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

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Version 1
Dec 23, 2020
In Development dx.doi.org/10.17504/protocols.io.bqyymxxw

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DOI
dx.doi.org/10.17504/protocols.io.bqyymxxw

PROTOCOL CITATION
Yin-Tse Huang 2020. Brad-seq mRNA (for Shotgun or DGE). **protocols.io** https://dx.doi.org/10.17504/protocols.io.bqyymxxw

CREATED
Dec 23, 2020

LAST MODIFIED
Dec 23, 2020

PROTOCOL INTEGER ID
45816

MATERIALS TEXT

- **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 H2O		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)

DISCLAIMER:

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 RNAlatter from tissue; Place **20 mg** of tissue in crushing tube with metal cone

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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

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

1st mRNA extraction 10m

10 Put **100 µl** lysed sample in each well of 8-strip (Keep another half in **-80 °C** just in case)

11 Add **6.25 µl** 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

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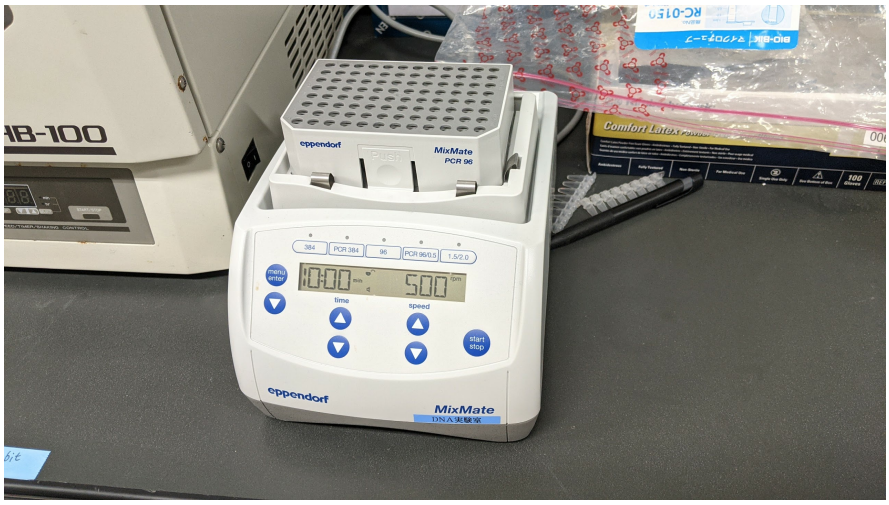
12.2 Dispense **20 µl** of Streptavidin beads into each well of a 8-strip (**1 mg** : **1 µl** beads/tissue ratio); Put 8-strip on magnetic rack and remove supernatant

12.3 Resuspend beads with **100 µl** 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

10m

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



15 Place 8-strip on magnet rack and remove supernatant

If DNA is needed, keep the supernatant

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

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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

Keep TTT away from AAA

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

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**

DANase 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

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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**

Keep TTT away from AAA

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

Supernatant here is GOOD for **SHO protocol**

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 GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTV
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

Fragmentation/Priming program: for DGE
(**25 °C 1s** , **94 °C 1.5 min** , **30 °C 1 min** , **20 °C 4 min** , **20 °C hold**)

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

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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

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

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

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

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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

15m

46 Sit at **Room temperature** for **00:15:00**

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

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

1m

51 Elute the pellet in **20 µl** of 10 mM Tris pH 8.0;

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Let it sit for **00:01:00**

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

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

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

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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 µl** 80% EtOH. Dry. 5m

64 Resuspend the beads by adding **22 µl** 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

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