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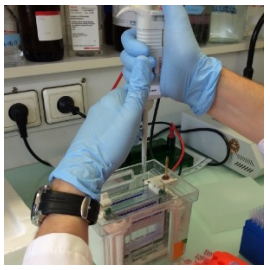
Nuclear Run On Transcription Assay



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Xavier Contreras[1], Rosemary Kiernan[1]

[1]Institut de Genetique Humaine, UMR9002, Montpellier, France

Rosemary Kiernan
Institut de Genetique Humaine, Montpellier, France

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

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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 cm^2 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 **Catalog #**035RA02825
- CTP **Catalog #**035RC02825
- GTP **Catalog #**035RG02825
- RNasin **Promega**
- sarkosyl **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**L5777
- sspe 20x **Merck MilliporeSigma (Sigma-Aldrich)**

Before start

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

	Swelling buffer: **Tris-HCl, pH 7.5** **$MgCl_2$** **$CaCl_2$** **Water**	**Stock** **1 M** **1 M** **1 M**	**Final Conc** **10 mM** **2 mM** **3 mM**	**Vol (for 50 ml)** **500 µl** **100 µl** **150 µl** **to 50 ml**
	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** **MgCl2** **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 :	Stock	Final Conc	Vol (for 5 ml)
	DTT	1 M	20 mM	100 µl
	NaCl	5 M	150 mM	150 µl
	Tris-HCl pH 7.5	1 M	50 mM	250 µl
	EDTA	0.5 M	1 mM	10 µl
	SDS	20 %	0.1 %	25 µl
	Water			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 cm^2 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