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fastGRO

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

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Materials

MATERIALS

- 1.5 mL Eppendorf tubes
- Chloroform
- Isopropanol
- PBS
- NEBNext Ultra II Directional RNA Library Prep Kit for Illumina - 24 rxns **New England Biolabs** **Catalog #**E7760S
- 0.5M EDTA **Catalog #**AM92606
- 2 mL Eppendorf
- Glycerol
- RNA Clean & Concentrator-5 Kit **Zymo Research** **Catalog #**R1015
- Corning® 15 ml Centrifuge Tubes **Corning**
- M280 streptavidin beads **Invitrogen - Thermo Fisher**
- 5M NaCl **Ambion** **Catalog #**AM9760G
- Capillary electrophoresis instrument (e.g. Agilent Tapestation 4200)
- 1M MgCl2 solution **Thermo Fisher Scientific** **Catalog #**AM9530G
- 50ml Falcon tubes **Corning** **Catalog #**352070
- Qubit RNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32852
- 1M Tris-HCl (pH 8.0) **Thermo Fisher Scientific** **Catalog #**15568025
- Tween-20
- Ethanol
- KCl 2M **Catalog #**AM9640G
- Sarkosyl **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**L7414
- 4-thiouridine (4sU) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T4509
- TRIzol™ LS Reagent **Thermo Fisher** **Catalog #**10296028
- ATP **Thermo Fisher** **Catalog #**18330019
- CTP **Thermo Fisher** **Catalog #**18331017
- GTP **Thermo Fisher** **Catalog #**18332015
- EZ-Link™ HPDP-Biotin, No-Weigh™ Format **Thermo Fisher** **Catalog #**A35390
- SUPERase• In™ RNase Inhibitor (20 U/μL) **Thermo Fisher** **Catalog #**AM2696

- Qubit™ 3 Fluorometer **Thermo Fisher** **Catalog #**Q33216
- IGEPAL® CA-630 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**I8896
- Glycogen **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**10901393001
- 1M DTT **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**43816-10ML
- 1M Tris-HCl pH 7.5 **Thermo Fisher Scientific** **Catalog #**15567027
- 1M CaCl2 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**21115
- NN-Dimethylformamide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**227056-100ML
- 4-Thiouridine-5-Triphosphate (4-thio-UTP) **Catalog #**N-1025-1
- RNA ScreenTape and Reagents **Agilent Technologies**
- Bioruptor USD-200 **Diagenode**

Before start

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.

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

Store at 4 °C.

1 mg/mL EZ-link HPDP Biotin

Resuspend 1 mg in 1 ml of DMF in polypropylene tubes, vortex and incubate at 36 °C for 30 min.
Store at -20 °C.

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

> Note
>
> 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 concentration of 2×10^7 nuclei per 10 µl of FB.

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

Nuclear Run On

10 Prepare fresh 2x Nuclear run-on buffer (NRO). (100 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)

> Note
>
> Per library, use 1.5-2 x10^7 nuclei

11 Warm the NRO buffer at 30 °C .

12 Thaw nuclei On ice .

> Note
>
> 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 100 µL of thawed nuclei solution with 100 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 600 µL Trizol LS.
Vortex.
Incubate 00:05:00 at Room temperature .

Note

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

Total RNA precipitation

16 Add 160 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 400 µL of isopropanol to precipitate RNA and incubate at Room temperature for 00:10:00 .

Note

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 150 µg of RNA to a 1.5 ml tube and add water up to 500 µL .

> Note
>
> 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

EZ-link HPDP-Biotinylation

31 Transfer 150 µg of fragmented RNA in one 2 ml Eppendorf tube.

Note

Use only polypropylene tubes during biotinylation.

32 Incubate RNA at 65 °C for 00:10:00 , then On ice for 00:05:00 .

33 Prepare Biotin-RNA mix in 2 ml tube. Follow the order:

- up to 150 µg of fragmented RNA in 500 µL
- 100 µL Biotinylation Buffer
- 200 µL DMF
- 200 µL EZ-link HPDP Biotin

34 Incubate in the dark at 24 °C and 800 rpm for 02:00:00 .

Precipitation of biotinylated RNA

35 Add appr. 800 µL of chloroform to the RNA-biotin in the 2 mL phase-lock tube and mix by manually shaking the tube.

36 Centrifuge at 4 °C and 16000 x g 00:05:00

37 Transfer upper phase into new tube (appr 1 mL).

38 Add 1/10 volume (100 µl) of 5 M NaCl and mix.

Note

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

39 Add 1 volume (1 ml) of isopropanol and mix for 00:00:15 manually.

40 Centrifuge 16000 x g, 4°C, 00:05:00 .

41 Remove supernatant.

42 Wash pellet with 1 mL of ice-cold 75% ethanol.
Centrifuge 10006 x g, 4°C, 00:30:00 .

43 Remove supernatant.

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

Note

Biotinylated RNA should NOT dry.

45 Resuspend RNA in 100 µL of nuclease-free water.

Note

Biotinylated RNA can be stored at -80 °C .

Enrichment of biotinylated RNA

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

47 Leave half volume of WB at Room temperature and heat the other half at 65 °C.

48 Prepare the M280 Streptavidin Dynabeads:

48.1 Take 100 µL of beads per sample.

48.2 Wash the beads twice with 2 volumes (200 µL per sample) of wash buffer.

48.3 Resuspend in 1 Volume (100 µL per sample) of wash buffer.

49 Increase the volume of the solution of RNA-biotin to 200 µL with nuclease-free water.

Note

Can also scale down to 100 µL if you have less than 150 µg and use 50uL of beads.

50 Incubate at 65 °C for 00:10:00.
Place on ice for 00:05:00.

51 Add 100 µL of prepared Invitrogen streptavidin beads to 200 µL of RNA-biotin.

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

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

54 Wash at least 3 times with 900 µL of warm (65 °C) WB.

55 Wash at least 3 times with 900 µL of room temperature WB.

56 Resuspend beads in in 100 µL of 100 mM DTT and incubate 00:05:00 .

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

58 Repeat steps 57-58 and collect the eluted RNA in the same tube (200 µL total volume).

Purification of labelled RNA with RNA Clean and

59 Use buffers provided with the RNA Clean and Purification kit-5 (Zymo Research). Add ethanol to wash and pre-wash buffers and resuspend DNAse in water.

60 Add 400 µL of RNA Binding Buffer to each sample and mix.

61 Add 600 µL of ethanol (95-100%) and mix.

62 Transfer the sample to the Zymo-Spin IC Column in a Collection Tube and centrifuge for 00:00:30 . Discard the flow-through.

63 Add 400 µL of RNA Wash Buffer to the column and centrifuge for 16000 x g, 00:00:30 . Discard the flow-through.

64 For each sample to be treated, prepare DNase I reaction mix in an RNase-free tube. Mix well by gentle inversion:

- 5 µL DNase I
- 35 µL DNA Digestion Buffer

65 Add 40 µL reaction mix directly to the column matrix. Incubate at Room temperature for 00:15:00 .

66 Add 400 µL RNA Prep Buffer to the column and centrifuge for 00:00:30 . Discard the flow-through.

67 Add 700 µL RNA Wash Buffer to the column and centrifuge for 00:00:30 . Discard the flow-through.

68 Add 400 µL RNA Wash Buffer to the column and centrifuge for 00:02:00 . Discard the flow-through.

69 Centrifuge for 00:01:00 at full speed to ensure complete removal of the wash buffer. Transfer the column carefully into an RNase-free tube.

70 Add 6 µL DNase/RNase-Free Water directly to the column matrix, incubate for 00:01:00 and centrifuge for 00:00:30

71 Measure concentration of labelled RNA by Qubit fluorometer.

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