6



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© Commercial automated scRNA-seq workflow

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1 Works for me

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Lesley Shirley

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
AMPure XP Beads	A63882	Beckman Coulter
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
EQUIPMENT		
NAME	CATALOG #	VENDOR
Bioanalyzer	G2991AA	

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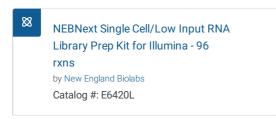
ABSTRACT

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.

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



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

Reagent	Volume (µI)
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.

- 4 Seal dispensed plates, centrifuge immediately \$\ext{log1000 x g, 4°C, 00:01:00}\$ and keep chilled on ice.
- 5 PAUSE POINT Lysis buffer plates can be stored at 8-80 °C prior to cell sorting. Plates can be stored for < 6 months.

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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 **31000** x g, 4°C, 00:01:00 and keep chilled on ice.

- 7 After FACS sorting, seal and centrifuge plates immediately **31000 x g, 4°C, 00:01:00** and keep chilled on ice.
- 8 **PAUSE POINT** Plates of sorted cells can be stored at 8 -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 (µI)
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
70°C	5 minutes
4°C	∞

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 (µI)
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.

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4
10/20/2020

17 Seal and transfer the plate to a thermocycler with the heated lid set to 8 100 °C and run the following program:

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

Purification of amplified cDNA

18



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

- 19 Centrifuged amplified cDNA plate \$\mathbb{3}1000 \text{ 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 (\square 24 μ I SPRI : \square 40 μ I 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.

i protocols.io 5 10/20/2020

- 20.5 Wash the beads with $\frac{180 \, \mu l}{80\%}$ freshly prepared ethanol for 0.00:00:30 remove ethanol and discard.
- 20.6 Repeat ethanol wash.
- 20.7 Allow beads to dry \bigcirc 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



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.

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AccuClear® Ultra High Sensitivity dsDNA Quantitation Kit with DNA Standards

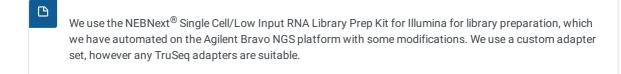
by Biotium

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.

- 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.
- Use the Agilent Bravo with a 384ST multichannel head to add 350 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.
- Dilute any samples >125 $ng/\mu l$ with nuclease free water so they are in the range of 10 125 $ng/\mu l$ 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/\mu l 125 ng/\mu l . This deviates from the standard kit SOP which has a stated range of 0.03 ng/\mu l 25 ng/\mu l .
- 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 8 -20 °C for several weeks prior to library preparation.

Illumina sequencing library preparation



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.
- 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 \odot 00:05:00 at \upday 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 $\Box 13 \mu I$ 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.

- The Bravo will add \blacksquare 3.6 μ I 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

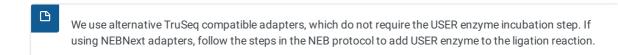
Reagent	Volume (µI)
NEBNext Ultra II Ligation Master Mix	1440
NEBNext Ultra II Ligation Enhancer	48
TruSeq Duplexed Adapter (100 μM)	12
Nuclease-Free Water	108
Total	1608

Mix well by pipetting.

⋈ protocols.io 9 10/20/2020

- 39 The Bravo will add 13.4 μl of ligation mix to each sample and mix by pipetting.
- The plate is incubated on deck at § 20 °C for © 00:15:00 , however this step may also be performed on a thermocycler.

41



42



Allow AMPure XP beads to equilibrate to room temperature (\sim 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 $\frac{180 \, \mu l}{80\%}$ freshly prepared ethanol for 0.00:00:30 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

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 (µI)
KAPA HiFi HotStart ReadyMix	3000
Total	3000

- The Bravo will add $25 \,\mu$ I PCR mix and $25 \,\mu$ I sample into a lyophilised plate of UDIs and mix thoroughly by pipetting. The final concentration of each UDI in the PCR reaction is $2 \,\mu$ M.
- 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	
62°C	15 seconds	8

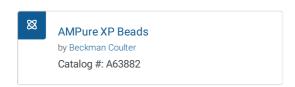
72°C	3 minutes	
72°C	5 minutes	1
4°C	∞	1

47 PAUSE POINT amplified libraries can be stored at 8 -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



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

- 49.1 Manually transfer **□400 µI** of the equivolume pool into a 1.5 ml Eppendorf tube
- 49.2 Add 0.95 X volume of SPRI beads (■380 μI SPRI: ■400 μI 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.
- 49.7 Repeat ethanol wash.

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

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

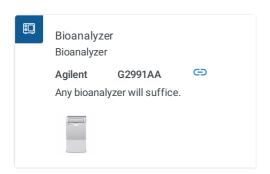
Transfer tube to magnet, allow **© 00:05:00** for the beads to form a pellet.

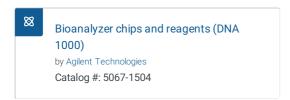
Transfer supernatant into a new tube, taking care not to disturb the pellet.

Quality control and normilisation of sequencing libraries

50







51 Run 11 μl of the library pool in triplicate on a Bioanalyzer using the DNA 1000 kit.

⋈ protocols.io 13 10/20/2020

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.