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Commercial automated scRNA-seq workflow

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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.

Guidelines

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

Note

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

MATERIALS

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

STEP MATERIALS

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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 **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.

Note

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

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

Note

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

	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
	42ºC	90 minutes
	70ºC	10 minutes
	4ºC	∞

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
	98ºC	45 seconds	1
	98ºC	10 seconds	16-25 depending on cell type
	62ºC	15 seconds	
	72ºC	3 minutes	
	72ºC	5 minutes	1
	4ºC	∞	1

Purification of amplified cDNA

18

AMPure XP Beads **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.

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

Note

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 **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.

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.

Note

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

Note

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 **New England Biolabs Catalog #**E6420L

AMPure XP Beads **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.

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.

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
	72ºC	15 minutes
	65ºC	30 minutes
	4ºC	∞

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

Note

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

AMPure XP Beads **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.

43

Note

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 **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
	98ºC	45 seconds	1
	98ºC	10 seconds	8
	62ºC	15 seconds	
	72ºC	3 minutes	
	72ºC	5 minutes	1
	4ºC	∞	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 AMPure XP Beads **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.

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

Quality control and normilisation of sequencing libraries

50

Note

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

Equipment

Bioanalyzer

NAME

Bioanalyzer

TYPE

Agilent

BRAND

G2991AA

SKU

https://www.agilent.com/en/product/bioanalyzer-automated-electrophoresis/bioanalyzer-instrument/2100-bioanalyzer-instrument-228250

LINK

Any bioanalyzer will suffice.

SPECIFICATIONS

Bioanalyzer chips and reagents (DNA 1000) **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

Note

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.