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fastGRO

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MATERIALS

NAME	CATALOG #	VENDOR
1.5 mL Eppendorf tubes		
Chloroform		
Isopropanol		
PBS		
NEBNext Ultra II Directional RNA Library Prep Kit for Illumina - 24 rxns	E7760S	New England Biolabs
0.5M EDTA	AM92606	
2 mL Eppendorf		
Glycerol		
RNA Clean & Concentrator-5 Kit	R1015	Zymo Research
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
50ml Falcon tubes	352070	Corning
Qubit RNA HS Assay Kit	Q32852	Thermo Fisher Scientific
1M Tris-HCl (pH 8.0)	15568025	Thermo Fisher Scientific
Tween-20		
Ethanol		

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NAME	CATALOG #	VENDOR
KCl 2M	AM9640G	
Sarkosyl	L7414	Sigma Aldrich
4-thiouridine (4sU)	T4509	Sigma Aldrich
TRIzol™ LS Reagent	10296028	Thermo Fisher
ATP	18330019	Thermo Fisher
CTP	18331017	Thermo Fisher
GTP	18332015	Thermo Fisher
EZ-Link™ HPDP-Biotin, No-Weigh™ Format	A35390	Thermo Fisher
SUPERase• In™ RNase Inhibitor (20 U/μL)	AM2696	Thermo Fisher
Qubit™ 3 Fluorometer	Q33216	Thermo Fisher
IGEPAL® CA-630	I8896	Sigma Aldrich
Glycogen	10901393001	Millipore Sigma
1M DTT	43816-10ML	Sigma
1M Tris-HCl pH 7.5	15567027	Thermo Fisher Scientific
1M CaCl2	21115	Sigma-aldrich
NN-Dimethylformamide	227056-100ML	Sigma Aldrich
4-Thiouridine-5-Triphosphate (4-thio-UTP)	N-1025-1	
RNA ScreenTape and Reagents		Agilent Technologies
Bioruptor USD-200		Diagenode

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.

- 40% glycerol

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- 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

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 2x10^7 nuclei per 10 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). (**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)

Per library, use 1.5-2 x10^7 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 **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** .

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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** .

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** .

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

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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.

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

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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.

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.

Biotinylated RNA should NOT dry.

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45 Resuspend RNA in **100 µl** of nuclease-free water.

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.

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** .

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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.

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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.

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