Profiling the binding sites of RNA-binding protein by LACE-seq

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Method Article

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Abstract

RNA-binding proteins (RBPs) directly interact with various RNAs in living cells to regulate their processing, translation, and stability. Identifying the precise binding sites of RBPs is critical for appreciating their physiological or pathological roles in germline and early embryo development. Current methods typically need millions of cells to map RBP binding positions, which prevents us from appreciating the crucial role of RBPs in early development. Here, we present the LACE-seq method for unbiased mapping of RBP-binding sites at single-nucleotide resolution in fewer cells or even single oocytes. LACE-seq depends on RBP-mediated reverse transcription termination, and linear amplification of the cDNA ends for deep sequencing. To further promote its application, we describe a step-by-step protocol about how to construct a successful LACE-seq library.

Introduction

The human genome encodes over 1,500 RNA-binding proteins (RBPs) that play crucial roles in the development or differentiation of somatic cells, germ cells, and early embryonic cells\(^1-3\). RBPs achieve such regulatory functions by directly interacting with various target RNAs via specific motifs or structural elements\(^1\). As a well-accepted working rule, RBPs usually regulate gene expressions in a position-dependent manner\(^4\). For example, the preferable bindings of RBPs at intronic regions usually suggest potential regulations in alternative splicing, while their bindings at 3'UTR tend to participate in regulating mRNA stability or translation\(^5-7\). As an emerging theme, many RBPs are intimately involved in transcriptional regulation by interacting with promoter and enhancer noncoding RNAs\(^7-9\). Such position effects and regulatory rules highlight the value of developing transcriptome-wide methods for mapping the precise binding positions of a defined RBP.

Methods currently available for profiling RBP binding landscapes in living cells include CLIP-seq (cross-linking and immunoprecipitation sequencing, also known as HITS-CLIP), iCLIP, and eCLIP\(^10-13\). These three state-of-the-art approaches use a specific antibody to pull down defined RBP-associated RNA fragments from millions of cells and sequence them unbiasedly. Conceptually different from CLIP-based methods, an elegant technique called TRIBE fuses the catalytic domain of adenosine deaminases ADAR with individual RBP and deduces RBP targets by directly calculating the in vivo RNA editing sites\(^14\). Though TRIBE can map RBP targets from hundreds of cells, significant challenges arose from the low editing efficiency and duplex bias of the ADARcd domain\(^15\), which tends to cause frequent A-to-I editing at distal positions far from the bonafide RBP binding sites. Moreover, ectopically expressed RBPs may perturb native RNA interaction networks. Therefore, mapping protein-RNA interactions in fewer cells under native conditions still represents a significant challenge.

To address this technical challenge, we have developed a LACE-seq method for unbiased mapping of precise RBP-binding positions in fewer cells or even single oocytes under native conditions. In brief, LACE-seq includes the following steps: (1) cells are cross-linked with UV-C light, and the interested RBP-RNA
complexes are immunoprecipitated from cell lysate and fragmented on the beads with micrococcal nuclease; (2) The 3’ ends of fragmented RNAs are dephosphorylated and ligated with a 5’ pre-adenylated linker containing four randomized nucleotides; (3) reverse transcription is performed on beads with a biotinylated primer containing the T7 promoter sequence; (4) the cDNA terminated at intact RBP-RNA cross-linking sites are poly (A) tailed and enriched for PCR amplification and subsequent in vitro transcription; (5) the linearly amplified RNAs are converted into libraries for single-end deep sequencing. Of note, the IVT steps can be omitted if starting with millions of cells. We have recently demonstrated the power of LACE-seq methods in determining the target repertoire and regulatory mechanisms of multiple RBPs in mouse oocytes\textsuperscript{16}.

### Reagents

#### Basic reagents

1. 10 × PBS buffer, pH 7.4 (Thermo Scientific, AM9624)
2. Protein A/G magnetic beads (Thermo Scientific, 26162)
3. SUPERase-In RNase Inhibitor (Thermo Scientific, AM2696)
4. RQ1 RNase-Free DNase (Promega, M6101)
5. Micrococcal Nuclease (Thermo Scientific, EN0181)
6. FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific, EF0651)
7. T4 RNA ligase 2, truncated (NEB, M0242)
8. Superscript II Reverse Transcriptase (Thermo Scientific, 18064014)
9. Ribolock RNase inhibitor (Thermo Scientific, E00381)
10. Exonuclease I (E. coli) (NEB, M0293)
11. RNase H (Thermo Scientific, EN0202)
12. Dynabeads MyOne Streptavidin C1 (Thermo Scientific, 650002)
13. Terminal transferase (NEB, M0315)
14. KAPA HiFi HotStart ReadyMix PCR Kit (KAPA Biosystems, KK2601)
15. Ampure XP beads (Beckman Coulter, A63881)
16. T7 RNA Polymerase (NEB, M0251)
17. TURBO DNase (Thermo Scientific, AM2238)
18. Agencourt RNAClean beads (Beckman Coulter, A63987)
19. Platinum Pfx DNA Polymerase (Thermo Scientific, C11708021)
20. MinElute Gel Extraction Kit (Qiagen, 28604)
21. Qubit RNA HS Assay Kit (Thermo Scientific, Q32852)
22. Qubit dsDNA HS Assay Kit (Thermo Scientific, Q32851)
23. dNTP Solution Mix (NEB, N0447)
24. Ribonucleotide Solution Mix (NEB, N0466)
25. dATP Solution (NEB, N0440)

Buffers

1. Blocking buffer (1 × PBS, 0.2 mg/ml glycogen, 0.2 mg/ml BSA)
2. Wash buffer (1 × PBS, 0.1% SDS, 0.5% NP-40, 0.5% sodium deoxycholate)
3. High-salt wash buffer (5 × PBS, 0.1% SDS, 0.5% NP-40, 0.5% sodium deoxycholate)
4. PNK buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl$_2$, 0.5% NP-40)
5. 1 × MN reaction buffer (50 mM Tris-HCl, pH 8.0, 5 mM CaCl$_2$)
6. 1 × PNK + EGTA buffer (50 mM Tris-HCl, pH 7.4, 20 mM EGTA, 0.5% NP-40)
7. 1 × B & W buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl)
8. 0.1 M Na-phosphate buffer, pH 8.0 (93.2 mM Na$_2$HPO$_4$, 6.8 mM NaH$_2$PO$_4$, 0.05% tween 20)
9. 6 × Orange G dye (10 mM Tris-HCl, pH 7.6, 0.15% orange G, 60% glycerol, 60 mM EDTA)

Oligos

1. 3’ linker:

/5rApp/NNNNAGATCGGAAGACGCTGTTAGGGAAAGAGTGT/3ddC/ (5rApp devotes pre-adenylated 5’ nucleotides, N represents randomized nucleotide, and 3ddC indicates 3’-dideoxycytidine)

2. T7-RT primer:
/Biotin/GATCACTAATACGACTCCTATAGGGACACTCTTTCCCTACACGACGCTCTTCGATCT

3. Second-strand primer:

CAAGCAGAAGACGGCATACGAGATTTTTTTTTTTTTTTTTTTTTTTTTTTVV

4. Primer A:

GATCACTAATACGACTCCTATAGG

5. P7 primer:

CAAGCAGAAGACGGCATACGAGAT

6. P5 index primer:

AATGATACGGCGACCACCGAGATCTACACNNNNNACACTCTTTCCCTACACGACGCTCTTCGATCT (NNNNN devotes index nucleotides)

**Equipment**

1. CL-1000 ultraviolet crosslinker (UVP)

2. 1.5 ml DNA LoBind tubes (Eppendorf, 022431021)

3. 2 ml DNA LoBind tubes (Eppendorf, 022431048)

4. LP Vortex Mixer (Thermo Scientific)

5. Tube Revolver (Crystal Industries, HYQ-2231)

6. ThermoMixer C (Eppendorf)

7. DynaMag-96 side magnet (Thermo Scientific)

8. DynaMag-2 magnet (Thermo Scientific)

9. Qubit fluorometer (Thermo Scientific)

10. Mini G centrifuge (IKA)

11. Cell lifter (Corning, 3008)

12. Thermal cycler (Bio-Rad, T100)

13. Sorvall ST16R centrifuge (Thermo Scientific)
14. Fusion FX (Vilber)

15. Ultra-low temperature freezer (Thermo Scientific, Forma 900 Series)

16. Blue light gel imager (Bio-Friend, BD-BGC1)

17. 10 cm Petri dishes (NEST, 752001)

**Procedure**

A. For low-input cells or single oocytes

1. UV cross-linking of cells

   1) Resuspend HeLa cells, K562 cells, or mouse oocytes in PBS and pick a specific number of cells to a 1.5 ml LoBind microcentrifuge tube using needles under a microscope.

   2) Spin down the cells to the bottom with an IKA mini G centrifuge and quickly put the tube on ice.

   3) Irradiate cells at 400 mJ with UV-C light on ice for two times. The cross-linked samples can be stored at -80 °C for two weeks until use or directly proceeded to the next step. (see **Note 1**)

2. Magnetic beads preparation

   4) Mix the protein A/G magnetic beads thoroughly before use. Place 10 µl of beads for each sample into a 1.5 ml microcentrifuge tube.

   5) Wash beads twice with 200 µl of BSA/PBS solution (0.1% BSA in 1×PBS) and block with 200 µl of blocking buffer at room temperature (RT) for 1 h by rotating at 20 rpm.

   6) Place the microcentrifuge tube into a magnetic stand for 1 min and discard the supernatant. Wash the blocked beads once with 200 µl of Na-phosphate buffer and resuspend with 40 µl of Na-phosphate buffer containing 2 µg specific antibody. Rotate the tube at 20 rpm for 1 h at RT. (See **Note 2** and **Note 3**)

   7) Spin down the beads to the bottom of the microcentrifuge tube with an IKA mini G centrifuge for 5 s. Place the tube into a magnetic stand for 1 min and discard the supernatant.

   8) Wash beads twice with 200 µl of wash buffer and resuspend in 10 µl of wash buffer for each sample. Leave the beads on ice for the following addition to the cross-linked lysate.

3. RNA immunoprecipitation and fragmentation

   9) Lyse cross-linked cells with 50 µl of wash buffer on ice for 10 min.

   10) Add 1 µl of SUPERase·In RNase inhibitor and 4 µl of RQ1 DNase to the cell lysate and digest genomic DNA at 37 °C for 3 min in ThermoMixer C. Then snap-chill the tube on ice for 3 min.
11) Aliquot 10 µl of antibody-coupled beads from Step 8 into cell lysate prepared at Step 10 and rotate at 20 rpm for 1 h at 4 °C.

12) Spin briefly and place the tube into a magnetic stand for 1 min. Discard the supernatant and wash the beads three times with 200 µl of ice-cold wash buffer, once with 200 µl of high-salt wash buffer, and once with 200 µl of PNK buffer.

13) Dilute MNase 1:1000 by mixing 0.5 µl of MNase with 499.5 µl of 1 × MN buffer. Sequentially dilute MNase 1:3000 to 1:6000 by mixing 0.5 µl of diluted MNase with corresponding volumes of 1 × MN buffer. Add 10 µl of the diluted MNase to the beads and incubate at 37 °C for 3 min. (See Note 4)

14) Add 200 µl of 1 × PNK + EGTA buffer directly to the tube and place into a magnetic stand for 1 min. Discard the supernatant and wash the beads once more with 1 × PNK + EGTA buffer, twice with 200 µl of wash buffer, and twice with 200 µl of PNK buffer.

4. RNA dephosphorylation and 3’ linker ligation

15) Resuspend the beads from Step 14 in 20 µl of FastAP mixture (2 µl 10 × FastAP buffer, 1 µl FastAP alkaline phosphatase, 17 µl water) and incubate at 37 °C for 10 min in ThermoMixer C with intermittent mixing for 15 s at 1000 rpm every 3 min.

16) Discard the supernatant and wash the beads twice with 200 µl of 1 × PNK + EGTA buffer, twice with 200 µl of PNK buffer, and twice with 200 µl of BSA solution (0.2 mg/ml BSA in DEPC-treated water).

17) Resuspend the beads in 20 µl of ligation mixture (12.5 µl water, 2 µl 10 × ligation buffer, 0.5 µl 3’ linker (1 µM), 1 µl T4 RNA ligase 2, 4 µl 50% PEG8000) and incubate at 25 °C for 2.5 h in ThermoMixer C with intermittent vortexing at 500 rpm for 15 s every 3 min.

18) Place the tube on a magnetic stand for 1 min and discard the supernatant. Wash beads three times with 200 µl of PNK buffer.

5. Reverse transcription on beads

19) Resuspend the beads in 8.5 µl of DEPC-treated water and 1 µl of T7-RT primer (0.5-100 nM). The amount of T7-RT primer is determined by the starting cell numbers. For example, we typically use 1 µl T7-RT primer with concentration of 0.5 nM for single cell, while 100 nM for dozens of cells. (see Note 5)

20) Transfer the 9.5 µl beads mixture to a new PCR tube, heat at 65 °C for 5 min, and snap-chill on ice for 2 min.

21) Add RT mixture (3 µl of 5 × first-strand buffer, 0.5 µl of 0.1 M DTT, 0.5 µl of Superscript II, 0.5 µl of RNase inhibitor, 1 µl of dNTP mix (10 mM)) to the PCR tube and mix by pipetting up and down for 20 times.
22) Incubate the PCR tube at 42 °C for 50 min, 70 °C for 15 min, and hold at 12 °C in a thermal cycler.

23) Add 2 µl of 10 × Exonuclease I buffer and 3 µl of Exonuclease I mixture directly into the tube. Mix and incubate at 37 °C for 1 h, 80 °C for 20 min.

6. First-strand cDNA capture by streptavidin beads

24) Release the first-strand cDNA from Protein A/G beads by adding 10 µl of RNase H reaction mixture (3 µl of 10 × RNase H buffer, 1 µl of RNase H, 6 µl of water) to the PCR tube from Step 23. Mix briefly and incubate the mixture at 37 °C for 30 min, 65 °C for 20 min in a thermal cycler.

25) Place the PCR tube on a magnetic stand for 1 min and transfer the supernatant to a new 1.5 ml LoBind microcentrifuge tube. Keep the tube on ice and waiting for the addition of Streptavidin beads.

26) Transfer 5 µl of Streptavidin beads to a new 1.5 ml tube per sample and wash three times with 100 µl of 1 × B & W buffer.

27) Resuspend Streptavidin beads in 5 µl of 1 × B & W buffer and transfer them to the supernatant from Step 25. Incubate the tube at RT for 30 min by occasionally mixing every 5 min.

28) Place the tube into a magnetic stand for 1 min and discard the supernatant.

29) Wash beads twice with 200 µl of 1 × B & W buffer and once with 200 µl of BSA solution.

7. Poly(A) tailing and pre-PCR

30) Resuspend Streptavidin beads in 9 µl of nuclease-free water and transfer to a new PCR tube.

31) Add 3.5 µl of TdT mixture (1.25 µl of 10 × Terminal transferase Buffer, 1.25 µl of CoCl₂, 0.5 µl of Terminal transferase, 0.5 µl of dATP (0.2 µM)) directly to the PCR tube and incubate at 37 °C for 8 min, 70 °C for 10 min in a thermal cycler. (See Note 6)

32) Add 0.5 µl of 10 µM second strand primer, 0.5 µl of 10 µM primer A, 12.5 µl of 2 × KAPA HiFi HotStart ReadyMix directly to the PCR tube and mix by pipetting up and down for 20 times.

33) Run PCR program: 98 °C for 3 min; 98 °C for 15 s, 50 °C for 20 s, and 72 °C for 30 s, 14-18 cycles; 72 °C for 5 min.

34) Place the PCR tube into a magnetic stand for 2 min and transfer the supernatant to a 1.5 ml LoBind microcentrifuge tube.

35) Add 46.8 µl of Ampure XP beads (1.8: 1 ratio) to the LoBind tube. Pipette samples up and down 20-30 times and incubate at RT for 5 min.
36) Place the tube into a magnetic stand for 5 min and discard the supernatant. Wash Ampure XP beads twice with 200 μl of freshly prepared 80% ethanol.

37) Discard the supernatant and resuspend beads in 13 μl of nuclease-free water and pipette samples up and down 20-30 times. Incubate at RT for 5 min.

38) Place the tube into a magnetic stand for 5 min and transfer the supernatant to a new PCR tube.

8. *In vitro* transcription (IVT) and RNA purification

39) Add IVT reaction mixture (2 μl of 10 × Reaction buffer, 2 μl of NTP mix (25 mM), 1 μl of 0.1 M DTT, 0.5 μl of RNase Inhibitor, 2 μl of T7 Polymerase) to the PCR tube and incubate at 37 °C for 24 h.

40) Add DNase mixture (3 μl of 10 × TURBO buffer, 1 μl of TURBO enzyme, 6 μl of water) to the IVT solution and incubate at 37 °C for 30 min. Transfer the reaction mixture to a new 1.5 ml LoBind tube.

41) Add 66 μl of Agencourt RNA Clean beads (2.2: 1 ratio) to the 1.5 ml tube. Pipette samples up and down 20-30 times and incubate at RT for 5 min.

42) Place the tube into a magnetic stand for 5 min and discard the supernatant. Wash RNA Clean beads twice with 200 μl of freshly prepared 80% ethanol.

43) Resuspend RNA Clean beads in 13 μl of nuclease-free water, pipette samples up and down 20-30 times, and incubate at RT for 5 min.

44) Place the LoBind tube into a magnetic stand for 5 min and transfer the supernatant to a new PCR tube.

45) Take 1 μl of sample for RNA quantification using Qubit RNA HS Assay Kit.

9. Reverse transcription, PCR barcoding, and deep sequencing

46) Add 1 μl of 10 μM P7 primer to the PCR tube and heat at 65°C for 5min. Snap-chill it on ice for 2 min.

47) Add RT mixture (4 μl of 5 × first strand buffer, 1 μl of 0.1 M DTT, 0.5 μl of Superscript II, 0.5 μl of RNase inhibitor, and 1 μl of dNTP mix (10 mM)) to the PCR tube.

48) Incubate at 42 °C for 50 min, 70 °C for 15 min, and hold at 12 °C.

49) Add PCR mixture (1 μl of 10 μM P7 primer, 1 μl of 10 μM P5 index primer, 3 μl of 10 × Pfx Buffer, 1 μl of 50 mM MgSO₄, 0.6 μl of dNTP (25 mM each), 0.8 μl of Pfx enzyme, 2.6 μl of water) to cDNA product.

50) Run PCR program: 94 °C for 3 min; 94 °C for 15 s, 62 °C for 30 s, and 72 °C for 30 s, 8-12 cycles; 72 °C for 10 min. (see *Note 7*)
51) Fractionate PCR product on a 2% agarose gel at 120 V for 2 h, until the Orange G dye reaches the bottom of the gel.

52) Cut out the fragments between 130 bp and 300 bp from the gel and transfer them to a new 2 ml LoBind microcentrifuge tube.

53) Extract DNA with Gel Extraction Kit (Qiagen) and elute in 16 µl of elution buffer.

54) Quantify the library by Qubit dsDNA HS Assay Kit.

55) Sequence the LACE-seq library with Illumina HiSeq 2500 or NextSeq500 using single-end mode.

B. For large amounts of cells (> 10^6 cells)

Please note that this is a modified protocol suitable for large amounts of cells, for which IVT is not necessary.

1. UV cross-linking of cells

1) Grow cells in 10 cm Petri dishes to reach 80% confluence, rinse twice with 5 ml of ice-cold 1× PBS.

2) Place the dish on ice with the cover off, irradiate cells with UV-C light at 400 mJ two times.

3) Scrape the cells off from the dish into 5 ml of 1× PBS, transfer cell suspension to a 50 ml centrifugation tube, and centrifugation at 500 g for 5 min at 4 °C.

4) Discard the supernatant. The cross-linked samples can be stored at -80 °C for six months until use or directly proceed to the next step.

2. Magnetic beads preparation

5) Mix the protein A/G magnetic beads thoroughly before use. Place 40 µl of beads for each sample into a 1.5 ml microcentrifuge tube.

6) Wash beads twice with 200 µl of BSA/PBS solution (0.1% BSA in 1×PBS) and block with 500 µl of blocking buffer at room temperature (RT) for 1 h by rotating at 20 rpm.

7) Place the microcentrifuge tube into a magnetic stand for 1 min and discard the supernatant. Wash the blocked beads once with 500 µl of Na-phosphate buffer and resuspend with 200 µl of Na-phosphate buffer containing 10-15 µg specific antibody. Rotate at 20 rpm for 1 h at RT.

8) Spin down the beads to the bottom of the microcentrifuge tube with an IKA mini G centrifuge for 5 s. Place the tube into a magnetic stand for 1 min and discard the supernatant.
9) Wash beads twice with 500 µl of wash buffer and resuspend in 40 µl of wash buffer per sample. Leave the beads on ice for the following addition to the cross-linked lysate.

3. RNA immunoprecipitation and fragmentation

10) Lyse cross-linked cells on ice using 500 µl of wash buffer for 10 min.

11) Add 10 µl of SUPERase·In RNase inhibitor and 50 µl of RQ1 DNase to the cell lysate for digesting genomic DNA at 37 °C for 3 min in ThermoMixer C. Snap-chill the tube on ice for 3 min.

12) Aliquot 40 µl of antibody-coupled beads from Step 9 into cell lysate prepared at Step 11 and rotate at 20 rpm/min for 1 h at 4 °C.

13) Spin briefly and place the tube into a magnetic stand for 1 min. Discard the supernatant and wash the beads three times with 500 µl of ice-cold wash buffer, once with 500 µl of high-salt wash buffer, and once with 500 µl of PNK buffer.

14) Dilute MNase 1:1000 by mixing 0.5 µl of MNase with 499.5 µl of 1 × MN buffer. Add 1 µl of the diluted MNase and 499 µl of 1 × MN buffer to the thoroughly washed beads and incubate at 37 °C for 10 min in ThermoMixer C with intermittent mixing for 15 s at 1000 rpm every 3 min.

15) Place the tube into a magnetic stand for 1 min and discard the supernatant. Wash the beads twice with 500 µl of 1 × PNK + EGTA buffer, twice with 500 µl of wash buffer, and twice with 500 µl of PNK buffer.

4. RNA dephosphorylation and 3’ linker ligation

16) Resuspend the thoroughly washed beads in 100 µl of FastAP mixture (10 µl of 10 × FastAP buffer, 8 µl of FastAP alkaline phosphatase, 2 µl of RNase inhibitor, 80 µl of water) and incubate at 37 °C for 10 min in ThermoMixer C with intermittent mixing for 15 s at 1000 rpm every 3 min.

17) Discard the supernatant and wash the beads twice with 500 µl of 1 × PNK + EGTA buffer, twice with 500 µl of PNK buffer, and twice with 500 µl of BSA solution (0.2 mg/ml BSA in DEPC-treated water).

18) Resuspend the beads in 30 µl of ligation mixture (16 µl of water, 3 µl of 10 × ligation buffer, 1 µl of 3’ linker (10 µM), 1 µl T4 RNA ligase 2, 1 µl of RNase inhibitor, 8 µl 50% PEG8000) and incubate at 25 °C for 2.5 h in ThermoMixer C with intermittent vortexing at 500 rpm for 15 s every 3 min.

19) Place the tube on a magnetic stand for 1 min and discard the supernatant. Wash beads three times with 500 µl of PNK buffer.

5. Reverse transcription on beads

20) Resuspend the beads in 20 µl of nuclease-free water and 1 µl of 10 µM T7-RT primer.
21) Transfer the 21 µl beads mixture to a new PCR tube, heat at 65 °C for 5min, and snap-chill on ice for 2 min.

22) Add RT mixture (6 µl of 5 × first-strand buffer, 0.5 µl of 0.1 M DTT, 1 µl of Superscript II, 0.5 µl of RNase inhibitor, 1 µl of dNTP mix (10 mM)) to the PCR tube and mix by pipetting up and down for 20 times.

23) Incubate the PCR tube at 42 °C for 50 min, 70 °C for 15 min, and hold at 12 °C in a thermal cycler.

24) Add 4 µl of 10 × Exonuclease I buffer, 3 µl of Exonuclease I, and 3 µl of nuclease-free water mixture directly into the tube. Mix and incubate at 37 °C for 1 h, 80 °C for 20 min.

6. First-strand cDNA capture by streptavidin beads

25) Release the first-strand cDNA from Protein A/G beads by adding 10 µl of RNase H reaction mixture (5 µl of 10 × RNase H buffer, 2 µl of RNase H, 3 µl of nuclease-free water) to the PCR tube from Step 24. Mix briefly and incubate at 37 °C for 30 min, 65 °C for 20 min in a thermal cycler.

26) Place the PCR tube on a magnetic stand for 1 min and transfer the supernatant to a new 1.5 ml LoBind microcentrifuge tube. Keep the tube on ice and waiting for the addition of Streptavidin beads.

27) Transfer 10 µl of Streptavidin beads to a new 1.5 ml tube per sample and wash three times with 200 µl of 1 × B & W buffer.

28) Resuspend Streptavidin beads in 10 µl of 1 × B & W buffer and transfer them to the supernatant from Step 26. Incubate at RT for 30 min by occasionally mixing every 5 min.

29) Place the tube into a magnetic stand for 1 min and discard the supernatant.

30) Wash beads twice with 200 µl of 1 × B & W buffer and once with 200 µl of BSA solution.

7. Poly(A) tailing and pre-PCR

31) Resuspend Streptavidin beads in 14.5 µl of nuclease-free water and transfer to a new PCR tube.

32) Add 5.5 µl of TdT mixture (2 µl of 10 × Terminal transferase Buffer, 2 µl of CoCl₂, 1 µl of Terminal transferase, 0.5 µl of dATP (1 mM)) directly to the PCR tube and incubate at 37 °C for 10 min, 70 °C for 10 min in a thermal cycler.

33) Add 1 µl of 10 µM second strand primer, 1 µl of 10 µM P5 index primer, 20 µl of 2 × KAPA HiFi HotStart ReadyMix directly to the PCR tube and mix by pipetting up and down for 20 times.

34) Run PCR program as below: 98 °C for 3 min; 98 °C for 15 s, 55 °C for 20 s, and 72 °C for 30 s, 10-14 cycles; 72 °C for 5 min.
35) Fractionate PCR product on a 2% agarose gel at 120 V for 2 h, until the Orange G dye reaches the bottom of the gel.

36) Cut out the fragments between 130 bp and 300 bp from the gel and transfer them to a new 2 ml LoBind microcentrifuge tube.

37) Extract DNA with Gel Extraction Kit (Qiagen) and elute in 16 µl of elution buffer.

38) Quantify the library by Qubit dsDNA HS Assay Kit.

39) Sequence the LACE-seq library with Illumina HiSeq 2500 or NextSeq500 using single-end mode.

**Troubleshooting**

**Notes:**

1. The concentration and complexity of LACE-seq library usually are higher if starting with freshly prepared cells rather than frozen cells stocked at -80 °C over two weeks.

2. The antibody usage is dependent on the expression level of specific RBP and the input cell numbers. Users should also consider the immunoprecipitation efficiency when determining the antibody amounts to add.

3. A successful LACE-seq library construction strictly depends on the specificity and quality of the chosen antibody. We highly recommend to use the antibody suitable for immunoprecipitation.

4. The appropriate concentration of Micrococcal nuclease is critical for LACE-seq library construction. The protein-associated RNA fragments will be too short to be ligated with adaptors if the concentration of MNase is too high. In contrast, the fragment size will be too long to discern the cDNA termination sites if its concentration is too low. The dilution factor titrated in our hand is suitable for low-input cells and single oocytes.

5. We found that 0.5-100 fmol T7-RT primers are suitable for constructing LACE-seq library started from dozens of cells. Exonuclease I digestion cannot completely remove the T7-RT primer. If an overdose of primer is used, the residual T7-RT primers will be poly(A) tailed and form primer dimers in the constructed library.

6. The poly (A) tail length should be strictly controlled since a long stretch of A may lead to failure in sequencing. We have systematically tested the reaction time and dATP concentration to ensure the optimal length of the poly (A) tail.

7. The cycle numbers of barcoding PCR strictly depend on the concentration of transcribed RNA at the IVT step, which typically can produce approximately 120 ng total RNA if start with 1-50 oocytes. Over amplification usually introduce too many PCR duplicates, thus decreasing the library complexity.
**Time Taken**

A LACE-seq library typically can be constructed within 2.5 days:

Day 1

UV cross-linking of cells typically takes 10 min, while magnetic beads preparation, RNA immunoprecipitation, and fragmentation steps usually need 4 h. In subsequent phases, RNA dephosphorylation and 3’ linker ligation can be completed within 3 h. In comparison, the reverse transcription on beads requires 2.5 h, first-strand cDNA capture by streptavidin beads needs 1.5 h, and poly(A) tailing and pre-PCR can be finished within 1.5 h.

Day 2

In vitro transcription and RNA purification usually need 24 h.

Day 3

Reverse transcription, PCR barcoding, and size selection typically can be completed within 5 h.

**Anticipated Results**

If a LACE-seq library construction is successful, we expect to see a smeared band ranged from 130 bp to 400 bp and peaked at 180 bp. After gel extraction, we typically will get 50-200 ng of PCR products at a final concentration of 5-10 ng/μl. As reverse transcription termination sites are a critical feature for deducing the precise RBP binding positions and prioritizing the targets, among a successful LACE-seq library, the percentage of cDNA end containing reads should be higher than 60%.

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