scEC&T-seq: a method for parallel sequencing of extrachromosomal circular DNAs and transcriptomes in single human cells

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Method Article

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Abstract

Extrachromosomal DNA amplifications are common in cancer and are associated with decreased patient survival. A key feature of extrachromosomal circular DNA is its ability to be randomly mis-segregated to daughter cells promoting rapid intercellular heterogeneity. Understanding how extrachromosomal circular DNA dynamics contribute to intercellular heterogeneity remains crucial to better understand its role in tumor evolution and adaptation to therapy. Here, we introduce scEC&T-seq (single cell extrachromosomal circular DNA and transcriptomic sequencing), a method for parallel detection of extrachromosomal circular DNAs and full-length mRNA in single cancer cells. In this protocol, a single cell's DNA is separated from its polyadenylated RNA as described by Macaulay et al. (2015)[1]. This is followed by removal of linear DNA through exonuclease digestion and further enrichment of circular DNA by rolling circle amplification with φ29 polymerase[2-4]. The separated mRNA from the same cell is processed using on-bead Smart-seq2[1]. The duration of the entire procedure from cell sorting to library preparation is approximately 8 days. Our scEC&T-seq protocol has been validated in single cancer cells from neuroblastoma cell lines and primary tumors, and in normal single T-cells isolated from patient's blood. Besides identifying large, oncogene-containing circular DNAs in cancer cells, our method also captures other smaller circular DNAs, which have been previously described in both cancer and non-malignant cells[5]. We envision that our method may enable the analysis of yet unknown prerequisites for the maintenance of both small and large circular DNA in cancers, but also in the context of other diseases and normal cellular development.

Introduction

Reagents

- Cell or tissue source for single-cell isolation.
- RNaseZap (Ambion, cat. no. AM9780)
- DNA-OFF (Takara Bio, cat. no. 9036)
- Nuclease-free water (Ambion, cat. no. AM9937)
- 10 M NaOH (Sigma-Aldrich, cat. no. 72068)
- 5 M NaCl (Ambion, cat. no. AM9760G)
- 0.5 M EDTA, pH 8.0 (Promega, cat. no. V4231)
- Tris-EDTA buffer solution, BioUltra for molecular biology (pH 8.0) (TE; Sigma-Aldrich, cat. no. 93283)
- UltraPure 1 M Tris–HCl Buffer, pH 7.5 (Thermo Fisher Scientific, cat. no. 15567027)
- 1 M $MgCl_2$ (Ambion, cat. no. AM9530G)
- 2 M KCl (Ambion, cat. no. AM9640G)
- 1 M DTT (Sigma-Aldrich, cat. no. 646563).
- 50% (vol/vol) Tween 20 (Invitrogen, cat. no. 003005)

- Polyethylene glycol (PEG)
- Buffer RLT Plus (Qiagen, cat. no. 1053393)
- SUPERase In (Ambion, cat. no. AM2696)
- Dynabeads MyOne Streptavidin C1 (Invitrogen, cat. no. 65001)
- SuperScript II reverse transcriptase (Life Technologies, cat. no. 18064071)
- 5× First-strand buffer (Life Technologies, cat. no. 18064071)
- DTT (Invitrogen, cat. no. 18064-014)
- 5 M Betaine solution (Sigma-Aldrich, cat. no. B0300-1VL)
- dNTP mix, 10 mM each (Life Technologies, cat. no. 18427-013)
- Kapa Hifi HotStart ReadyMix (Kapa, cat. no. KK2601)
- Agencourt AMPure XP Beads (Beckman Coulter, cat. no. A63881)
- Buffer EB (Qiagen, cat. no. 19086)
- Ethanol ROTIPURAN ≥99,8 %, (Roth, cat. no. 9065.4)
- NEBNext Ultra II FS DNA Library Prep Kit for Illumina (New England Biolabs, cat. no. E7805L)
- NEBNext Multiplex Oligos for Illumina (New England Biolabs, cat. no. E6440S)
- Biotinylated Oligo-dT30VN (5-Biotin-TEG-AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3; IDT)
- Template Switch Oligo (5-AAGCAGTGGTATCAACGCAGAGTACATrGrGrG-3; IDT)
- ISPCR oligo (5-AAGCAGTGGTATCAACGCAGAGT-3; IDT)
- REPLI-g Single Cell Kit (Qiagen, cat. no. 150345)
- Plasmid-Safe ATP-dependent DNase (Epicentre, cat. no. 161100)
- Plasmid-Safe 10x reaction buffer (Epicentre, cat. no. 161100)
- ATP (1mM; Epicentre, cat. no. 161100)
- MssI (PmeI) (5 U/µl; Thermo Scientific, cat. no. ER1341)
- High Sensitivity D5000 ScreenTape (Agilent, HS-D5000, cat. no. 5067-5592)
- High Sensitivity D5000 Reagents (Agilent, HS-D5000, cat. no. 5067-5593)
- High Sensitivity D1000 ScreenTape (Agilent, HS-D1000, cat. no. 5067-5584)
- High Sensitivity D1000 Reagents (Agilent, HS-D5000, cat. no. 5067-5585)

REAGENT SETUP

Dynabead solution A

Dynabead solution A is NaOH (0.1 M) and NaCl (0.05 M) in nuclease-free water. To prepare 10mL of this solution, add 100 µl of 10M NaOH and 100 µl of 5 M NaCl to 9.8 mL of nuclease-free water. Prepare this

buffer in a clean preamplification UV PCR cabinet in the pre-PCR room. Treat 15-ml Falcon tubes with UV irradiation for 60 minutes prior to buffer preparation. This buffer can be prepared in bulk and stored at room temperature.

Dynabead solution B

Dynabead solution B is NaCl (0.1 M) and 0.01% (vol/vol) Tween20 in nuclease-free water. To prepare 10mL of this solution, add 200 µl of 5M NaCl and 2 µl of 5% (vol/vol) Tween to 9.8 mL of nuclease-free water. Prepare this buffer in a clean preamplification UV PCR/laminar flow cabinet in the pre-PCR room. Treat 15-ml Falcon tubes with UV irradiation for 60 minutes prior to buffer preparation. This buffer can be prepared in bulk and stored at room temperature, but it should be supplemented with 0.01% (vol/vol) Tween20 prior to use.

Dynabead 2× 'Binding and Wash' buffer

Dynabead 2× 'Binding and Wash' buffer is Tris-HCl (10 mM, pH 7.5), NaCl (2M), EDTA (1mM), and 0.02% (vol/vol) Tween20 in nuclease-free water. To prepare 10mL of this solution, add 4 ml of 5M NaCl, 100 µl of 1M Tris-HCl (pH 7.5), 20 µl of 0.5M EDTA (pH 8.0) and 4 µl of 5% (vol/vol) Tween to 5.88 ml of nuclease-free water. Prepare this buffer in a clean preamplification UV PCR/laminar flow cabinet in the pre-PCR room. Treat 15-ml Falcon tubes with UV irradiation for 60 minutes prior to buffer preparation. This buffer can be prepared in bulk and stored at room temperature, but it should be supplemented with 0.02% (vol/vol) Tween20 prior to use.

Dynabead 1× 'Binding and Wash' buffer

To prepare this buffer, dilute Dynabead 2x ‘Binding and Wash’ buffer in a 1:1 ratio with nuclease-free water. Prepare this buffer in a clean preamplification UV PCR/laminar flow cabinet in the pre-PCR room. Treat 15-ml Falcon tubes with UV irradiation for 60 minutes prior to buffer preparation. This buffer should be prepared after supplementing the Dynabead 2x ‘Binding and Wash’ buffer with 0.02% (vol/vol) Tween20.

Biotinylated Oligo-dT30VN

When ordering this primer choose the options: RNAse-free and HPLC-purified. The primer should be resuspended at 100 µM in nuclease-free water in a clean preamplification UV PCR/laminar flow cabinet in the pre-PCR room and store at −20 °C.

Template-switching and ISPCR oligos

Both oligos should be resuspended to 100 µM in nuclease-free water in a clean preamplification UV PCR/laminar flow cabinet in the pre-PCR room. Resuspended oligos must be stored at −80 °C in single-use 10-µl aliquots to avoid multiple freeze-thaw cycles.

PEG buffer

PEG buffer is 18% (wt/vol) polyethylene glycol (PEG), NaCl (2.5 M), Tris-HCl (10mM, pH 8), EDTA (1 mM) and 0.05% (vol/vol) Tween20. To prepare 10mL of this solution, add 4.5 ml of 40% (wt/vol) PEG, 5 ml of 5M NaCl, 100 µl of 1M Tris-HCl (pH 8), 20 µl of 0.5 M EDTA and 10 µl of 5% (vol/vol) Tween20. Prepare this buffer in a clean preamplification UV PCR/laminar flow cabinet in the pre-PCR room. Treat 15-ml Falcon tubes with UV irradiation for 60 minutes prior to buffer preparation. Sterilize through a 0.20-µm filter and treat the buffer with UV irradiation for 60 min. This buffer can be stored at 4ºC.

Equipment

- UV PCR Workstation (e.g., UVP UV PCR Workstation, model no. 95-0367-02) and dedicated preamplification pipettes
- Microplate centrifuge, PCR Plate Spinner (VWR, cat. no. 521-1649)
- 1.5-ml Microcentrifuge Safe-Lock tubes, polypropylene (Sigma-Aldrich, cat. no. T9661)
- 0.2-ml Eppendorf PCR tubes (Sigma-Aldrich, cat. no. Z316121)
- 15-ml and 50-ml Polypropylene Falcon tubes (BD, cat. nos. 352096 and 352070)
- DynaMag PCR Magnet (Life Technologies, cat. no. 492025)
- DynaMag-2 Magnet (Thermo Fisher Scientific, cat. no. 12321D)
- Magnum FLX Universal Magnet Plate (Alpaqua, cat. no. A000400)
- Eppendorf MixMate (Merck, cat. no. EP022674200)
- FrameStar 96-well skirted PCR plates (4titude, cat. no. 4ti-0960/C)
- Multidispensing pipette (e.g., Thermo Fisher Scientific Multipette Xstream, cat. no. 4986 000.025)
- 4Titude aluminium foil seal 125 x 78mm (4titude, cat. no. 4AJ-9776006)
- ThermalSeal A Sealing Films (Excel Scientific, cat. no. Z723304-100EA)
- Thermocycler (Biorad)
- Handheld electronic 96-channel pipette (Biorad)
- Agilent 4200 TapeStation system (Agilent, cat. no. G2991BA)
- Qubit 4 Fluorometer (Invitrogen, cat. no. Q33238)

- MANTIS Liquid Dispenser (Formulatrix)
- BioMek FXP Laboratory Automation Workstation (Beckman, cat no. A31842)

EQUIPMENT SETUP

Contamination must be cautiously avoided in single-cell work, especially in all steps prior to cDNA and gDNA amplification. For this reason, pre and postamplification workspaces must be physically separated and each work area should include its own separate equipment.

Preamplification work setup

All steps prior to single cell cDNA or DNA amplification (Steps 1-32 and 45-79) must be performed in a clean preamplification room. The preamplification room should include a UV PCR/laminar flow working station with designated P2, P20, P200, P1000, multidispensing and multichannel pipettes. The surface of the UV PCR/laminar flow cabinet should be properly decontaminated with DNA-OFF, RNaseZAP and treated with UV irradiation for 60 minutes before and after use. All 96-well plates, microcentrifuge tubes and Falcon tubes used in the preamplification work setup should be treated with UV irradiation for 60 minutes prior to use. For Steps 20-24; 47-51 and 64-69 of the scEC&T-seq protocol, we use a liquid-handling robot: BioMek FXP Laboratory Automation Workstation. Additional equipment required in the preamplification room includes a thermomixer for 96-well plates (MixMate), a vortex mixer, a low elution magnet for 96-well plates and a PCR plate spinner.

Postamplification work setup

All steps after PCR amplification (Steps 33-45 and 80-150) must be performed in a designated and clean postamplification room. The essential equipment in the postamplification room includes: P2, P20, P200, P1000, multidispensing and multichannel pipettes; a thermomixer for 96-well plates (MixMate); a low elution magnet for 96-well plates; a vortex mixer; microcentrifuge tubes and Falcon tubes; 96-well plates; and a PCR plate spinner.

Procedure

Single-cell sorting using fluorescence-activated cell sorting (FACS)

Steps 1 and 2 must be performed in the preamplification room.

1. In a clean PCR laminar flow cabinet, prepare 96-well plates containing 2.5 µl of RLT Plus solution per well. Avoid creating bubbles. We recommend preparing the plates immediately before FACS sorting of the single-cell samples, as the 2.5 µl of RLT Plus buffer solidify if left at room temperature overnight.

2. Seal and spin down the plates for 15 seconds in a PCR plate spinner at room temperature. Check that the lysis buffer is at the bottom of each well.

3. After preparation of a single-cell or a single-nuclei suspension, deposit single cells or single nuclei directly into the RLT Plus solution contained in each well by FACS. At least one well per plate should be left empty as a negative control. The site of deposition of the FACS-sorting beads in a well of a plate, must be previously calibrated to ensure accurate deposition in the center of the 96-well plates.

4. Immediately after deposition, seal the plate using an aluminium foil seal and transfer the 96-well plate to a metal rack on dry ice and store at −80 °C. Plates can be stored at -80 °C for >6 months.

Preparation of oligo-dT30VN-labeled beads

All steps (5-15) should be performed in the preamplification room and in the PCR laminar flow cabinet (except Step 10).

5. Add 50 µl of Dynabeads to a 0.2-ml Eppendorf tube and place it on a DynaMag PCR magnet for 30 seconds. Without removing the tube from the magnet, discard the supernatant.

6. Remove the tube from the magnet and gently resuspend in 200 µl of Dynabead solution A. Avoid creating bubbles when resuspending. Place the tube back into the magnet allowing the magnetic beads to separate for 30 seconds. Remove and discard the supernatant.

7. Repeat Step 6 once.

8. Remove the tube from the magnet and gently resuspend in 200 µl of Dynabead solution B. Place the tube back into the magnet allowing the magnetic beads to separate for 30 seconds, then remove and discard the supernatant.

9. Remove the tube from the magnet and gently resuspend in 50 µl of 2× B&W buffer. Then, add 50 µl of 100 µM Biotinylated Oligo-dT30VN to the beads and gently resuspend the mixture.

10. Incubate the beads and Biotinylated Oligo-dT30VN for 20 min in a rotator in the preamplification room.

11. After incubation, place the tube on the magnet for 30 seconds. While keeping the tube on the magnet, remove and discard the supernatant.

12. Remove the tube from the magnet and wash the beads by resuspending in 200 µl of 1× B&W buffer. Return the tube to the magnet and, after 30 seconds, remove and discard the supernatant.

13. Repeat washing Step 12, three times.

14. Prepare 1 ml of bead “Resuspension buffer” by mixing the reagents in the table below.

- Superscript II first-strand buffer (5×) -> 200 µl (Final concentration: 1x)

- SUPERase In RNAse inhibitor (20 U/µl) -> 50 µl (Final concentration: 1 U/µl)

- Tween 20 (5%) -> 2 µl (Final concentration: 0.01%)

- Nuclease-free water -> 748 µl

15. Remove the tube from the magnet and resuspend the beads in 1ml of bead “Resuspension buffer”. Mix by vortexing. Note that once the SUPERase IN inhibitor is added to the beads, they must be used within 30 min.

Physical separation of polyadenylated mRNA and gDNA

All manual steps must be performed in the PCR laminar flow cabinet also located in the preamplification room. We performed Steps 20-24 using a Biomek liquid-handling robot in the preamplification room. The volumes described in the following section are enough to process one 96-well plate containing single-cell lysates.

16. Prepare 1000 µl of RT mastermix in a 1.5 ml Eppendorf tube in the preamplification laminar flow cabinet by combining the reagents indicated in the table below. Mix by vortexing and store on ice until use.

- dNTP mix (10mM) -> 100 µl (1mM of each (dATP, dCTP, dGTP and dTTP))

- $MgCl_2$ (1 M) -> 6 µl (6 mM)

- Betaine (5 M) -> 200 µl (1 M)

- Superscript II first-strand buffer (5×) -> 200 µl (1x)

- DTT (100 mM) -> 25 µl (2.5 mM)

- SUPERase In RNAse inhibitor (20 U/µl) -> 50 µl (1 U/µl)

- Superscript II reverse transcriptase (200 U/µl) -> 50 µl (10 U/µl)

- Template Switch Oligo (TSO; 100 µM) -> 10 µl (1 µM)

- Nuclease-free water -> 359 µl

17. In the preamplification laminar flow cabinet, prepare the G&T-seq wash buffer by combining the reagents indicated in the table below in a 15 ml Falcon tube. Then, in a new 96-well plate, add 55 µl of G&T-seq wash buffer per well. Seal the plate and spin down for 15 s in a plate spinner. Store on ice or at 4 ºC until use.

- Superscript II first-strand buffer (5×) -> 1160 µl (1×)

- Tween 20 (5%) -> 58 µl (0.05%)

- DTT (100 mM) -> 580 µl (10 mM)

- SUPERase In RNAse inhibitor (20 U/ µl) -> 116 µl (0.4 U/µl)

- Nuclease-free water -> 3886 µl

18. Add 10 µl of Oligo-dT30VN beads per well to the 96-well plate containing the lysed single-cells (Step 4), seal, and briefly spin down in a plate spinner.

19. Mix the plate on a MixMate at 2100 rpm for 15 s. Then incubate with mixing at 800 rpm for 20 min. After this step, do not spin down the plate to prevent bead aggregation.

20. Program and prepare the liquid-handling robot by placing the required equipment in the correct layout of the robot deck. This includes: tips, a low-elution magnet for 96-well plates, the 96-well plate containing the G&T-seq buffer (Wash-plate; Step 17) and a new empty 96-well plate for collecting the gDNA (gDNA-plate).

21. After the 20-min incubation with the beads, place the plate on the low-elution magnet placed in the robot and allow the beads to separate for 30 s. Use the liquid-handling robot to transfer 13.5 µl of supernatant containing the gDNA of the single-cells to the gDNA-plate. To minimize gDNA loss, do not discard the tips used in this step and reuse them in Steps 22 - 24.

22. Using the liquid-handling robot, transfer 15 µl of G&T-seq wash buffer from the Wash-plate to the plate containing the beads. Seal the plate and remove it from the robot deck, mix at 2100 rpm for 15 s on a MixMate and visually inspect if the beads are resuspended. Depending on the well shapes of the PCR

plate type and the low-elution magnet design, this may happen at slightly different rpm. Then incubate at 2000 rpm for 5 min. Do not spin down the plate after incubation to avoid bead aggregation.

23. Return the bead-resuspended 96-well plate to the low-elution magnet and allow the beads to settle for 30 seconds. Use the liquid-handling robot to transfer 15µl of supernatant to the gDNA-plate using the same tips as in Step 21.

24. Repeat Steps 22 and 23 twice to wash the beads three times in total. In the final supernatant transfer step, increase the transferring volume from 15 µl to 17 µl to remove any remaining G&T-seq washing buffer from the bead-containing plate.

Reverse Transcription

25. Immediately after Step 24, transfer the plate to the PCR laminar flow cabinet. Dispense 10 µl of RT mastermix (prepared in Step 16) per well and briefly spin in a plate spinner. This plate is now referred to as the mRNA-plate. On a MixMate, mix the plate at 2100 for 15 s or until beads are fully resuspended. Do not spin at any point.

26. Place the mRNA-plate in a thermal cycler and run the following program:

Cycle	Temperature	Time (min)
1	42 ºC	60 min
2	50 ºC	2 min
3	42 ºC	2 min
4	Return to cycle 2	9×
5	60 ºC	10 min

27. Collect the gDNA-plate from the liquid-handling robot, seal it and spin down in a plate spinner for 15 s. The gDNA-plate can be stored at 4ºC until further processing. We recommend proceeding with gDNA purification and exonuclease digestion (Step 45) on the same day, to avoid freezing-thawing cycles that could fragment the DNA.

PCR amplification of cDNA

All steps must be performed in the preamplification room.

28. Prepare the following PCR mastermix by combining and mixing the reagents in the table below. Store the master mix on ice until use.

- Kapa Hifi HotStart ReadyMix (2×) -> 1100 µl (1×)

- ISPCR oligo (2 µM) -> 100 µl (0.1 µM)

29. After reverse transcription, remove the cDNA-plate (Step 26) from the thermal cycler and spin down for 15s on a PCR plate spinner.

30. Add 12 µl of PCR reaction mastermix (Step 28) directly to each well of the cDNA-plate, resulting in a total volume of 22µl. Seal the plate and spin down in a plate spinner for 15 s. On a MixMate, mix at 2100 rpm for 15 s and spin down again for 15 s.

31. Perform the cDNA amplification on a thermal cycler and run the program described on the table below. Note that the number of cycles can be optimized for different cell types depending on the mRNA content of the cell. For high-mRNA-content cells, we recommend between 19-21 repetitions of cycle 5. For low-mRNA-content cells or nuclei, we recommend 24 repetitions of cycle 5.

Cycle	Temperature	Time (min)
1	98 ºC	3 min
2	98 ºC	20 s
3	67 ºC	15 s
4	72 ºC	6 min
5	Return to cycle 2	19-24×
6	72 ºC	5 min
7	4 ºC	Hold

32. The plate containing the amplified cDNA can be stored at -20 ºC until further processing.

Purification of amplified cDNA and quality control

The purification of amplified cDNA should be performed in a clean postamplification room separated from the location of the preamplification room.

33. AMPure XP beads should be warmed up to room temperature before use (approximately for 15 min) and mixed by vortexing until fully resuspended. In the meantime, if needed, thaw the amplified cDNA-containing plate from Step 32 and spin down for 15 s in a PCR plate spinner.

34. Add 20 µl of the mixed AMPure XP beads to each well of the 96-well plate containing the amplified cDNA (1:0.9 ratio) at room temperature. Seal the plate and mix on a MixMate at 2100 rpm for 15 s. Let the mixture stand for 5 min at room temperature.

35. Transfer the amplified cDNA-containing plate to a low-elution magnet, and allow the beads to settle for 2 min.

36. Remove and discard the supernatant without disturbing the beads. For this step, we used a handheld electronic 96-channel pipette.

37. Without removing the plate from the magnet, wash the AMPure XP beads with 100 µl of freshly prepared 80% (vol/vol) ethanol for 30 s. Then carefully, without disturbing the beads, remove and discard the ethanol using a handheld 96-channel pipette.

38. Repeat washing Step 37 once.

39. After the second wash, remove any remaining ethanol from the well, and allow the AMPure XP beads to dry for 2-5 min. Avoid over drying the beads as this would decrease DNA elution yield.

40. Remove the plate from the magnet and elute the cDNA by adding 20 µl of elution buffer (EB) to the AMPure XP beads. Seal the plate and resuspend the beads by mixing on a MixMate for 15 s at 2100 rpm.

41. Allow the mixture to stand off the magnet for 2 min at room temperature.

42. Place the plate back into the magnet and allow the beads to settle for 5 min.

43. Remove the supernatant containing the purified cDNA and transfer it to a new 96-well plate. At this point, the plate containing the amplified cDNA can be sealed and stored at -20 ºC.

44. Prior to library preparation, check the cDNA quality in some representative wells by using an Agilent High Sensitivity D5000 screentape (HS-D5000) on an Agilent Bioanalyzer. Amplified cDNA should range between 0.5 and 2 kb in size, reaching a maximum at 1–1.5 kb. Failed amplifications are generally due to degraded RNA or 'empty' wells in which no cell was deposited during FACS.

Purification of gDNA

The purification of gDNA should be performed in the preamplifiation room. We performed Steps 47-51 using a Biomek liquid-handling robot placed in the preamplification room. All manual steps must be performed in the PCR laminar flow cabinet.

Note that the purified gDNA will be directly eluted into the exonuclease digestion buffer. Therefore, we recommend preparing the exonuclease master mix (Step 54) during the incubation time in Step 46 and keep the mixture on ice until use.

45. AMPure XP beads should be warmed up to room temperature before use (approximately for 15 min) and mixed by vortexing until fully resuspended. In the meantime, spin down the gDNA-plate (Step 27) in a plate spinner for 15s.

46. Add 47.5 µl of mixed AMPure XP beads to each well of the gDNA-plate (1:0.8 ratio) at room temperature. Use non-sticky foil to seal the plate, briefly spin in a plate spinner, and mix on a MixMate for 15s at 2100rpm. Afterwards, incubate for 20 min with mixing at 800 rpm.

47. In the meantime, program and prepare the liquid-handling robot by placing the required equipment in the correct layout of the robot deck. This includes: the tips, a low-elution magnet, and a half reservoir (75 ml) for ethanol. We recommend preparing the exonuclease master mix described in Step 54 at this point and keep it on ice.

48. After the 20-min incubation, transfer the gDNA-plate to the low-elution magnet placed on the robot's deck, and allow the beads to settle for 2 min. In the meantime, add 40 ml of freshly prepared 80 % (vol/vol) ethanol solution to the half reservoir placed on the Biomek liquid-handling robot's deck.

49. Using the liquid-handling robot, remove and discard the supernatant without disturbing the beads.

50. Without removing the plate from the magnet and using the liquid-handling robot, wash the AMPure XP beads by adding 150 µl of freshly prepared 80% (vol/vol) ethanol to each well and incubate for 30 s. Remove and discard the ethanol using the liquid-handling robot without disturbing the beads.

51. Repeat Step 50 once.

52. After the second wash, remove any remaining ethanol from the well, and allow the AMPure XP beads to dry for 2-5 min at room temperature. Avoid over drying the beads as this would decrease DNA elution yield.

53. Continue immediately with exonuclease digestion of linear DNA.

Exonuclease digestion of linear DNA

The exonuclease digestion of linear DNA should be performed in the preamplification room. All steps must be performed in the PCR laminar flow cabinet.

54. Prepare the exonuclease master mix as described in the table below. Mix well and keep on ice until use.

- Plasmid-Safe 10× Reaction Buffer -> 200 µl (1×)

- ATP (20mM) -> 100 µl (1 mM)

- Plasmid-Safe ATP-Dependent DNAse (10 U/ µl) -> 200 µl (1 U/ µl)

- Nuclease-free water -> 1500 µl

55. Take the plate containing the beads from Step 52 and add 20 µl of exonuclease master mix to each well. Seal the plate, briefly spin in a plate spinner, and mix on a MixMate at 2100 rpm for 15s. Shortly spin down the plate in a PCR plate spinner for 15 s.

56. **(Optional: endonuclease control)** In some wells, besides the exonuclease master mix, we added 1 µl of MssI (PmeI) enzyme (5 U/µl), as control for linearization of circular DNA.

57. In a thermal cycler, incubate the plate at 37°C for a total of 5 days (120 hours).

58. Every 24 hours, 10 units of the Plasmid-Safe ATP-Dependent DNase were added and ATP was refreshed. After each 24-hour incubation period, prepare the master mix described below:

- Plasmid-Safe 10× Reaction Buffer -> 50 µl (1×)

- ATP (20mM) -> 100 µl (4 mM)

- Plasmid-Safe ATP-Dependent DNAse (10 U/ µl) -> 100 µl (2 U/ µl)

- Nuclease-free water -> 250 µl

59. After each 24-hour incubation time, remove the plate containing the exonuclease digestion mixture from the thermal cycler and add 5 µl of the master mix described above (Step 58). Seal the plate, briefly

spin in a plate spinner, and mix on a MixMate at 2100 rpm for 15s. Shortly spin down the plate in a PCR plate spinner for 15 s. When endonuclease controls are included, do **not** refresh MssI (PmeI) enzyme.

60. After 120 hours (5 days) incubation, heat inactivate the exonuclease enzyme by incubating at 70 ºC for 30 min in the thermal cycler.

61. Immediately, proceed with the purification of exonuclease-digested DNA and MDA amplification.

Purification of exonuclease-digested DNA

This purification step is adapted from Clark et al., 2017[6]. The purification of exonuclease-digested DNA should be performed in the preamplification room. We performed Steps 64-69 using a Biomek liquid-handling robot placed in the preamplification room. All manual steps must be performed in the PCR laminar flow cabinet.

62. Add 32 µl of PEG buffer to each well of the exonuclease-digested DNA plate. Seal the plate, briefly spin in a plate spinner, and mix on a MixMate for 15 s at 2100 rpm.

63. Incubate the mixture for 20 min at room temperature.

64. In the meantime, program and prepare the liquid-handling robot by placing the required equipment in the correct layout of the robot deck. This includes: the tips, a low-elution magnet, and a half reservoir (75 ml) for ethanol. We recommend preparing the master mix described in Step 71 at this point and keep it on ice.

65. After the 5-min incubation, transfer the exonuclease-digested DNA plate to a low-elution magnet placed on the robot's deck, and allow the beads to settle for 2 min. Add 40 ml of freshly prepared 80 % (vol/vol) ethanol solution to the half reservoir placed on the Biomek liquid-handling robot's deck.

66. Using the liquid-handling robot, remove and discard the supernatant without disturbing the beads.

67. Without removing the plate from the magnet and using the liquid-handling robot, wash the AMPure XP beads by adding 150 µl of freshly prepared 80% (vol/vol) ethanol to each well and incubate for 30 s. Remove and discard the ethanol using the liquid-handling robot without disturbing the beads.

68. Repeat Step 67 once.

69. After the second wash, remove any remaining ethanol from the well, and allow the AMPure XP beads to dry for 2-5 min at room temperature. Avoid over drying the beads as this would decrease DNA elution yield.

70. Continue inmediately with rolling circle amplification of circular DNA by Multiple Displacement Amplification (MDA)

Rolling circle amplification of exonuclease-digested DNA by Multiple Displacement Amplification (MDA)

The amplification of exonuclease-digested DNA by MDA should be performed in the designated preamplification room. All steps must be performed in the PCR laminar flow cabinet.

71. Prepare sufficient buffer D2 (denaturation buffer) for one 96-well plate (100×) by mixing the reagents described in the table below.

- DLB (reconstitute with 500 µl nuclease-free water) -> 275 µl

- DTT (1 M) -> 25 µl

- PBS -> 400 µl

72. Take the plate containing the beads from Step 69 and add 7 µl of buffer D2 per well. Seal the plate, briefly spin in a plate spinner, and mix on a MixMate at 2100 rpm for 15 s or until fully resuspended. Shortly, spin down on a PCR plate spinner.

73. Incubate the reaction at 65 ºC for 10 min in a thermal cycler for 96-well plates.

74. After the 10-min incubation, add 3 µl of STOP solution per well. Seal the plate, briefly spin in a microplate centrifuge, and mix on a MixMate at 2100 rpm for 15 s. Shortly, spin down on a PCR plate spinner and store on ice.

75. Prepare the reaction master mix by adding the components listed in the table below. Store on ice until use.

- Nuclease-free water -> 900 µl

- REPLI-g sc Reaction Buffer -> 2900 µl

- REPLI-g sc DNA Polymerase -> 200 µl

76. Add 40 µl of reaction master mix to each well of the plate containing the denatured exonuclease-digested DNA. Seal the plate, briefly spin in a plate spinner, and mix on a MixMate at 2100 rpm for 15 s. Briefly spin down to ensure that the mixture is at the bottom of the well.

77. Incubate the reaction at 30 ºC for 8 hours in a thermal cycler. Set the lid temperature to 70 ºC.

78. Inactivate REPLI-g sc DNA polymerase at 65 ºC for 3 min in the thermal cycler.

79. The amplified exonuclease-digested DNA can be stored at 4ºC for short-term storage or at -20 ºC for long-term storage.

Purification of amplified exonuclease-digested DNA

The purification of amplified exonuclease-digested DNA should be performed in the postamplification room separated from the location of the preamplification room to avoid contamination.

80. AMPure XP beads should be warmed up to room temperature before use (approximately for 15 min) and mixed by vortexing until fully resuspended. In the meantime, spin down the amplified exonuclease-digested DNA plate from Step 79 in a plate spinner.

81. Add 40 µl of the mixed AMPure XP beads to each well of the 96-well plate (1:0.8 ratio) at room temperature, seal the plate, briefly spin in a plate spinner, and mix on a MixMate at 2100 rpm for 15 s. Let the mixture stand for 5 min at room temperature.

82. Transfer the amplified exonuclease-digested DNA plate to a low-elution magnet and allow the beads to settle for 5 min.

83. Remove and discard the supernatant without disturbing the beads. For this step, we used a handheld electronic 96-channel pipette.

84. Without removing the 96-well plate from the magnet, wash the AMPure XP beads with 100 µl of freshly prepared 80% (vol/vol) ethanol for 30 s. Then carefully, without disturbing the beads, remove and discard the ethanol using a handheld electronic 96-channel pipette.

85. Repeat washing Step 84 once.

86. After the second wash, remove any remaining ethanol from the well, and allow the AMPure XP beads to dry for 2-5 min. Avoid over drying the beads as this would decrease DNA elution yield.

87. Remove the plate from the magnet and elute the amplified exonuclease-digested DNA by adding 100 µl of elution buffer (EB) per well to the AMPure XP beads. Seal the plate and resuspend the beads by mixing on a MixMate for 15 s at 2100 rpm or until the beads are fully resuspended.

88. Allow the mixture to stand off the magnet for 2 min at room temperature.

89. Place the 96-well plate back into the magnet and allow the beads to settle for 5 min.

90. Remove the supernatant containing the purified, amplified, exonuclease-digested DNA and transfer it to a new 96-well plate using a handheld electronic 96-channel pipette. At this point, the plate can be stored at -20 ºC.

91. Prior to library preparation, check the quality of the DNA in some representative wells by using an Agilent Genomic DNA screentape on an Agilent Bioanalyzer. Amplified DNA should peak around 10 kb size.

Library preparation: amplified cDNA and exonuclease-digested DNA

This part of the protocol was performed using the NEBNext Ultra II FS and adapted from the original manufacturer's protocol (<100ng). The volumes of all reactions have been reduced to ¼, in order to reduce the cost of library preparation and are enough to process one 96-well plate. All steps must be performed in the postamplification room. The protocol is identical for both amplified cDNA and exonuclease-digested DNA plates from Steps 43 and 90, respectively. Sequencing libraries may alternatively be prepared via tagmentation using Nextera XT or in-house Tn5.

Fragmentation/End Prep

94. If stored at -20 ºC, thaw plates from Step 43 or 90 on ice. Once completely thawed, spin down on a PCR plate spinner for 15s.

95. We recommend adjusting the concentration of the cDNA/exonuclease-digested DNA to 1-5 ng/ml in order to start with around 5-30 ng of input material per well. Transfer 6.5 µl of the diluted sample to a new 96-well plate.

96. Prepare enough fragmentation buffer for one 96-well plate (x100) by adding the components listed on the table below. Store on ice until use. Note that a precipitate might be observed in the Ultra II FS Reaction Buffer after thawing. If that is the case, mix by vortexing and pipetting up and down.

- NEBNext Ultra II FS Reaction Buffer -> 175 µl

- NEBNext Ultra II FS Enzyme Mix ->50 µl

97. Vortex the fragmentation buffer and add 2.25 µl to each well. Seal the plate and spin down on a PCR plate spinner. Then, mix on a MixMate at 2100 rpm for 15 s, and spin down again.

98. In a thermal cycler, run the program described in the table below to incubate the fragmentation reaction. Set the lid temperature to 75 ºC. Depending on the desired fragment size, the fragmentation time (37ºC) can be adjusted following the manufacturer's instructions.

Cycle	Temperature	Time (min)
1	37 ºC	25 min
2	65 ºC	30 min
3	4 ºC	Hold

Adaptor Ligation

99. Prepare 100 ml of a 1:25 dilution of the NEBNext Adaptor for Illumina in 10 mM Tris-HCl, pH 7.5-8.0 with 10 mM NaCl.

100. Add 1 ml of diluted Adaptor to each well and seal the plate. Spin down the plate on a PCR plate spinner, mix on a MixMate at 2100 rpm for 15 s, and spin down again.

101. Prepare the Ligation master mix by adding the components listed in the table below. Store on ince until use.

- NEBNext Ultra II Ligation Master Mix -> 750 µl

- NEBNext Ultra II FS Ligation Enhancer -> 25 µl

102. Add 7.75 µl of Ligation master mix to each well and seal the plate. Spin down in a PCR plate spinner, mix on a MixMate at 2100 rpm for 15s, and spin down again.

103. In a thermal cycler, incubate the reaction at 20 ºC for 15 min with the heated lid off.

104. During the incubation time, prepare a 3:4 dilution of the USER enzyme in nuclease-free water.

105. Add 1 mL of diluted USER enzyme to each well and seal the plate. Spin down in a PCR plate spinner, mix on a MixMate at 2100 rpm for 15s, and spin down again.

106. Incubate at 37 ºC for 15 min in a thermal cycler with the heated lid set to 47ºC.

Cleanup of adaptor-ligated DNA

107. AMPure XP beads should be warmed up to room temperature before use (approximately for 15 min) and mixed by vortexing until fully resuspended.

108. Spin down the adaptor-ligated DNA plate in a PCR plate spinner and add 15 µl of the mixed AMPure XP beads to each well (1:0.8 ratio) at room temperature. Seal the plate and mix on a MixMate at 2100 rpm for 15 s. Let the mixture stand for 5 min at room temperature.

109. Transfer the plate to a low-elution magnet for 96-well plates and allow the beads to settle for 5 min.

110. Remove and discard the supernatant without disturbing the beads. For this step, we used a handheld electronic 96-channel pipette.

111. Without removing the 96-well plate from the magnet, wash the AMPure XP beads with 100 µl of freshly prepared 80% (vol/vol) ethanol for 30 s. Then carefully, without disturbing the beads, remove and discard the ethanol wash using a handheld 96-electronic channel pipette.

112. Repeat washing Step 111 once.

113. After the second wash, remove any remaining ethanol from the well, and allow the AMPure XP beads to dry for 2-5 min. Avoid over drying the beads as this would decrease DNA elution yield.

114. Remove the plate from the magnet and elute the adaptor-ligated DNA by adding 5 µl of elution buffer (EB) per well. Seal the plate and resuspend the beads by mixing on a MixMate for 15 s at 2100 rpm or until the beads are fully resuspended. Briefly spin down on a plate spinner to ensure the mixture is at the bottom of the well.

115. Allow the mixture to stand off the magnet for 5 min at room temperature.

116. Place the 96-well plate back into the magnet and allow the beads to settle for 5 min.

117. Transfer the 5 µl of supernatant containing the purified adaptor-ligated DNA to a new 96-well plate using a handheld electronic 96-channel pipette and seal the plate.

PCR enrichment of adaptor-ligated DNA

118. Spin down the plate in a PCR plate spinner. Using a multichannel pipette, add 2.5 ml of pre-mixed unique dual index primer pairs to the adaptor-ligated DNA (NEBNext Multiplex Oligos for Illumina). Different index primer pairs must be added to each well of the plate when planning on pooling the entire plate for downstream sequencing.

119. Spin down the plate in a PCR plate spinner.

120. Add 6.25 mL of NEBNext Ultra II Q5 Master Mix to each well of the plate and seal it. Spin down in a PCR plate spinner, mix on a MixMate at 2100 rpm for 15 s and spin down again.

121. Run the PCR program described in the table below in a thermal cycler.

Cycle	Temperature	Time (min)
1	98 ºC	30 s
2	98 ºC	10 s
3	65 ºC	75 s
4	Return to cycle 2	7x
5	65 ºC	5 min
6	4 ºC	Hold

Cleanup of PCR reaction

122. AMPure XP beads should be warmed up to room temperature before use (approximately for 15 min) and mixed by vortexing until fully resuspended.

123. Spin down the PCR-amplified library plate from Step 121 and add 10 µl of the mixed AMPure XP beads to each well (1:0.8 ratio) at room temperature. Seal the plate and mix on a MixMate at 2100 rpm for 15 s. Let the mixture stand for 5 min at room temperature.

124. Transfer the plate to a low-elution magnet and allow the beads to settle for 5 min.

125. Remove and discard the supernatant without disturbing the beads. For this step, we used a handheld electronic 96-channel pipette.

126. Without removing the 96-well plate from the magnet, wash the AMPure XP beads with 100 µl of freshly prepared 80% (vol/vol) ethanol for 30 s. Then carefully, without disturbing the beads, remove and

discard the ethanol wash using a handheld 96-channel pipette.

127. Repeat washing Step 126 once.

128. After the second wash, remove any remaining ethanol from the well, and allow the AMPure XP beads to dry for 2-5 min. Avoid over drying the beads as this would decrease DNA elution yield.

129. Remove the plate from the magnet and elute the libraries by adding 30 µl of elution buffer (EB) per well. Seal the plate and resuspend the beads by mixing on a MixMate for 15 s at 2100 rpm or until the beads are fully resuspended.

130. Allow the mixture to stand off the magnet for 2 min at room temperature.

131. Place the 96-well plate back into the magnet and allow the beads to settle for 5 min.

132. Transfer the 30 µl of supernatant containing the libraries to a new 96-well plate using a handheld electronic 96-channel pipette and seal the plate. The library plate can now be stored at -20 ºC until further processing.

Pooling, quantification of libraries and sequencing

The pooling and quantification of the final libraries should be performed in the postamplification work area. We often pool 96 libraries of one 96-well plate.

133. Quantify the DNA concentration of each library using a Qubit fluorometer. We recommend using a fluorescence microplate reader to measure the entire plate at once. Include two wells as standards.

134. After quantification, transfer 5 ml of each library into a new 96-well plate. The remaining library can be store at -20 ºC and kept for re-sequencing or deep sequencing, if required.

135. Calculate and add the required volume of elution buffer to adjust the concentration of each well to 1 ng/ml. This step is performed to avoid the overrepresentation of some libraries in the final pool. For this step, we used a MANTIS liquid dispenser to facilitate the pipetting of different volumes of elution buffer into each well.

136. Spin down the plate using a PCR plate spinner and mix on a MixMate at 2100 rpm for 15 s.

137. Using a multichannel pipette, pool 3ml of each column (1-12) into a single column of a new 96-well plate. Seal the plate and mix on a MixMate at 2100 rpm for 15 s. Spin down in a PCR plate spinner.

138. Pool 10 ml of each pooled row into a 1.5 ml Eppendorf tube.

139. Perform AMPure XP beads cleanup of the pooled libraries by adding 64 ml of beads (0.8x). Mix by vortexing and, briefly, spin down to bring all liquid to the bottom of the tube.

140. Let the mixture stand for 5 min at room temperature.

141. Place the tube on a DynaMag magnet for 1.5 ml tubes. Let it stand for 2 min or until the solution is clear.

142. Remove and discard the supernatant. Then, wash the beads by adding 600 ml of freshly prepared 80% (vol/vol) ethanol. After 30 s wash, and while keeping the tube on the magnet, carefully remove and discard the ethanol without disturbing the beads.

143. Repeat Step 142 once.

144. Remove any remaining ethanol and allow the beads to airdry for 2-5 min. Avoid over drying the beads.

145. Remove the tube from the magnet and elute the DNA libraries in 20 ml of elution buffer (EB). Mix by vortexing and spin down the tube briefly to bring all liquid to the bottom of the tube. Let the mixture stand for 2 min at room temperature.

146. Place the tube back in the magnet and allow the beads to settle for 5 min.

147. Transfer the 20 ml containing the pooled libraries to a new 1.5 Eppendorf tube.

148. Measure the concentration of the final pool using a Qubit fluorometer.

149. Assess the quality of the pooled libraries using an Agilent High Sensitivity D1000 screentape (HS-D5000) on an Agilent Bioanalyzer.

150. Sequence the library pools. We recommend using a HiSeq 4000 instrument (Illumina) or a NovaSeq 6000 instrument with 2× 150bp paired-end reads for circular DNA libraries and 2× 75 bp paired-end reads for cDNA libraries.

Troubleshooting

Time Taken

This protocol takes approximately 8 days per 96-well plate, from single cell sorting to library preparation of both cDNA and DNA.

Anticipated Results

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