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scPLATE-seq: simple, easily automated single-cell 3′ transcriptome profiling

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Protocol status: In development
We have used this protocol successfully for several years.

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Abstract

Since 2012, numerous single-cell RNA sequencing (scRNA-seq) technologies have been introduced. Most involve either significant startup cost, an expert operator, or expensive library preparation. We developed a plate-based library prep that incorporates pre-amplification pooling, unique molecular identifiers (UMIs), and mRNA enrichment using silanol coated magnetic beads. Our method, which builds off of **Bush et al.** (Nature Communications, 2017) and **Picelli et al.** (Nature Protocols, 2014), uses readily available equipment, is easy to automate, and costs a fraction of commercial kits. In addition, our method has been optimized to reduce barcode cross contamination that can occur in pooled pre-amplification reactions. This method allows for plate-based 3′ scRNA-seq with UMIs at relatively low cost, using standard lab equipment.

NOTE (2018/08/29): we are currently testing optimizations described by **Bagnoli et al.** (*Nature Communications*, 2018). This protocol may be updated accordingly.

Troubleshooting

Reagents: Lysis Buffer

1
- SUPERaseIn RNase inhibitor, 20U/µl (Ambion, #AM2696)
- Triton X-100, 10% (Sigma-Aldrich, #93443)
- dNTP set, 100mM each (Thermo Scientific, #R0181)
- HyClone water (GE Healthcare, #SH30538.01)
- Oligo(dT) mRNA capture primers, AAGCAGTGGTATCAACGCAGAGTAC[8 bp barcode]$N_{(8)}T_{(29)}V$, (IDT).
- Template switch oligo (TSO), AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG (IDT)

Reagents: RT Mix

2 RT Mix
- Betaine, ultrapure (Alpha Aesar, #J77507)
- ProtoScript II reverse transcriptase (NEB, #M0368X)
- $MgCl_2$, 2M, cell culture grade (Fisher Scientific, #BP9741-10X5)
- Dithiothreitol (DTT), ultrapure (Invitrogen, #15508013)
- HyClone water (GE Healthcare, #SH30538.01)

Reagents: Exo I digestion Mix

3
- Exonuclease I, 20U/µl (Thermo Scientific, #EN0582)
- HyClone water (GE Healthcare, #SH30538.01)

Reagents: Pooling and Concentration

4
- Dynabeads MyOne Silane (ThermoFisher, #37002D)
- Buffer RLT Plus (Qiagen, #1053393)
- Ethanol, 200 proof (Sigma-Aldrich, #E7023)

Reagents: Pre-amplification PCR Mix

5
- KAPA HiFi HotStart ReadyMix, 2X (Kapa Biosystems, #KK2612)
- SMART PCR primer, AAGCAGTGGTATCAACGCAGAGT

Reagents: Nextera amplification

6
- Nextera PCR primer: AATGATACGGCGACCACCGAGATCTACACGCCTGTCCGCGGAAGCAGTGGTATCAACGC AGAGT*A*C

Equipment:

7

- Eppendorf tec PCR Plate 96, semi-skirted (Eppendorf, #951020303)
- Microseal 'F' PCR Plate Seal, foil, (Biorad, #MSF1001)
- Speedball soft rubber brayer, 3.5'' (Speedball, #004174)
- Eppendorf DNA LoBind microcentrifuge tubes, 1.5ml and 2ml (Eppendorf, #022431021 and #022431048)
- Agilent 2100 Bioanalyzer
- Agilent high-sensitivity DNA kit (Agilent, #5067-4626)
- Qubit 2.0 fluorometer (ThermoFisher)
- Qubit dsDNA high-sensitivity kit (ThermoFisher, #Q32854)
- Magnetic separators for 1.5ml and 15ml tubes (Invitrogen, #12321D and #12301D, or equivalent)
- Vortex mixer (Fisher #02-215-414, or equivalent)
- Multi-channel Pipettes (Integra Viaflow II, #4011, or similar)
- Eppendorf cold blocks (#022510541)

Steps 1-6: Lysis plate preparation

8

1. **Critical:** At all steps, be meticulous to avoid contamination. Work in a PCR hood whenever possible. Wear disposable Tyvek sleeves or coat in the hood prior to amplification. All reagents must be free of DNA, RNA, and nucleases. Single-cell library quality can be significantly affected by small changes in reagents (concentration, age, or manufacturer). Avoid multiple freeze-thaw cycles (aliquot reagents). Prior to beginning, clean all surfaces to remove nucleic acid and nuclease contamination.

1. Prepare lysis buffer mix. For each sample prepare 6µl of lysis mix: 5.33µl Hyclone H2O, 0.15µl of 10% Triton X-100, 0.38µl of SUPERaseIN, and 0.15µl of 100mM dNTP mix. Make at least 1.2X the volume required.
- In the lysis and binding steps, the concentration of Triton X-100, SUPERaseIN, and dNTP mix will be 0.2%, 1U/µl, and 2mM, respectively.

1. Load 6µl of lysis mix into each well of a 96-well plate. (This, and subsequent steps, should be carried out with multi-channel pipettes or using a liquid handling robot.)

1. Add 1.5µl of 10µM mRNA capture primers into each well. Mix thoroughly.

1. Seal plates. If plates will be stored frozen, meticulously seal using Biorad Microseal F foil or -80C safe film.

1. Centrifuge plate briefly at 4C to collect liquid. Plates can be kept at 4C for same-day use or stored at -80C for up to 1 month.

Steps 7-9: Loading samples into lysis plates

9 7. Centrifuge plate briefly to defrost plates and collect liquid. Put plates in Eppendorf cold blocks.

8. Load samples into plates. NOTE: If using FACS to load plates: 1) carefully align sorter using same model plates. One method of alignment is to sort a visible number of droplets onto a plate sealed with optical film. Align sorter so the droplet is dead center at all four corner wells. 2) observe stream stability. Large or irregular cells can destabilize the stream and lead to empty wells.

9. Thoroughly seal plates with -80C safe foil (or optical seal) and spin down for 2min at full speed, 4C. NOTE: plates can be stored at -80C for up to two weeks.

Steps 10-15: Reverse transcription

10 10. Prepare reverse transcription (RT) mix. For each sample, prepare 7.5µl of RT mix. Combine 3 µl 5X RT buffer, 3 µl of 5M betaine, 0.45µl of 200mM $MgCl_2$, 0.075 µl of 1M DTT, 0.2 µl of 200U/µl ProtoScript II reverse transcriptase, 0.2 µl of 20U/µl SUPERaseIN, 0.15µl of 100µM template switch oligo, and 0.43µl of Hyclone water. Make at least 1.2X the volume required.

- In the final 15µl reaction, the concentrations will be: 1M betaine, 1X RT buffer, 6mM MgCl2, 5mM DTT, 40U RT enzyme, 7.5U SUPERaseIN, and 1µM template switch oligo.
- NOTE: DTT should be fresh or from frozen aliquot that has not been repeatedly freeze-thawed. 1M DTT may precipitate in the freezer, but can be resuspended by warming for a few minutes at room temperature or 37C, and then vortexing. The template switch oligo should be aliquoted and stored at -80C.

11. Centrifuge at room temperature, full speed, for 2min.
12. Run primer anneal program on thermal cycler. 72C (3min)à4C (infinite)
13. Add RT mix to plate and mix thoroughly. As much as possible, keep mix and plate near 0C throughout.
14. Apply seal and quickly spin plate at 4C.
15. Run RT program.

- Stage 1: 42C (90min)
- Stage 2: 10 cycles of: 50C (2min), 42C (2min)
- Stage 3: 75C (10min), 4C (infinite)

Steps 16-18: Exonuclease I cleavage

11 16. Centrifuge plate briefly at 4C.

17. Dilute Exonuclease I to 7.5U/µl in 1X Exo I buffer and add 2µl (15U) to each sample. Mix thoroughly.
18. Run Exo I program on thermal cycler. 37C (30min)à80C (15min)à4C (infinite).

Steps 19-32: Pooling and cleanup

12

19. Centrifuge plate briefly at 4C.
20. Pool samples in 15ml polypropylene tube.
21. Clean up pooled samples with silane beads. In 15ml tube, combine sample (e.g., 1400µl) with cleanup mix:
- 5 volumes RLT Plus (e.g., 4,900µl)
- EtOH to 33% final (e.g., 2,771µl)
- 3µl silane beads for each 300µl solution (e.g., 91µl).

22. Vortex to mix. Incubate at room temperature for 20min on rotator.
23. Collect beads on magnetic stand. Remove most supernatant, but leave ~1ml remaining.
24. Resuspend beads in remaining 1ml supernatant and transfer to 1.5ml tube.
25. Collect beads on magnetic stand. Remove supernatant.
26. Wash beads 2X with 500µl 80% EtOH.
27. Remove remaining EtOH with p10 tip and dry pellet in hood. Do not over dry.
28. Elute in 100µl Hyclone H2O. Vortex tube for 1min, and then flick or very briefly pulse the sample in a microcentrifuge. Incubate 1min at room temperature and then collect beads on magnet and remove the supernatant into two PCR tubes (47.5µl each).
29. Add 50µl 2X Kapa HiFi Hotstart and 2.5µl 10µM SMART PCR primer into each tube. Mix and quickly spin down PCR mix.
30. Run SMART PCR program on thermal cycler. NOTE: The number of amplification cycles required can vary from 13-20, depending on cell type and state. Because over amplification can lead to both PCR bias and recombination, it is important to use the least amount of amplification that yields a sufficient library. To determine this, run the first plate for either 14 cycles (large cell lines) or 18 cycles (small primary cells); clean up, and determine concentration using Qubit DNA HS. Adjust the number of cycles for the remaining plates.
- Stage 1: 98C (3min)
- Stage 2: 98C (20seconds), 67C (15seconds), 72C (5min)
- Stage 3: 72C (5min), 4C (infinite)

31. Pool the two PCR reactions into a 1.5ml tube. Measure the total volume (slightly less than 200µl).
32. Clean up using Ampure XP with 0.7:1 ratio. Elute in 22µl Hylcone water. Remove 20µl to a new tube. Take care not to transfer any beads, which can ruin the Bioanlyzer run.

Steps 33-35: Library quantification and sequencing

13 33. Determine library concentration using 3µl of sample for Qubit HS analysis.

- NOTE: in some cases libraries can successfully be sequenced from low concentration samples (e.g., less than 0.07ng/µl), but such libraries may not be visible in the Bioanalyzer, and the Nextera tagmentation reaction will have to be loaded blind. It is generally preferable to *optimize the preamplification PCR to have a Qubit concentration of 0.1-0.4ng/µl*. Do not over amplify.
- NOTE: low concentration samples can be concentrated using a Speedvac. However, do not dry the sample—reduce the volume to ~10µl.

34. Determine average library size using Bioanalyzer DNA HS. Average fragment size should be 1,600-1,900bp.

- NOTE: When running low concentration samples, the Bioanalyzer is very sensitive to contaminants, including beads, reagents, and air bubbles. Take extra care and include a blank well.
- NOTE: Average fragment sizes below ~1,600bp often contain degradation. If the protocol has been followed closely, it is likely that such contamination was present in the cells prior to lysis. In some cases, such as tumor samples, such degradation may be unavoidable. Libraries with average fragment sizes as low as ~1,100bp have been successfully sequenced, but data quality is affected.

35. Perform Nextera XT DNA Library Prep with the following modifications:

- Load 0.5ng of sample into the tagmentation reaction instead of the stated 1ng.
- Use 5µl of Index 1 (i7) adapter/primer and 5µl of 5µM Nextera PCR primer
- Following tagmentation and amplification, cleanup with 0.6X:1 ratio Ampure XP, followed by an additional cleanup using 1:1 ratio Ampure XP. Elute in 22µl RSB.
- Run sample on Bioanalyzer DNA HS. Average fragment size should be between 300bp and 600bp. No primer dimers should be present.