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# Plate based scRNA-seq Illumina library construction

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1 Works for me

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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

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	

**CATALOG #** 

**VENDOR** 

## Preparation of lysis buffer plates and FACS

NAME

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

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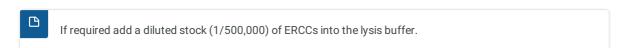


Prepare the cell lysis buffer, which will provide sufficient volume for one 96-well plate 8 On ice

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.



- 4 Seal dispensed plates, centrifuge immediately \$\pi 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.</p>

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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 @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 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

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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** μ**I** of primer annealing mix with **2** μ**I** 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 8 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

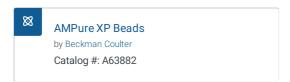
Reagent	Volume (µI)
NEBNext Single Cell cDNA PCR Master Mix	2500
NEBNext Single Cell cDNA Primer Mix	100
Nuclease-Free Water	1400
Total	4000

Mix well by pipetting.

- 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
62°C	15 seconds	depending on
72°C	3 minutes	cell type
72°C	5 minutes	1
4°C	∞	1

Purification of amplified cDNA



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 **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 (  $\square$ 24  $\mu$ I SPRI :  $\square$ 40  $\mu$ 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.
  - 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.

20.10 Transfer the plate to the magnet, allow  $\bigcirc$  **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

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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.
- 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  $\text{ng/}\mu\text{l}$  125  $\text{ng/}\mu\text{l}$ . This deviates from the standard kit SOP which has a stated range of 0.03  $\text{ng/}\mu\text{l}$  25  $\text{ng/}\mu\text{l}$ .
- Taking an average across the plate. Transfer  $\sim 10$  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

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- We use the NEBNext<sup>®</sup> 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.

- 33 Centrifuged plate containing ~ 10 ng of purified cDNA @1000 x g, 00:01:00 Use the Agilent Bravo with a 96 LT multichannel head to perform the following steps: 34 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  $\,\,\,\,\,\,\,\,\,\,\,$  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 (µI)
NEBNext Ultra II FS Reaction Buffer	336
NEBNext Ultra II FS Reaction Enzyme	96
Total	432

Mix well by pipetting.

- 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 (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.

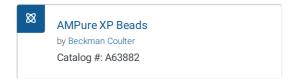
- The Bravo will add  $\Box 13.4 \, \mu l$  of ligation mix to each sample and mix by pipetting.
- The plate is incubated on deck at § 20 °C for © 00:15:00 , however this step may also be performed on a thermocycler.

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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.

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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  $\square 180 \ \mu I$  80% freshly prepared ethanol for  $\bigcirc 00:00:30$  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  $\bigcirc$  **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.

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.

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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  $\mathbf{25} \, \mu \mathbf{l}$  PCR mix and  $\mathbf{25} \, \mu \mathbf{l}$  sample into a lyophilised plate of UDIs and mix thoroughly by pipetting. The final concentration of each UDI in the PCR reaction is  $2 \, \mu M$ .
- 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

In a post-PCR lab, use the Hamilton STAR or Beckman NX-8 to combine  $\Box 5 \mu I$  of each sample per plate to form an equivolume pool of 96 samples.

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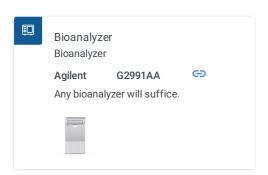
Allow AMPure XP beads to equilibrate to room temperature ( $\sim$ 30 minutes). Ensure solution is homogenous prior to use, mixing gently by inversion.

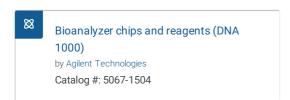
- 49.1 Manually transfer **□400 µI** of the equivolume pool into a 1.5 ml Eppendorf tube
- 49.2 Add 0.95 X volume of SPRI beads ( □380 μI SPRI: □400 μI amplified libraries), mix well by pipetting.
- 49.3 Incubate for © 00:05:00 at & Room temperature
- 49.4 Transfer the tube to a magnet, allow © 00:05:00 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.
- 49.8 Allow beads to dry  $\bigcirc$  **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.

Quality control and normilisation of sequencing libraries

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- 51 Run  $\Box 1 \mu I$  of the library pool in triplicate on a Bioanalyzer using the DNA 1000 kit.
- Taking an average of the readings add nuclease-free water to the library pool to produce a final concentration of 2.8 nM.

Sequencing

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