20 μl 50 μl to 10 ml
High Salt Wash Buffer: SSPE EDTA Tween20 NaCl Water	Stock 20 x 0.5 M 10 % 5 M	Final Conc 0.25 x 1 mM 0.05 % 100 mM	Vol (for 10 ml) 125 μl 20 μl 50 μl 200 μl to 10 ml
Elution Buffer : DTT NaCl Tris-HCl pH 7.5 EDTA SDS Water	Stock 1 M 5 M 1 M 0.5 M 20 %	Final Conc 20 mM 150 mM 50 mM 1 mM 0.1 %	Vol (for 5 ml) 100 μl 150 μl 250 μl 10 μl 25 μl to 5 ml

TET:
Tris.EDTA + 0.5 % Tween20

Add 2 µl of RNasin to all buffers (40 U/µl) unless otherwise stated

Isolation of nuclei

- 1 Wash cells 3 times directly in 15 cm² dish with ice cold PBS
Add 10ml of ice cold swelling buffer directly onto the cells and incubate on ice for 5 min
Scrape cells and transfer to a 15 ml conical tube and pipette up and down several times with cut-off pipette tips
Centrifuge at 4°C, at 1000 rpm for 10 min
Remove supernatant, add 1 ml of lysis buffer and pipette up and down several times with cut-off pipette tips (pellet contains isolated nuclei)
Make the volume up to 10ml with buffer
Wash nuclei once with lysis buffer
Resuspend in 1 ml of freezing buffer
Centrifuge at 1000 xg for 10 mins
Resuspend in 200 µl of freezing buffer (It will be a bit difficult to resuspend, so use cut-off tips)

Run on transcription reaction

- 2 Divide the samples into two tubes, one for BrUTP and one for UTP
Add 100 µl of Assay Buffer (assay Buffer contains all NTPs except BrUTP/UTP)
*Add Sarkosyl to individual tubes (20 µl of 10% stock/200 µl reaction) after the run on reaction buffer is added
Add BrUTP/UTP
Incubate the reaction at 31°C for 15 min with shaking every 5 min

RNA purification

- 3 Extract RNA with Trizol by adding 0.8 ml of Trizol to 200 µl run on reaction
Pipette up and down with tips
Add chloroform and precipitate with isopropanol (as for Trizol extraction)
Dissolve RNA with 90 µl water, incubate at 65°C for 5min
Add 10 µl 10x DNase buffer
Add 2 µl DNase1
Incubate for 20 min at 37°C (in a thermomixer if possible)
Heat inactivate DNase (65°C for 10 min or according to manufacturer's instructions)
Add 100 µl water, 300 mM NaCl, 200 µl isopropanol
Centrifuge 15 min at 13x g
Wash with 70% ethanol
Dissolve RNA in 50 µl DEPC water

Immunoprecipitation

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Equilibrate 40 µl of 25% slurry of BrdU/BrU conjugated beads in 500 µl binding buffer 2x
Add buffer, rotate tubes for 5 min on a rotating wheel (8 rpm) and centrifuge at 1000 x g for 2 min
Place on ice for 1 min before removing the supernatant
Block the beads in 5x volume of blocking buffer for 2 hours at 4°C
(Add extra 2 µl RNasin for every 1 ml of blocking buffer at this step)
Wash 2x with 500 µl of binding buffer
Resuspend beads in 400 µl binding buffer/ reaction
Bring NRO RNA to 100 µl and heat sample at 90°C for 5 min then place on ice for 2-3 mins, add
300 µl binding buffer, add 5 mM EDTA
Bind RNA to beads for 1 h* at 4°C. (* test times between 30 mins and 2 h)
Perform washes as follows: rotate tube for 3-5 min on a rotating stand, centrifuge at 1000 x g for 3
min, discard the supernatant
Wash 1x in 500 µl binding buffer
Wash 1x in 500 µl low salt buffer
Wash 1x in 500 µl high salt buffer
Wash 2x in 500 µl TET buffer
Elute 4x in 125 µl of elution buffer that has been heated to 42°C. Vortex the tubes every few
minutes
Perform acid phenol chloroform extraction once
Add 1 µl glyco blue
Add 0.3 M (final conc) of ammonium/sodium acetate (stock 5 M)
Add 3 vol of 100% ethanol
Precipitate at -20°C for 20 min
Spin and wash with 70% ethanol
Resuspend in 30 µl of DEPC water
Quantify by nanodrop