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Plate based scRNA-seq Illumina library construction

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1 Works for me dx.doi.org/10.17504/protocols.io.bi4fkgtn

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ABSTRACT

This SOP describes the procedure for plate based scRNA-seq performed with a commercial available kit from New England BioLabs. Following library construction, samples are pooled in equivolume and quantified, prior to sequencing on the Illumina HiSeq 4000 platform.

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GUIDELINES

It is vital all steps prior to cDNA amplification are performed in a designated RNase free and pre-cDNA amplification laboratory.

Throughout the protocol we have indicated the liquid handling in use at Sanger for specific parts of the process. However, these steps can be performed on alternative liquid handlers.

MATERIALS

NAME	CATALOG #	VENDOR
KAPA HiFi HotStart ReadyMixPCR Kit	KK2602	Kapa Biosystems
NEBNext Single Cell/Low Input RNA Library Prep Kit for Illumina - 96 rxns	E6420L	New England Biolabs

NAME	CATALOG #	VENDOR
AccuClear® Ultra High Sensitivity dsDNA Quantitation Kit with DNA Standards	#31028	Biotium
Bioanalyzer chips and reagents (DNA 1000)	5067-1504	Agilent Technologies
AMPure XP Beads	A63882	Beckman Coulter

STEPS MATERIALS

NAME	CATALOG #	VENDOR
NEBNext Single Cell/Low Input RNA Library Prep Kit for Illumina - 96 rxns	E6420L	New England Biolabs
AccuClear® Ultra High Sensitivity dsDNA Quantitation Kit with DNA Standards	#31028	Biotium
2x Kapa HiFi Hotstart Readymix	KK2602	Kapa Biosystems
Bioanalyzer chips and reagents (DNA 1000)	5067-1504	Agilent Technologies
AMPure XP Beads	A63882	Beckman Coulter

EQUIPMENT

NAME	CATALOG #	VENDOR
Bioanalyzer	G2991AA	

Preparation of lysis buffer plates and FACS

1 **Important!** This step must be performed in a designated RNAse free and pre-cDNA amplification area, keeping reagents chilled at all times

2

NEBNext Single Cell/Low Input RNA Library Prep Kit for Illumina - 96 rxns
by New England Biolabs
Catalog #: E6420L

Prepare the cell lysis buffer, which will provide sufficient volume for one 96-well plate **On ice**

Reagent	Volume (µl)
NEBNext Cell Lysis Buffer (10x)	24
Murine RNase Inhibitor	12
Nuclease-Free Water	204
Total	**240**

Mix well by pipetting.

3 Use the Formulatrix Mantis microfluidic liquid handler to dispense **2 µl** of lysis buffer into a 96-well PCR plate.

If required add a diluted stock (1/500,000) of ERCCs into the lysis buffer.

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4 Seal dispensed plates, centrifuge immediately **1000 x g, 4°C, 00:01:00** and keep chilled on ice.

5 **PAUSE POINT** Lysis buffer plates can be stored at **-80 °C** prior to cell sorting. Plates can be stored for < 6 months.

6

When FACS sorting, take care of plate calibration/priming prior to single-cell deposition. If many plates are deposited in parallel, repeat the calibration/priming at least every 8 plates.

Defrost lysis buffer plates prior to cell sorting, centrifuge **1000 x g, 4°C, 00:01:00** and keep chilled on ice.

7 After FACS sorting, seal and centrifuge plates immediately **1000 x g, 4°C, 00:01:00** and keep chilled on ice.

8 **PAUSE POINT** Plates of sorted cells can be stored at **-80 °C** for < 6 months. the quality of the data depends on the cell type and duration of storage.

Primer Annealing for first-strand synthesis

9

Prepare the primer annealing mix, which will provide sufficient volume for one 96-well plate **On ice**

Reagent	Volume (µl)
NEBNext Single Cell RT Primer Mix	50
Nuclease-Free Water	250
Total	**300**

Mix well by pipetting.

10 The Agilent Bravo with 96 ST head will combine **1.6 µl** of primer annealing mix with **2 µl** of lysed cells and mix by pipetting.

11 Seal and transfer the plate to a thermocycler with the heated lid set to **100 °C** and run the following program:

Temperature	Time
70ºC	5 minutes
4ºC	∞

Reverse transcription (RT) and template switching

12 Prepare the RT mix, which will provide sufficient volume for one 96-well plate **On ice**

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Reagent	Volume (µl)
NEBNext Single Cell RT Buffer	250
NEBNext Template Switching Oligo	50
NEBNext Single Cell RT Enzyme	150
Nuclease-Free Water	100
Total	**550**

Mix well by pipetting.

13 The Bravo will add **4.4 µl** of RT mix to each sample and mix by pipetting.

14 Seal and transfer the plate to a thermocycler with the heated lid set to **100 °C** and run the following program:

Temperature	Time
42°C	90 minutes
70°C	10 minutes
4°C	∞

15 Prepare the cDNA amplification mix, which will provide sufficient volume for one 96-well plate **On ice**

Reagent	Volume (µl)
NEBNext Single Cell cDNA PCR Master Mix	2500
NEBNext Single Cell cDNA Primer Mix	100
Nuclease-Free Water	1400
Total	**4000**

Mix well by pipetting.

16 The Bravo will add **32 µl** of cDNA amplification mix to each sample and mix by pipetting.

17 Seal and transfer the plate to a thermocycler with the heated lid set to **100 °C** and run the following program:

Temperature	Time	Cycles
98°C	45 seconds	1
98°C	10 seconds	16-25 depending on cell type
62°C	15 seconds	
72°C	3 minutes	
72°C	5 minutes	1
4°C	∞	1

Purification of amplified cDNA

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18

AMPure XP Beads
by Beckman Coulter
Catalog #: A63882

Allow AMPure XP beads to equilibrate to room temperature (~30 minutes). Ensure solution is homogenous prior to use, mixing gently by inversion.

19 Centrifuged amplified cDNA plate **1000 x g, 00:01:00**

20 Use the Agilent Bravo with a 96 LT multichannel head to perform the following steps:

20.1 Add 0.6 X volume of SPRI beads per sample (**24 µl** SPRI : **40 µl** amplified cDNA), mix well by pipetting.

20.2 Incubate for **00:05:00** at **Room temperature**

20.3 Transfer the plate to the magnet, allow **00:02:00** for the beads to settle.

20.4 Carefully remove and discard the supernatant without disturbing the bead pellet.

20.5 Wash the beads with **180 µl** 80% freshly prepared ethanol for **00:00:30** remove ethanol and discard.

20.6 Repeat ethanol wash.

20.7 Allow beads to dry **00:05:00**

20.8 Remove the plate from the magnet, add **50 µl** nuclease-free water and resuspend by mixing well.

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20.9 Incubate for **00:02:00** at **Room temperature**

20.10 Transfer the plate to the magnet, allow **00:05:00** for the beads to settle.

20.11 Transfer supernatant into a new 96-well PCR plate, taking care not to disturb the pellet.

Quality control of amplified cDNA

21

Purified amplified cDNA is quantified with a fluorescence based assay. We use the AccuClear Ultra High Sensitivity dsDNA Quantitation kit with 7 DNA standards (Biotium) according to manufacturer's instructions.

To streamline the workflow, we do not normalise sample input for library preparation. Instead, we calculate an average concentration and transfer a fixed volume such that 5-25 ng of each successfully amplified cDNA sample enters library preparation.

AccuClear® Ultra High Sensitivity dsDNA Quantitation Kit with DNA Standards
by Biotium
Catalog #: #31028

22 Pipette **20 µl** of each DNA standard into wells A1 - G1 of a 96-well PCR plate. Add nuclease-free water to H1.

23 Dilute the AccuClear dye (100X) to working concentration by mixing **300 µl** dye with **30 mL** AccuClear buffer in a 50 ml Falcon. Mix thoroughly by vortexing and transfer to a 384-well reservoir.

24 Use the SPT Labtech Mosquito LV to stamp **200 nl** of amplified cDNA and **1 µl** of known standards in triplicate into a 384-well assay plate. Immediately proceed to the next step.

25 Use the Agilent Bravo with a 384ST multichannel head to add **50 µl** 1 X AccuClear dye from the reservoir to the assay plate, mix thoroughly by pipetting.

26 Measure fluorescence values on a BMG FLUOstar Omega plate reader calibrated for use with AccuClear dye.

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27 Confirm known standards are performing as expected.

28 Dilute any samples >125 ng/µl with nuclease free water so they are in the range of 10 - 125 ng/µl and repeat quantitation.

We use 5X the volume of standard vs sample in our assay setup, which should allow a quantitative range of 0.15 ng/µl - 125 ng/µl. This deviates from the standard kit SOP which has a stated range of 0.03 ng/µl - 25 ng/µl.

29 Taking an average across the plate. Transfer ~ 10 ng of cDNA into a new 96-well PCR plate for sequencing library preparation.

30 **PAUSE POINT** Purified amplified cDNA can be stored at **-20 °C** for several weeks prior to library preparation.

Illumina sequencing library preparation

31

We use the NEBNext® Single Cell/Low Input RNA Library Prep Kit for Illumina for library preparation, which we have automated on the Agilent Bravo NGS platform with some modifications. We use a custom adapter set, however any TruSeq adapters are suitable.

NEBNext Single Cell/Low Input RNA Library Prep Kit for Illumina - 96 rxns
by New England Biolabs
Catalog #: E6420L

AMPure XP Beads
by Beckman Coulter
Catalog #: A63882

32 Allow AMPure XP beads to equilibrate to room temperature (~30 minutes). Ensure solution is homogenous prior to use, mixing gently by inversion.

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33 Centrifuged plate containing ~ 10 ng of purified cDNA **1000 x g, 00:01:00**

34 Use the Agilent Bravo with a 96 LT multichannel head to perform the following steps:

34.1 Add 0.9 X volume of SPRI beads per sample, mix well by pipetting.

34.2 Incubate for **00:05:00** at **Room temperature**

34.3 Transfer the plate to the magnet, allow **00:02:00** for the beads to settle.

34.4 Carefully remove and discard the supernatant without disturbing the bead pellet.

34.5 Wash the beads with **180 µl** 80% freshly prepared ethanol for **00:00:30** remove ethanol and discard.

34.6 Repeat ethanol wash.

34.7 Allow beads to dry **00:05:00**

34.8 Remove the plate from the magnet, add **13 µl** TE pH 8.0 and resuspend by mixing well.

34.9 Incubate for **00:02:00** at **Room temperature**

34.10 Transfer the plate to the magnet, allow **00:05:00** for the beads to settle.

34.11 Transfer **12.4 µl** into a new 96-well PCR plate, taking care not to disturb the pellet.

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https://dx.doi.org/10.17504/protocols.io.bi4fkgtn

35 Prepare fragmentation/end prep mix, which will provide sufficient volume for one 96-well plate **On ice**

Reagent	Volume (µl)
NEBNext Ultra II FS Reaction Buffer	336
NEBNext Ultra II FS Reaction Enzyme	96
Total	**432**

Mix well by pipetting.

36 The Bravo will add **3.6 µl** of fragmentation/end prep mix to each sample and mix by pipetting.

37 Seal and transfer the plate to a thermocycler with the heated lid set to **100 °C** and run the following program:

Temperature	Time
72°C	15 minutes
65°C	30 minutes
4°C	∞

38 Prepare adapter ligation mix, which will provide sufficient volume for one 96-well plate **On ice**

Reagent	Volume (µl)
NEBNext Ultra II Ligation Master Mix	1440
NEBNext Ultra II Ligation Enhancer	48
TruSeq Duplexed Adapter (100 µM)	12
Nuclease-Free Water	108
Total	**1608**

Mix well by pipetting.

39 The Bravo will add **13.4 µl** of ligation mix to each sample and mix by pipetting.

40 The plate is incubated on deck at **20 °C** for **00:15:00** , however this step may also be performed on a thermocycler.

41

We use alternative TruSeq compatible adapters, which do not require the USER enzyme incubation step. If using NEBNext adapters, follow the steps in the NEB protocol to add USER enzyme to the ligation reaction.

42

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AMPure XP Beads
by Beckman Coulter
Catalog #: A63882

Allow AMPure XP beads to equilibrate to room temperature (~30 minutes). Ensure solution is homogenous prior to use, mixing gently by inversion.

42.1 Add 0.7 X volume of SPRI beads per sample (**20 µl** SPRI : **29.4 µl** amplified cDNA), mix well by pipetting.

42.2 Incubate for **00:05:00** at **Room temperature**

42.3 Transfer the plate to the magnet, allow **00:02:00** for the beads to settle.

42.4 Carefully remove and discard the supernatant without disturbing the bead pellet.

42.5 Wash the beads with **180 µl** 80% freshly prepared ethanol for **00:00:30** remove ethanol and discard.

42.6 Repeat ethanol wash.

42.7 Allow beads to dry **00:05:00**

42.8 Remove the plate from the magnet, add **25 µl** nuclease-free water and resuspend by mixing well.

42.9 Incubate for **00:02:00** at **Room temperature**

42.10 Transfer the plate to the magnet, allow **00:05:00** for the beads to settle.

42.11 Transfer supernatant into a new 96-well PCR plate, taking care not to disturb the pellet.

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43

We use KAPA HiFi HotStart ReadyMix and unique dual indexed (UDI) tag plates for library PCR.
Note: this deviates from the standard NEB protocol which uses NEBNext Ultra II Q5 Master Mix and different cycling conditions.

2x Kapa HiFi Hotstart Readymix
by Kapa Biosystems
Catalog #: KK2602

44 Prepare PCR mix, which will provide sufficient volume for one 96-well plate **On ice**

Reagent	Volume (µl)
KAPA HiFi HotStart ReadyMix	3000
Total	**3000**

45 The Bravo will add **25 µl** PCR mix and **25 µl** sample into a lyophilised plate of UDIs and mix thoroughly by pipetting. The final concentration of each UDI in the PCR reaction is 2 µM.

46 Seal and transfer the plate to a thermocycler with the heated lid set to **100 °C** and run the following program:

Temperature	Time	Cycles
98°C	45 seconds	1
98°C	10 seconds	8
62°C	15 seconds	
72°C	3 minutes	
72°C	5 minutes	1
4°C	∞	1

47 **PAUSE POINT** amplified libraries can be stored at **-20 °C** for several weeks prior to library purification.

Pooling and purification of amplified libraries

48 In a post-PCR lab, use the Hamilton STAR or Beckman NX-8 to combine **5 µl** of each sample per plate to form an equivolume pool of 96 samples.

49

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AMPure XP Beads
by Beckman Coulter
Catalog #: A63882

Allow AMPure XP beads to equilibrate to room temperature (~30 minutes). Ensure solution is homogenous prior to use, mixing gently by inversion.

49.1 Manually transfer **400 µl** of the equivolume pool into a 1.5 ml Eppendorf tube

49.2 Add 0.95 X volume of SPRI beads (**380 µl** SPRI : **400 µl** amplified libraries), mix well by pipetting.

49.3 Incubate for **00:05:00** at **Room temperature**

49.4 Transfer the tube to a magnet, allow **00:05:00** for the beads to form a pellet.

49.5 Carefully remove and discard the supernatant without disturbing the bead pellet.

49.6 Wash the beads with **180 µl** 80% freshly prepared ethanol for **00:00:30** remove ethanol and discard.

49.7 Repeat ethanol wash.

49.8 Allow beads to dry **00:05:00**

49.9 Remove the tube from the magnet, add **400 µl** nuclease-free water and resuspend by mixing well.

49.10 Incubate for **00:02:00** at **Room temperature**

49.11 Transfer tube to magnet, allow **00:05:00** for the beads to form a pellet.

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49.12 Transfer supernatant into a new tube, taking care not to disturb the pellet.

Quality control and normilisation of sequencing libraries

50

Library sequencing pools are quantified on an Agilent Bioanalyzer. Pools are then diluted to 2.8 nM for sequencing.

Bioanalyzer
Bioanalyzer
Agilent **G2991AA**
Any bioanalyzer will suffice.

Bioanalyzer chips and reagents (DNA 1000)
by Agilent Technologies
Catalog #: 5067-1504

51 Run **1 µl** of the library pool in triplicate on a Bioanalyzer using the DNA 1000 kit.

52 Taking an average of the readings add nuclease-free water to the library pool to produce a final concentration of 2.8 nM.

Sequencing

53

We sequence samples on an Illumina HiSeq 4000 instrument (paired-end, 75-bp reads) according to the manufacturer's protocol. We typically aim for an average depth of 1 million reads per cell, plexing up to 384 samples per run.

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