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 We are still developing and optimizing this protocol

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Brad-seq mRNA (for Shotgun or DGE) V.1

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MATERIALS

■ Lysis/binding buffer (LBB)

A	B	C
Component	Stock	Volumes for 50 ml
100 mM Tris-HCl	1 M pH 8	5 ml
1M LiCl	8 M	6.25 ml
10 mM EDTA	500 mM pH 8	1 ml
1% SDS (or LiDS)	5% w/v	10 ml
5 mM DTT	0.5 M	500 µl
Antifoam A		750 ul
RNase-free H2O		Make up to 50 ml

1. Add 5 µl/ml 2-Mercaptoethanol per ml before use.
2. Store at 4 C; warm up to RT by placing at 37 C before use (make sure salt crystals are all dissolved)
3. Shelf life: 1-2 months

■ **Washing Buffer A (WBA)**

A	B	C
Component	Stock	Volumes for 50 ml
10 mM Tris-HCl	1 M pH 8	500 µl
150 mM LiCl	8 M	940 µl
1 mM EDTA	500 mM pH 8	100 µl
0.1% SDS	5% w/v	500 µl
RNase-free H ₂ O		Make up to 50 ml

Store at 4 C and keep on ice prior to use

■ **Washing Buffer B (WBB)** (Store at 4 C and keep on ice prior to use)

■ **Low-salt Buffer (LSB)** (Store at 4 C and keep on ice prior to use)

10 mM Tris-HCl pH 8 (Store at room temperature)

Tissue Lysis

5m

- 1 Add 5 µL of 2-Mercaptoethanol (2-ME) to 1 mL of LBB (for ratio, adjust it for the amount LBB used)
- 2 Wipe off RNA later from tissue; Place 20 mg of tissue in crushing tube with metal cone

- 3 Add 200 µL 100/10: LBB/Tissue ratio in tube
- 4 Crush sample with multi-beads shocker at 2000 rpm , 2-4 times
- 5 Sit in Room temperature for 00:05:00 and remove metal cone 5m
- 6 Centrifuge at 14000 rpm, 00:01:00
- 7 Transfer all the lysate to a new 1.5 mL tube
- 8 Centrifuge at 14000 rpm, 00:10:00
- 9 Carefully transfer the supernatant to a new tube. Be careful not to carry over cell debris

Note

Stop here and store samples in -80 °C if needed

1st mRNA extraction

10m

10 Put 100 μ L lysed sample in each well of 8-strip (Keep another half in -80 $^{\circ}$ C just in case)

11 Add 6.25 μ L of biotin-20nt-20T oligo; Mix well by pipetting

12 Sit in Room temperature for 00:10:00 for incubation

10m

12.1 While waiting, prepare NEB magnetic Streptavidin beads
Resuspend beads well before use

12.2 Dispense 20 μ L of Streptavidin beads into each well of a 8-strip (1 mg : 1 μ L beads/tissue ratio); Put 8-strip on magnetic rack and remove supernatant

12.3 Resuspend beads with 100 μ L LBB to wash the beads;
Place 8-strip on magnet rack and remove supernatant
Beads are ready for use

13 Add biotin-incubated samples to washed beads

14 Slowly stir at Room temperature at 500 rpm for 00:10:00

10m

- 15 Place 8-strip on magnet rack and remove supernatant

Note

If DNA is needed, keep the supernatant

Safety information

2-ME in the solution

- 16 Wash with 150 μL of cold WBA (keep it as cool as possible) and Place 8-strip on magnet rack and remove supernatant
- 17 Wash with 150 μL of cold WBB (keep it as cool as possible) and Place 8-strip on magnet rack and remove supernatant
- 18 Wash with 150 μL of cold LSB (keep it as cool as possible) and Place 8-strip on magnet rack and remove supernatant

19 RNA elution buffer prep.

A	B	C
Component	Stock conc.	Volume for 1 mL
Tris-HCl	10 mM	999 µl
2-Mercaptoethanol	1 M	1 µl

Freshly make every time before use

20 Resuspend beads in 17 µL RNA elution buffer

21 Warm at 80 °C for 00:02:00 in a thermal cycler. After that, cool it quickly on ice for 00:05:00

7m

Note

Keep TTT away from AAA

22 Place 8-strip on magnet rack and **transfer supernatant** (17 µL) to a new 8-strip

Note

Supernatant here is GOOD for **DGE protocol**

22.1 DNAase treatment for Secondary mRNA Recovery (for SHO protocol)

22.2

RNase-Free DNase Set Qiagen Catalog
#79254

DNase prep.

A	B
Component	Volume
RDD buffer	1.85 uL
DNAase I	0.46 uL
total	2.31 uL

DNAase (1500 K units): add 550 uL DEPC water, divided in small amount in tubes for use;
shelf life 9 months

22.3

Add 2.31 μL DNAase to 1st RNA supernatant (17 μL) = 19.31 μL in total

22.4

Sit at Room temperature for 00:15:00

15m

22.5

Kill the activity of DNAase at 70 °C for 00:10:00

10m

2nd mRNA recovery

10m

23

Add 150 μL DEPC water to re-suspend the used beads;
Place 8-strip on magnet rack and remove supernatant

24

Add 5 μL of 2-Mercaptoethanol (2-ME) to 1 mL of SBB (for ratio, adjust it for the amount SBB used)

- 25 Add 150 μL SBB and Place 8-strip on magnet rack and remove supernatant (Wash beads)
- 26 Add in DNAase treated RNA (19.31 μL) to the washed beads + 130 μL SBB
- 27 Sit at Room temperature for 00:10:00 10m
- 28 Place 8-strip on magnet rack and remove supernatant
- 29 Wash with 150 μL of cold WBA (keep it as cool as possible) and Place 8-strip on magnet rack and remove supernatant
- 30 Wash with 150 μL of cold WBB (keep it as cool as possible) and Place 8-strip on magnet rack and remove supernatant
- 31 Wash with 150 μL of cold LSB (keep it as cool as possible) and Place 8-strip on magnet rack and remove supernatant
- 32 Resuspend beads in 17 μL RNA elution buffer

- 33 Warm at 80 °C for 00:02:00 in a thermal cycler. After that, cool it quickly on ice for 00:05:00

Note

Keep TTT away from AAA

- 34 Place 8-strip on magnet rack and **transfer supernatant** (17 µL) to a new 8-strip

Note

Supernatant here is GOOD for **SHO protocol**

Note

Can be stored at -20 °C if needed

RNA fragmentation & 3-prime adapter cDNA priming

5m

- 35 Make 3 strand priming 2.5 µL

A	B
Component	Volume
5X Thermo Scientific RT buffer	1.5 µl
3-prime priming adapter	1 µl

DGE 3' priming adaptor L-3ILL-20TV.2

GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTT

Shotgun 3' priming adaptor L-3ILL-N8.2

GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNN

- 36 Mix 2.5 µL 3 strand priming with 7.5 µL extracted RNA = 10 µL in total
Keep the remaining at -20 °C)

37 10 µL of mixed in thermo cycler for RNA fragmentation

Note

Fragmentation/Priming program: for DGE

(25 °C 1s , 94 °C 1.5 min , 30 °C 1 min , 20 °C 4 min , 20 °C hold)

Note

Fragmentation/Priming program: for DGE

(25 °C 1s , 94 °C 1.5 min , 30 °C 1 min , 20 °C 4 min , 20 °C hold)

38 5 µL master mix + 10 µL fragmented RNA = 15 µL mixed solution 1st strand master mix

A	B
Component	vol/rxn
5X Thermo Scientific RT buffer	1.5 µl
0.5M DTT	0.3 µl
H2O	2.2 µl
25mM dNTPs	0.5 µl
RevertAid RT enzyme	0.5 µl

39 Mixed solution in thermo cycler for reverse transcription

Note

Condition:

25 °C 10min , 42 °C 50min , 50 °C 10min , 70 °C 10min , 4 °C hold

- 40 Use Ampure beads solution for cDNA purification;
🧪 35 µL Ampure bead solution + 🧪 20 µL cDNA
Ampure bead solution

A	B
Component	Volume
50 mM EDTA pH 8.0	5 µl
Ampure beads	30 µl

Ampure bead = 1.5X sample (3:2)

- 41 Vortex for ⌚ 00:05:00 at 🌡️ Room temperature

5m

- 42 Place 8-strip on magnet rack and remove supernatant

- 43 Wash with 🧪 200 µL ethanol without suspending; Dry the pellet
Repeat this process twice

Note

Don't dry the pellet too much at 2nd time, otherwise it's hard to elute

Note

Not recommend to store at 🌡️ -20 °C at this stage

5-prime adapter sequence addition

15m

44 Add 4 µL of 10 µM 5-prime adapter directly to the pellet at Room temperature to resuspend the bead pellet

45 Prepare the master mix in advance during cDNA synthesis, and add the enzyme just before using 6 µL mater mix/rxn + 4 µL suspended cDNA pellet
mater mix recipe

A	B
Component	Volume
H2O	3.5 µl
10X Poll buffer	1 µl
250 mM MgCl2	1 µl
25 mM dNTPs	0.25 µl
DNA Pol I	0.25 µl

46 Sit at Room temperature for 00:15:00

15m

47 40 µL Ampure bead solution + 10 µL cDNA
Ampure bead solution recipe

A	B
Component	Volume
50 mM EDTA pH 8.0	10 µl
Ampure beads	30 µl

Ampure bead = 1.5X sample (3:2)

48 Vortex for 00:05:00 at Room temperature

49 Place 8-strip on magnet rack and remove supernatant

50 Wash with 200 μL ethanol without suspending; Dry the pellet
Repeat this process twice

Note

Don't dry the pellet too much at 2nd time, otherwise it's hard to elute

51 Elute the pellet in 20 μL of 10 mM Tris pH 8.0;
Let it sit for 00:01:00

1m

52 Transfer the supernatant to new tubes

Enrichment and adapter extension

53 12.2 μL Enrichment master mix + 2 μL 1 μM ILL-INDEX primer + 5.8 μL cDNA
Enrichment master mix recipe

A	B
Component	Volume
2 X KAPA	10 μl
2 μM PE1 primer	1 μl
8 μM each EnrichS1 + S2 primers	1 μl
25mM dNTPs	0.2 μl

54 Mixed solution in thermo cycler

Note

98 °C 5 min, (98 °C 20 s, 65 °C 15 s, 72 °C 15 s) * 18 cycles,
 72 °C 3 min, 10 °C hold

Final Cleanup

55 24 µL Ampure beads + 20 µL of enrichment product;
Mix well

56 Vortex for 00:05:00 at Room temperature

57 Place 8-strip on magnet rack and remove supernatant

58 Wash with 200 µL ethanol without suspending; Dry the pellet
Repeat this process twice

Note

Don't dry the pellet too much at 2nd time, otherwise it's hard to elute

59 Elute the pellet in 12 µL of H₂O

60 Use Bioanalyzer for quantification for measuring the concentration of each sample

Library preparation

10m

- 61 Mix equimolar of samples in a 1.5 mL tube
- 62 Add same amount of AMPure XP as the DNA, mix well, and let it stand for 00:05:00 5m
- 63 Place on magnet rack for 00:05:00, remove supernatant and wash twice with 70 μ L 5m
80% EtOH. Dry.
- 64 Resuspend the beads by adding 22 μ L of water, leave them in the magnet rack for 00:05:00 5m
Transfer the supernatant to a new 0.5 mL low bind tube.
- 65 Quantification with Qbit or Bioanalyzer