Oct 07, 2020

fastGRO-LI (Low-input fastGRO)

Forked from fastGRO

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1 Works for me dx.doi.org/10.17504/protocols.io.bkdtks6n

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DOI

dx.doi.org/10.17504/protocols.io.bkdtks6n

PROTOCOL CITATION

Elisa Barbieri, Connor Hill, Alessandro Gardini 2020. fastGRO-LI (Low-input fastGRO) . **protocols.io** https://dx.doi.org/10.17504/protocols.io.bkdtks6n

FORK FROM

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CREATED

Aug 27, 2020

LAST MODIFIED

Oct 07, 2020

PROTOCOL INTEGER ID

41107

MATERIALS

NAME	CATALOG #	VENDOR
IGEPAL-CA630	I3021 SIGMA-ALDRICH	Sigma Aldrich
1.5 mL Eppendorf tubes		
Chloroform		
Isopropanol		
PBS		
NEBNext Ultra II Directional RNA Library Prep Kit for Illumina - 24 rxns	E7760S	New England Biolabs
ATP	035RA02825	
0.5 M EDTA	AM9261	Ambion
Glycerol		
Eppendorf tubes (1.5 & 2.0 ml)		
Corning® 15 ml Centrifuge Tubes		Corning
M280 streptavidin beads		Invitrogen - Thermo Fisher
5M NaCl	AM9760G	Ambion
Capillary electrophoresis instrument (e.g. Agilent Tapestation 4200)		
1M MgCl2 solution	AM9530G	Thermo Fisher Scientific

NAME	CATALOG #	VENDOR
50ml Falcon tubes	352070	Corning
Qubit RNA HS Assay Kit	Q32852	Thermo Fisher Scientific
4-Thiouridine	T4509-25MG	Sigma Aldrich
1M Tris-HCl (pH 8.0)	15568025	Thermo Fisher Scientific
ethanol		
Tween-20		
Sarkosyl	L7414	Sigma Aldrich
TRIzol™ LS Reagent	10296028	Thermo Fisher
CTP	18331017	Thermo Fisher
GTP	18332015	Thermo Fisher
SUPERase• In™ RNase Inhibitor (20 U/μL)	AM2696	Thermo Fisher
Qubit™ 3 Fluorometer	Q33216	Thermo Fisher
NN-Dimethylformamide	D4551	Sigma Aldrich
2M KCl	AM9640G	Invitrogen - Thermo Fisher
Glycogen	10901393001	Millipore Sigma
1M DTT	43816-10ML	Sigma
1M Tris-HCl pH 7.5	15567027	Thermo Fisher Scientific
1M CaCl2	21115	Sigma-aldrich
4-Thiouridine-5-Triphosphate (4-thio-UTP)	N-1025-1	
RNA ScreenTape and Reagents		Agilent Technologies
Bioruptor USD-200		Diagenode
MTSEA-biotin-XX	#90066	Biotium

BEFORE STARTING

Prepare spike-in RNA or Nuclei

Prepare 50 mM of 4-thiouridine (4sU) in DEPC-treated water. Aliquot and store in the dark at -20 °C.
Incubate drosophila cells for 5 minutes with 50mM of 4sU in their growing medium. Wash cells with 1X PBS, lyse in Trizol reagent. Extract RNA, aliquot, snap-freeze in liquid nitrogen and store at -80 °C.
Can also prepare drosophila nuclei to control for the Nuclear run-on. Can be done using same nuclei extraction protocol (steps 1-9) and drosophila nuclei can be added to your sample at steps 8 or 12 to 5-10% of amount of nuclei from your sample.

Prepare buffers and solutions.

Swelling Buffer (SB) - Add 2 U/ml Superare-In before use.

- 10 mM Tris-HCL pH 7.5
- 2 mM $MgCl_2$
- 3 mM $CaCl_2$

Store at 4 °C.

Swelling Buffer + 10% Glycerol (GSB) - Add 2 U/ml Superare-In before use. Store at 4 °C.

Lysis Buffer (LyB) - Add 2 U/ml Superare-In before use.

- 10 mM Tris-HCL pH 7.5
- 2 mM $MgCl_2$
- 3 mM $CaCl_2$
- 10% Glycerol
- 1% Igepal

Store at 4 °C.

Freezing Buffer (FB) - Add 2 U/ml Superare-In before use.

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- 40% glycerol
- 5 mM $MgCl_2$
- 0.1 mM EDTA
- 50 mM Tris-HCL pH8

Store at 4 °C.

1 mg/mL MTS-Biotin
Prepare 1 mg/ml MTS-Biotin (Biotium) in DMF (store at -20C, stable for up to 3 months).

10x Biotinylation Buffer
100 mM Tris pH 7.5
10 mM EDTA pH 8.0
Store at 4 °C.

Nuclei isolation

1 Harvest cells and wash in cold 1X PBS

2 Resuspend cells in **10 mL** of ice-cold SB.
Incubate for **00:05:00** .
Spin **400 x g, 00:10:00** .

3 Remove supernatant and resuspend in **10 mL** GSB

Volume of GSB should be at least 5 times the volume of cell pellet

4 Vortex lightly while adding **10 mL** of LyB

5 Incubate on ice for **00:05:00**
Add **25 mL** of LyB and centrifuge **600 x g, 00:05:00** .

6 Flick to loosen pellet and resuspend in **25 mL** of LyB.
Centrifuge **600 x g, 00:05:00**

7 Remove supernatant and resuspend in **10 mL** of FB.
Take **10 µl** for cell count.

8 Centrifuge **900 x g, 00:06:00** and resuspend using wide-end pipette tips in FB to a maximum concentration of 5x10^6 nuclei per **25 µl** of FB.

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9 Nuclei can be stored at **-80 °C** for months.

Nuclear Run On

10 Prepare fresh 2x Nuclear run-on buffer (NRO). (**25 µl** /sample)

- 10 mM Tris-HCl ph8
- 5 mM $MgCl_2$
- 300 mM KCl
- 1 mM DTT
- 500 µM ATP
- 500 µM GTP
- 500 µM 4-thio-UTP
- 2 µM CTP
- 200 µ/ml Superase-in
- 1% Sarkosyl (N-Laurylsarcosine sodium salt solution)

Per library, use 0.5-5x10^6 nuclei

11 Warm the NRO buffer at **30 °C** .

12 Thaw nuclei **On ice** .

5-10% drosophila nuclei can be added to your sample as spike-in if not using 4S-U labelled drosophila RNA in step 26.

13 Mix **25 µl** of thawed nuclei solution with **25 µl** of 2xNRO buffer.
Pipette up and down 15 times using end-cut pipette tip.

14 Incubate **00:07:00** at **30 °C** .

15 Add **150 µl** Trizol LS.
Vortex.
Incubate **00:05:00** at **Room temperature** .

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STOP POINT: Freeze with liquid nitrogen, and store at -80 °C

Total RNA precipitation

16 Add **40 µl** of chloroform, shake vigorously by hand for **00:00:15**

17 Incubate **00:02:00** at **Room temperature** .

18 Centrifuge at **12000 rpm, 4°C, 00:15:00** .

19 Transfer upper, aqueous phase into new 1.5 mL centrifuge tube.

20 Add **100 µl** of isopropanol to precipitate RNA and incubate at **Room temperature** for **00:10:00** .

Can add 1-2 ul of 2 µg/µL glycogen to allow for visualization of pellet with lower RNA concentrations.

21 Centrifuge at **12000 rpm, 4°C, 00:10:00** .

22 Wash RNA pellet using **1 mL** of cold 75 % ethanol
Centrifuge at **12000 rpm, 4°C, 00:10:00** .

23 Completely remove ethanol and air-dry pellet.
Dissolve in **100 µl** of nuclease-free water.

24 Determine concentration by Nanodrop or Qubit.

RNA fragmentation

25 Transfer **30 µg** of RNA to a 1.5 ml tube and add water up to **100 µl** .

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Save 5 µl of unfragmented RNA to be run on TapeStation as a control for fragmented RNA and to check quality of RNA.

26 Add 5-10% of labelled spike-in RNA if using instead of drosophila nuclei.

27 Fragment RNA using Bioruptor with the following settings using: 1 cycle: 30 sec / 30 sec ON / OFF at high settings.

28 Transfer fragmented RNA to 2 ml tube.

29 Analyze fragmentation efficiency of fragmented versus unfragmented RNA on Agilent 2200 TapeStation.

30 Sonicated RNA can be snap-frozen in liquid nitrogen and stored at **-80 °C**

MTS-Biotinylation

31 Incubate RNA at **65 °C** for **00:02:00** , then **On ice** for **00:05:00** .

32 Prepare Biotin-RNA mix in 1.5 ml tube. Follow the order:

- up to **30 µg** of fragmented RNA in **100 µl**
- H20 up to **140 µl** µl
- **5 µl** HEPES pH 7.4
- **5 µl** 0.5M EDTA
- **50 µl** Diluted MTS-Biotin

33 Incubate in the dark at **24 °C** and **800 rpm** for **00:30:00** .

Precipitation of biotinylated RNA

34 Add **160 µl** of chloroform to the RNA-biotin in the 2 mL phase-lock tube and mix by manually shaking the tube.

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35 Centrifuge full speed **21000 x g, 4°C, 00:05:00** .

36 - Transfer upper phase into new tube (appr **200 µl**).

37 If needed at 1-2µl of 2 µg/µl Glycogen to allow for visualization of pellet with lower RNA concentrations.

38 Add 1/10 volume (20 µl) of 5 M NaCl and mix.

39 Add 1 volume (200 µl) of isopropanol and mix for 15 sec manually.

40 Centrifuge **16000 x g, 4°C, 00:30:00** .

41 Remove supernatant.

42 Wash pellet with **1 mL** of ice-cold 75% ethanol.
Centrifuge **10006 x g, 4°C, 00:05:00** .

43 Remove supernatant.

44 Spin quickly at **4 °C** and remove remaining supernatant with 200 µl and 10 µl pipettes.

Biotinylated RNA should NOT dry.

45 Resuspend RNA in **44 µl** of nuclease-free water.

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Biotinylated RNA can be stored at **-80 °C** .

DNAse treatment

46 Add **5 µl** of TURBO DNase buffer to the RNA-biotin.

47 Add **1 µl** of TURBO DNAse.

48 Incubate at **37 °C** for **00:30:00** .

49 Add **1 µl** of inactivation reagent.

50 Incubate at **Room temperature** for **00:05:00** .

51 Centrifuge **10000 x g, Room temperature , 00:02:00** .

52 Transfer RNA-biotin solution in a new tube.

Enrichment of biotinylated RNA

53 Prepare Wash Buffer (WB):
- 100 mM Tris pH 7.5
- 10 mM EDTA pH 8.0
- 1M NaCl
- 0.1% (vol/vol) Tween-20

54 Leave half volume of WB at room temperature and heat the other half at **65 °C** .

55 Prepare the beads:

55.1 Take **25 µl** of beads per sample.

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55.2 Wash the beads twice with 2 volumes (**50 µl** per sample) of wash buffer.

55.3 Resuspend in 1 Volume (**25 µl** per sample) of wash buffer.

56 Incubate at **65 °C** for **00:10:00** .

Place on ice for **00:05:00** .

57 Add **25 µl** of prepared Invitrogen streptavidin beads to **50 µl** of RNA-biotin.

58 Incubate at **4 °C** for **00:15:00** in rotation.

59 Transfer tubes to a magnetic rack.
Remove supernatant.
Do not disturb beads.

60 Wash at least 3 times with **200 µl** of warm (**65 °C**) WB.

61 Wash at least 3 times with **200 µl** of room temperature WB.

62 Resuspend beads in **25 µl** of 100 mM DTT and incubate **00:05:00** .

63 Transfer tubes to the magnetic rack.
Collect the 4-thio-labeled RNA in a new tube.

64 Repeat steps 62-63 and collect the eluted RNA in the same tube (50 µl total volume).

RNA precipitation of labelled RNA

65 Add **150 µl** nuclease-free water to the samples to final volume of 200 µl.

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66 Add **160 µl** of chloroform to the labelled RNA and mix

67 Centrifuge **21000 x g, 4°C, 00:05:00** .

68 Transfer upper, aqueous phase into new 1.5 mL centrifuge tube.

69 Add **2 µl** of 2 µg/µl glycogen and mix.

70 Add 1/10 volume (20 µl) of 5 M NaCl and mix.

71 Add 1 volume (200 µL) of isopropanol and mix.

72 Centrifuge **21000 x g, 4°C, 00:30:00** .

73 Remove supernatant.

74 Wash pellet with **1 µl** of ice-cold 75 % ethanol.

75 Centrifuge **21000 x g, 4°C, 00:05:00** .

76 Remove supernatant.

77 Quick spin **21000 x g, 4°C, 00:00:15** and remove remaining supernatant with 10 µL pipette.

78 Resuspend RNA in **6 µl** DEPC-treated H2O.

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79 Use **1 µl** to measure concentration of labelled RNA by Qubit fluorometer.

80 Libraries can be prepared with NEBNext Ultra II Directional RNA Library Prep or other library prep kits.

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