

# High-throughput single cell sequencing using CEL-Seq2 on a nanoliter dispensing robot

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## Method Article

**Keywords:** CEL-Seq2, single cell sequencing, nanoliter pipetting, high-throughput, mosquito, mCEL-Seq2, 384-well plate

**Posted Date:** May 24th, 2018

**DOI:** <https://doi.org/10.1038/protex.2018.069>

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# Abstract

For the detection of subtle transcriptome changes reflecting fate biases, an scRNA-seq method is required that maximizes both sensitivity and accuracy. Recent benchmarking has demonstrated that CEL-Seq2 optimized both of these factors, but comes with high costs per cell (Hashimshony, T. et al. Genome Biol. 17, 77, 2016; Svensson, V. et al. Nat. Methods 14, 381–387, 2017). To enable high-throughput sequencing by the CEL-Seq2 method we here establish a robotic version of this method that permits sequencing of thousands of cells at substantially reduced costs.

## Introduction

Studying tissues and organs at the single cell level offers the possibility of unprecedented insights into the differentiation dynamics and the cellular heterogeneity of the tissue or organ of interest. In this protocol we describe a down-scaled version of CEL-Seq2 for the use with a nanoliter pipetting robot that allows us to sequence thousands of cells at low cost.

## Reagents

1. SuperScript II™ Reverse Transcriptase (Invitrogen, 18064014)
2. RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, 10777019)
3. Second Strand Buffer (Invitrogen, 10812014)
4. E. coli DNA Polymerase I (Invitrogen, 18010025)
5. E. coli DNA Ligase (Invitrogen, 18052019)
6. E. coli RNase H (Invitrogen, 18021071)
7. MEGAscript T7 Transcription Kit (Invitrogen, AM1334)
8. ExoSAP-IT For PCR Product CleanUp (Applied Biosystems™, 75001.1.ML)
9. NEBNext® Magnesium RNA Fragmentation Module (New England Biolabs, E6150S)
10. Agencourt® AMPure XP beads (Beckman Coulter, A63881)
11. Agencourt® RNAClean XP beads (Beckman Coulter, A63987)
12. Agilent RNA 6000 Pico Kit
13. Phusion® High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs)
14. 10 µM randomhexRT primer (GCCTTGGCACCCGAGAATTCCANNNNNN)
15. 10 µM Illumina RNA PCR (RP) primers
16. Hydrophobic encapsulation barrier (Vapor-Lock, Qiagen, 981611 or Mineral oil, Sigma, M8410)
17. Triton® X-100 (Sigma, T8787)
18. Qubit dsDNA HS (High Sensitivity) Assay Kit
19. Agilent High Sensitivity DNA kit
20. 192 barcoded primers

## Equipment

1. mosquito®HTS (TTP Labtech)
2. Cell sorter capable of sorting into 384-well plates (e.g. BD Influx™, BD FACSAria™ Fusion)
3. Laboratory centrifuge suitable for well plates
4. Mini-centrifuge to spin tubes
5. 384 and 96-well plates, LoBind, DNase/RNase-free
6. Aluminium plate sealers, temperature range: -80°C to 105°C
7. 384-well and 96-well thermal cycler or hybridization oven and fridge with adjustable temperature (16°C)
8. 96-well plate magnetic stand for bead clean-up
9. Qubit® Fluorometer (Invitrogen)
10. Agilent 2100 Bioanalyzer
11. DNA LoBind tubes, PCR Clean – 0.5 ml, 1.5 ml and 2 ml
12. DNA Low Binding filter tips, DNase and RNase-free
13. RNase decontamination solution
14. RNase-free water
15. RNase-free ethanol, molecular biology grade
16. Single-channel and multi-channel pipettes

# Procedure

**General Remarks:** Before starting the experiment make sure to clean your bench and pipets using RNase decontamination solution. Use RNase-free DNA-low binding tubes and tips. Each time well plates are centrifuged they should be covered using aluminium plate sealers. Perform all centrifugation steps at 4°C.

**Plate preparation for single cell sorting** The following steps describe how to prepare 8 x 384-well plates for single cell sorting.

**Prepare barcoded primer source plate:**

1. Prepare a mastermix of the following reagents (enough for 8 plates): a. 840 µl water with 0.35% Triton® X-100 b. 120 µl 10mM dNTP c. 240 µl water with 1:100000 ERCC mix 1 or 2
2. Pipet 5 µl of the solution into each of the 192 wells of a 384-well plate using a multi-channel pipet (columns 1-12, rows A-P). Avoid bubbles.
3. Centrifuge the plate at 2200g for 1 min
4. Dispense 1000 nl of each 192 barcoded primers at 1µM working concentration from a 384-well plate into the prepared 384-well plate containing 5 µl buffer using the mosquito®HTS. (mosquito from here on)
5. Add a mixing routine for every dispense step in the program (see Advanced Options for dispense step) and mix the solution 10 times and by moving the tips up by 2 mm.
6. Centrifuge the plate at 2200g for 1 min

**Preparation of a source plate containing hydrophobic encapsulation barrier** (Vapor-Lock)

7. Pipet 25µl of Vapor-Lock into each of the 192 wells of a 384-well plate
8. Centrifuge the plate at 2200g for 1 min

**Preparation of plates for single cell sorting**

9. Place the primer source plate into the first plate position of the mosquito
10. Put 4 empty plates into the remaining bays.
11. Dispense 240 nl of primer mix from the first column of your primer source plate into each first column and column 13 of your empty plates
12. Change the mosquito pipets
13. Dispense the remaining columns in the same manner and change the mosquito pipets every time you dispense into a new column i.e. dispense from column 2 of the source plate to columns 2 and 14 in the empty plates, from column 3 of the source plate to columns 3 and 15 in the empty plates etc. Like this one of the 192 barcodes is used twice per 384-well plate.
14. After the primers are dispensed change the primer source plate with the plate containing Vapor-Lock and dispense 1.2 µl of Vapor-Lock in the same manner, i.e. dispense 1.2 µl from columns 1, 2, 3. to columns 1 and 13, 2 and 14, 3 and 15 etc. of the plates containing the primer mix. Change pipets when pipetting from a new column of the source plate.
15. Centrifuge the plates at 2200g for 1 min and freeze them at -20°C until they are used. (Note: Instead of using Vapor-Lock one can use mineral oil. In this regard it is possible to first dispense 1.2 µl mineral oil into every well of the empty 384-well plates without changing the pipets and then dispense the primer mix as described in steps 11. - 13.)

**Cell Sorting**

16. Use an appropriate nozzle size for your cells of interest (min. 5 times the cell size, 100µm nozzles can be used if the sorted cells are heterogenous in size)
17. Include dead live staining, if possible, especially with extended cell extraction procedures.
18. Exclude doublets using FSC-W to FSC-H and SSC-W to SSC-H ratios as well as the single cell mode of the cell sorter.
19. Centrifuge the plates at 2200g for 10 min at 4°C and store them at -80°C until further processing using CEL-Seq2.

**Amplified RNA preparation from single cells** The following steps describe how to prepare one 384-well plate using mCEL-Seq2.

20. Incubate the plate containing your cells (sample plate) at 90°C for 3 min in a thermocycler (lid: 105°C)
21. Centrifuge the plates at 2200g for 1 min at 4°C and transfer plates on ice.

**Reverse transcription**

22. Prepare a mastermix for the reverse transcriptase reaction (the recipe includes additional volume to account for dead volume in the

mosquito pipet tips): a. First Strand Buffer 70  $\mu$ l b. DTT 0.1 M 35  $\mu$ l c. RNaseOut 17.5  $\mu$ l d. Superscript II 17.5  $\mu$ l total 140  $\mu$ l 23. Pipet 8.5  $\mu$ l into each well of one column of a new 384-well plate and centrifuge the plate briefly. This plate will serve as your buffer source plate. 24. Dispense 160 nl of First Strand Reaction mix from the buffer source plate to every column of your sample plate and change the pipets after every dispense step. 25. Centrifuge the plates at 2200g for 1 min at 4°C 26. Incubate the sample plate at 42°C for 1h (lid: 50°C). 27. Heat-inactivate the reverse transcriptase at 70°C for 10 min. 28. Chill the plate on ice and centrifuge the plates at 2200g for 1 min at 4°C. Second Strand Synthesis 29. Prepare a mastermix for second strand synthesis: a. Ultrapure water: 631.4  $\mu$ l b. Second Strand Buffer: 205  $\mu$ l c. dNTP 20.5  $\mu$ l d. E. coli Ligase 7.38  $\mu$ l e. E. coli DNA Polymerase 28.7  $\mu$ l f. RNase H 7.38  $\mu$ l total 900.36  $\mu$ l 30. Pipet 14  $\mu$ l into each well of four new columns of your buffer source plate. This approach is used when pooling 96 cells per library (see below). (Alternatively it is possible to pipet 28  $\mu$ l into each well of 2 columns when 192 cells are to be pooled into one library.) 31. Dispense 2196 nl Second Strand Reaction mix in two steps (2 x 1098 nl) from the buffer source plate into every well of your sample plate. 32. Pipets have to be changed four times after dispensing into columns 1-6, 7-12, 13-18, 19-24. This is the case when 96 cells should be pooled together. (If 192 cells should be pooled, change pipets after dispensing into columns 1-12 and 13-24) 33. Centrifuge the sample plate at 2200g for 1 min at 4°C 34. Incubate the sample plate at 16°C for 2 h. **cDNA cleanup** 35. Prewarm AMPure XP beads to room temperature (takes app. 30 min) 36. Vortex beads until dispersed 37. Pool columns 1-6, 7-12, 13-18, 19-24 each into a single tube. Pool the columns that should be pooled into a single column using a multichannel pipet and then transfer the contents of this column into a tube. 38. Centrifuge the four tubes at 10000g for 1 min to separate the aqueous phase from the oil. 39. For every sample transfer the aqueous phase (app. 200  $\mu$ l) into two wells (app. 100  $\mu$ l each) of a DNA low-binding 96-well plate. 40. Add 0.8 volumes of AMPure XP beads to every well containing a sample. 41. Mix by resuspending 5 times using a multichannel pipet 42. Incubate at room temperature for 10 min 43. Place on magnetic stand and let the solution clear 44. Remove and discard the supernatant 45. Add 180  $\mu$ l of freshly prepared 80% ethanol 46. Incubate 40 seconds and remove the ethanol completely 47. Repeat the washing step once 48. Air dry beads for 10 min 49. Resuspend the sample in the two wells with a total of 7  $\mu$ l water. 50. Incubate at room temperature for 2 min 51. Place on magnetic stand until solution is clear 52. Transfer app. 6.4  $\mu$ l of the supernatant into a fresh PCR tube In vitro transcription 53. Add 9.6  $\mu$ l of in vitro transcription mix, consisting of 1.6  $\mu$ l of ATP, GTP, CTP, UTP, 10x T7 Buffer and T7 enzyme each (mastermix: 7  $\mu$ l of every component). Mix well. 54. Incubate at 37°C for 13 h (lid:70°C) **aRNA cleanup** 55. Add 6  $\mu$ l EXO-SAP to every sample and incubate at 37°C for 15 min 56. Add 2.44  $\mu$ l 10x Fragmentation Buffer and mix 57. Incubate at 94°C for 3min 58. Immediately after move the samples to ice and add 2.44  $\mu$ l 10x Fragmentation Stop Buffer 59. Prewarm RNAClean XP beads to room temperature (takes app. 30 min) 60. Vortex beads until dispersed 61. Add 21.5  $\mu$ l of beads to every sample and mix well 62. Incubate at room temperature for 10 min 63. Place on magnetic stand and let the solution clear 64. Remove and discard the supernatant 65. Add 180  $\mu$ l of freshly prepared 70% ethanol 66. Incubate 40 seconds and remove the ethanol completely 67. Repeat wash 2 times 68. Air dry beads for 10 min 69. Resuspend each amplified RNA (aRNA) sample with 7  $\mu$ l water. 70. Perform a quality check using 1 $\mu$ l of aRNA using the Agilent RNA 6000 Pico Kit and the Bioanalyzer. The average

fragment size of the aRNA should be between 500 bp ( $\pm 50$  bp). **Library preparation** **Reverse Transcription of aRNA** 71. Add 1.5  $\mu$ l of the following solution to 5  $\mu$ l of aRNA: a. 1  $\mu$ l random Hexamers (5 x MM: 5  $\mu$ l) b. 0.5  $\mu$ l dNTP (5 x MM: 2.5  $\mu$ l) 72. Incubate at 65°C for 5 min and chill on ice thereafter 73. Add 4  $\mu$ l of First Strand Synthesis Solution a. 2  $\mu$ l First Strand Buffer (5 x MM: 10  $\mu$ l) b. 1  $\mu$ l 0.1M DTT (5 x MM: 5  $\mu$ l) c. 0.5  $\mu$ l RNaseOUT (5 x MM: 2.5  $\mu$ l) d. 0.5  $\mu$ l Superscript II (5 x MM: 2.5  $\mu$ l) 74. Incubate at 25°C for 10 min 75. Incubate at 42°C for 1 h (lid: 50°C) **PCR amplification** 76. Add to every sample 2  $\mu$ l of one uniquely indexed Illumina RPI primer 77. Add to every sample 38  $\mu$ l of the following solution and mix: a. Ultra pure water 11  $\mu$ l b. Phusion HF PCR Master Mix 25  $\mu$ l c. Illumina primer RP1 2  $\mu$ l 78. Amplify DNA in a PCR cycler using the following conditions: Denaturation 98°C 30 s 11 cycles (up to 15 cycles with low aRNA concentration) 98°C 10 s 60°C 30 s 72°C 30 s Final extension 72°C 10 min 4°C  $\infty$  **1st Bead Cleanup of PCR products** 79. Transfer samples (each 50  $\mu$ l into a low binding 96-well plate 80. Prewarm AMPure XP beads to room temperature (takes app. 30 min) 81. Vortex beads until dispersed 82. Add 50  $\mu$ l to each 50  $\mu$ l PCR reaction 83. Incubate at room temperature for 10 min 84. Place on magnetic stand and let the solution clear 85. Remove supernatant 86. Wash with 180  $\mu$ l freshly prepared 80% EtOH 87. Incubate for 40 s and discard supernatant without disturbing the beads 88. Repeat washing step once 89. Air dry beads for 10 min 90. Resuspend with 25  $\mu$ l water, mix thoroughly 91. Incubate at room temperature for 2 min 92. Place on magnetic stand and let the solution clear 93. Transfer supernatant (25  $\mu$ l) to a new well **2nd Bead Cleanup of PCR products** 94. Prewarm AMPure XP beads to room temperature (takes app. 30 min) 95. Vortex beads until dispersed 96. Add 25  $\mu$ l to each 25  $\mu$ l solution 97. Incubate at room temperature for 10 min 98. Remove supernatant 99. Wash with 180  $\mu$ l freshly prepared 80% EtOH 100. Incubate for 40 s, and discard supernatant without disturbing the beads 101. Repeat washing step once 102. Air dry beads for 10 min 103. Resuspend with 10  $\mu$ l water, mix thoroughly 104. Incubate at room temperature for 2 min 105. Place on magnetic stand and let the solution clear 106. Transfer supernatant (10  $\mu$ l) to a new tube 107. Library is ready for sequencing and can be stored at -20°C 108. Measure concentration and fragment size of each library and pool equimolar amounts of libraries according to the used unique RPI guidelines (Illumina). Sequence cells at ~200000 reads per cell.

## Timing

**Preparation of plates for single cell sorting:** 3 hours **Cell Sorting:** 1 hour - 8 hours + **Reverse Transcription - in vitro transcription of cDNA:** 1 day **RNA cleanup - Library preparation and purification:** 1 day

## Supplementary Files

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- [supplement0.txt](#)