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Onepot-seq

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protocol .

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Onepot-seq protocol follows steps below:
1. Cells and beads preparation
2. Scatteration of beads and cells in well
3. Cell lysis and beads isolation
4. cDNA synthesis
5. cDNA Library amplification
6. NGS preparation

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Onepot-seq is single cell RNA sequencing(scRNA-seq) experiment method.
The main idea of Onepot-seq is to do single cell experiment in continuous medium without compartmentalization.
Therefore, we focus on temperature control and not disturbing the solutions due to transient mRNA localization.

Onepot-seq protocol follows steps below:
1. Cells and beads preparation
2. Scatteration of beads and cells in well
3. Cell lysis and beads isolation
4. cDNA synthesis
5. cDNA Library amplification
6. NGS preparation

From the 4th step onwards, since it is a process of dealing with beads, it basically follows the Dropseq protocol (Macosko et al., 2015) and there are some modifications.

Macosko EZ, Basu A, Satija R, Nemesh J, Shekhar K, Goldman M, Tirosh I, Bialas AR, Kamitaki N, Martersteck EM, Trombetta JJ, Weitz DA, Sanes JR, Shalek AK, Regev A, McCarroll SA (2015). Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets.. Cell.
https://doi.org/10.1016/j.cell.2015.05.002

- Poly T beads (Chemgene, MACOSKO-2011-10(V+))

- PBSB : PBS supplemented with 0.1% BSA

- incubation buffer : 6% Ficoll PM-400, 20mM EDTA, 0.2M Tris pH 7.5

A	B
1ml of Incubation buffer composition	
H20	460 µl
20% Ficoll PM-400 (GE healthcare)	300 µl
500mM EDTA (Life Technologies)	40 µl
1M Tris pH 7.5 (Sigma)	200 µl
Total	1000 µl

- Lysis buffer : 1:1 mixture of incubation buffer and 20% Sarkosyl

- 6X SSC

- TE-SDS : TE buffer + 0.5% SDS

- TE-TW : TE buffer + 0.01% Tween-20

- 10mM Tris pH8.0

- DNase free Water (DW)

- Reverse Transcription mix (RT mix)

A	B
RT mix composition	
H20	80 µl
Maxima 5X RT buffer	40 µl
20% Ficoll PM-400	40 µl
10mM dNTPs	20 µl
100uM TSO	5 µl
RNase inhibitor	5 µl
Maxima H- RTase	10 µl
Total	200 µl

- Exonuclease I mix (Exo I mix)

A	B
Exonuclease mix composition	
10X ExoIbuffer	20 µl
H20	170 µl
ExoI	10 µl
Total	200 µl

- AMPure XP beads

Cells and beads preparation

1 Suspend cells in PBS (1,000 cells/µl of concentration is recommended)

2 Wash Poly T beads 3 times with **1 mL** PBSB
(PBSB : PBS supplemented with 0.1% BSA)

3 Suspend beads in incubation buffer (20,000 beads in **100 µL** incubation buffer)

(incubation buffer : 6% Ficoll PM-400, 20mM EDTA, 0.2M Tris pH 7.5)

Scatteration of beads and cells in well (※ Do experiments in 4C room)

4 Add incubation buffer to 12 well plate

5 Add 1,000 cells (suspended in PBS) to incubation buffer, let the sum of solution volume be **900 µL** .

5.1 If you are testing more than 1,000 cells, increase the number of wells or increase the area of the wells according to cells and beads number.

6 Add 20,000 beads (suspended in **100 µL** incubation buffer)

7 Gently pipette **1 mL** incubation buffer to spread the beads and cells evenly

8 Wait **00:15:00** to sink the cells and beads down to the bottom 15m
(Depending on the cell type, it may take more time to sink)

Cell lysis and beads isolation (※ Do experiments in 4C room)

9 Gently stack up **200 µL** of lysis buffer on top of incubation buffer
(※ avoid the beads being affected by current flow)

10 Incubate for **00:15:00** 15m

11 Quickly disrupt 1.2ml of solutions with **1 mL** of 6X SSC

12 Quickly transfer 2.2ml of solutions to **30 mL** of 6X SSC (in 50 ml falcon tube)

12.1 If multiple wells or larger area wells are used to increase the number of cells, collect beads corresponding to 5,000 cells in one falcon tube.
(RT mix is effective up to 5,000 cells)

13 Pipette 4~5 times to avoid mRNA cross contamination

14 Centrifuge beads (1000 X g / **00:01:00**) 1m
After centrifugation, discard supernatant

15 Add **1 mL** of 6X SSC

16 Transfer beads to 1.5ml tube

17 Wash beads 3 times with **1 mL** 6X SSC

18 Wash beads with ~ **300 µL** of 5X RT buffer

cDNA synthesis (※ similar with Drop-seq protocols) 2h

19 Add **200 µL** Reverse Transcription mix (RT mix)

A	B
RT mix composition	
H20	80 µl
Maxima 5X RT buffer	40 µl
20% Ficoll PM-400	40 µl
10mM dNTPs	20 µl
100uM TSO	5 µl
RNase inhibitor	5 µl
Maxima H- RTase	10 µl
Total	200 µl

20 Incubate for **00:30:00** at **Room temperature** using rotator

30m

21 Incubate for **01:30:00** at **42 °C** using rotator

1h 30m

22 Wash the beads once with **1 mL** TE-SDS

23 Wash the beads twice with **1 mL** TE-TW

24 Wash the beads once with **1 mL** of **10 millimolar (mM)** Tris pH8.0

25 Add **200 µL** of Exonuclease I mix (Exo I mix)

A	B
Exonuclease mix composition	
10X ExoIbuffer	20 µl
H20	170 µl
ExoI	10 µl
Total	200 µl

26 Incubate for **00:45:00** at **37 °C** using rotator 45m

27 Wash the beads once with **1 mL** TE-SDS

28 Wash the beads twice with **1 mL** TE-TW

29 Wash the beads twice with **1 mL** DW

cDNA Library amplification (※ similar with Drop-seq protocols) 2h

30 Resuspend beads with DW
(2,000-2,500 beads in 10 µL of DW)

31 Aliquot **10 µL** beads containing solutions to 8-strip tube

32 Add polymerase and PCR primer to each tube
1 reaction of PCR mix composition follows below :

A	B
cDNA amplification PCR composition	
DW	14.6 µl
beads in DW	10 µl
SMART PCR Primer (100uM)	0.4 µl
2X KAPA	25 µl
Total	50 µl

(50µL PCR reaction for 100~125 cells / 2,000~2,500 beads)

33 Run PCR following the protocol below:

A	B
	95C 3min
Denature	98C 20 sec
Annealing	65C 45 sec
Elongation	72C 3 min
	4 cycles
Denature	98C 20 sec
Annealing	67C 20 sec
Elongation	72C 3 min
	9 cycles
Final extension	72C 5 min
Hold	4C forever

34 Collect PCR cDNA library solutions to 1.5ml tube

35 0.6X Ampure bead purification
elute cDNA library into **20 µL** DW

NGS library preparation

36 For NGS library prep, follow the Nextera XT DNA Library Preparation Kit (#FC-131-1024) manufacturer's instructions.