Cell sorting and library preparation using Smartseq2:

1) Prepare lysis buffer mix (2µl per well)

NOTE: Reagents are prepared on ice, working quickly. ERCC is stored in single-use aliquots at -80 °C , thawed on ice and added last.

Reagent - µl per reaction
H20 - 1.31µl
RNase Inhibitor - 0.05µl
ERCC (1:600 000) - 0.05µl
Triton-X100 (10% solution) - 0.04µl
10mM dNTP - 0.5µl
100uM dT primer - 0.05µl
Total - 2µl

```
1:REAGENT name=H2O, vol=1.31ul
2:REAGENT name=ERCC(1:600000), vol=0.05ul
3:ADD %1 %2
4:REAGENT name=Triton-X100(10%solution), vol=0.05ul
5:ADD %3 %4
6:REAGENT name=10mM dNTP, vol=0.05ul
7:ADD %5 %6
8:REAGENT name=10mM dT primer, vol=0.05ul
9:ADD %7 %8 #final lysis buffer mix
```

Add 2µl **lysis buffer mix** to each well. Cover with appropriate lids. Spin down.

```
10:DISPENSE %9 vol=3ul
#dispense指令为“点板”，即将试剂转移至孔板内
#Add...per well/each well一律是dispense
#dispense和add的区别是，前者加入孔板（可以为空），
后者加入有试剂的容器
#dispense和transfer的区别是，前者一般是从大容器
（如烧杯）转移到小容器（如孔板）,后者一般是大容器之间转移
11:COVER %10 lid=appropriate lids
12:SPIN %11
```

Snap freeze on **dry ice**. Store until use at -80 °C.

```
13:SNAPFREEZE %12 refrigerant=dry ice
14:STORE %13 temp=-80C
```

2) Sort single cells and multiplets
(aggregates of multiple cells)

Sort into wells containing 2 µl **lysis buffer mix.** Multiplets can be discerned from singlets by gating on the basis of FSC-W (Forward scatter - Width) and FSC-H (Forward scatter - Height) (see **Figure 1**).

```
15:REAGENT name=cell
#推测出这里对待测序细胞进行分选，放入裂解液中
16:CELLSORT %15 %14
```

Following sort, immediately seal with appropriate seals (approved for -80°C)and centrifuge at 2000 x g,4°C,5 min, Snap freeze on **dry ice**. Store until use at -80 °C.

```
17:SEAL %16 seal=appropriate seals, temp=-80C
18:SPIN %17 force/rpm=2000xg, temp=4C, time=5min
19:SNAPFREEZE %18 Refrigerant=dry ice
20:STORE %19 temp=-80C
```

3) Primer annealing

NOTE: Perform steps 3 and 4 as quickly as possible in order to prevent RNA degradation

Thaw plate and spin down briefly. Incubate in thermocycler at 72°C for 3 min to anneal primers. Place on ice immediately.

```
21:THAW %20
22:SPIN %21 time=briefly
23:INCUBATE %22 place=thermocycler, method=(72C:3min)
24:PLACE %23 holder=ice
```

4) Reverse transcription

Prepare RT master-mix:
Reagent - Reagent volume
SmartScribe - 0.475µl
RNase Inhibitor - 0.125µl
5x First Strand buffer - 1µl
DTT (100mM) - 0.25µl
Betaine (5M) -\t1µl
MgCl2 (1M) - 0.03µl
TSO (100uM) - 0.05µl
H20 - 0.07µl
Total - 3µl

```
#默认体积最大的第一个取，其他的试剂加入其中
25:REAGENT name=5x First Strand buffer, vol=1ul
26:REAGENT name=SmartScribe, vol=0.475ul
27:ADD %25 %26
28:REAGENT name=RNase Inhibitor, vol=0.125ul
29:ADD %27 %28
30:REAGENT name=DTT(100mM), vol=0.25µl
31:ADD %29 %30%
32:REAGENT name=Betaine(5M), vol=\t1µl
33:ADD %31 %32
34:REAGENT name=MgCl2(1M), vol=0.03µl
35:ADD %33 %34
36:REAGENT name=TSO(100uM), vol=0.05µl
37:ADD %35 %36
38:REAGENT name=H20, vol=0.07µl
39:ADD %37 %38
```

Dispense 3µl per well, cover plate with new film and spin down.

```
40:DISPENSE %39 vol=3ul
41:COVER %40 lid=new film
42:SPIN %41
```

Incubate in thermocycler:
42°C for 90min.
70°C for 5 min.
4°C hold.

```
43:INCUBATE %42 place=thermocycler, method=(42C:90min,
70C:5min, 4C:hold)
```

4) cDNA preamplification:

Prepare cDNA preamplification mix:
Reagents - Reagent volume
H2O - 1.0688µl
Kapa HiFi HotStart ReadyMix (2x) - 6.25µl
IS_PCR primer (10uM) - 0.125µl
Lambda Exonuclease - 0.05625µl
Total - 7.5µl

```
44:REAGENT name=Kapa HiFi HotStart ReadyMix(2x), vol=6.25ul
45:REAGENT name=H2O, vol=1.0688ul
46:ADD %44 %45
47:REAGENT name=IS_PCR primer(10uM), vol=0.125ul
48:ADD %46 %47
49:REAGENT name=Lambda Exonuclease, vol=0.05625µl
50:ADD %48 %49
```

Dispense 7.5 µl per well. Total reaction volume will be 12.5µl. Spin down and cover with new lid.

```
51:DISPENSE %50 vol=7.5ul
52:SPIN %51
53:COVER %52 lid=new lid
```

Incubate in thermocycler with the following program:
1 cycle:
37°C for 30 min.
95°C for 3 min.
Repeat 18-24 cycles (depending on initial RNA concentration):
98°C for 20 sec.
67°C for 15 sec.
72°C for 4 min.
1 final elongation cycle:
72°C 5 min.
4°C min. hold

```
#括号内表示一个cycle，括号后乘号表示重复次数，重复一次可省略。括号内使用若干“temp:time”表示，hold表示保温到取出反应为止
#所有的参数均可以直接在机器上输入
54:INCUBATE %53 place=thermocycler, method=(37C:30min,
95C:3min), (98C:20sec, 67C:15sec, 72C:4min)x(18-24),
(72C:5min, 4C:hold)
```

5) cDNA cleanup

For cDNA cleanup we prepare SPRI-beads in 20% PEG-8000 solution as described in: https://openwetware.org/wiki/SPRI_bead_mix#Ingredients_for_50_mL_2

Using 20% SPRI-bead solution:
i. Add 0.7x the reaction volume of SPRI beads per well. Mix well by pipetting. (i.e 8.75 µl SPRI-bead solution for 12.5 µl reaction volume)
ii. Incubate 5 min. Room temperature
iii. Place on magnetic stand for 3 min.
iv. Carefully remove supernatant
v. Add 40 µl 80% EtOH and incubate 30 sec.
vi. Remove EtOH (without disturbing the beads)
vii. Wash again with EtOH. Make sure to remove as much ethanol as possible.
viii. Allow beads to air-dry for 10 - 15 min.
ix. Remove plate from magnetic stand
x. Elute beads in 15 µl EB or TE buffer. Mix well by pipetting
xii. Incubate 5 min. Room temperature
xiii. Place on magnetic plate for 3 min.
xiv. Optional: Carefully transfer supernatant to a new plate

```
55:REAGENT name=20%SPRI-bead solution, vol=?
56:DISPENSE %55 vol=0.7x the reaction volume
57:INCUBATE %56 method=(Room temperature:5min)
58:PLACE %57 holder=magnetic stand, time=3min
59:REMOVE %58 substance=supernatant
60:REAGENT name=80%EtOH vol=40ul
61:ADD 59% 60%
62:INCUBATE %61 method=(:7min)
63:REMOVE %62 substance=EtOH
64:LOOP begin=60, end=63, time=1
65:DRY %64 dryer=air, time=10-15min
66:PLACE %66 holder=????
67:REAGENT name=EB or TE buffer, vol=15ul
68:ELUTE %66 %67
69:MIX %68 method=pipetting
70:INCUBATE %69 method=(Room temperature:5min)
71:PLACE %70 holder=magnetic stand, time=3min
*72:TRANSFER %71 substance=supernatant, container=new
plate
```

6) cDNA quantification

In order to normalize cDNA amounts, measure concentration of randomly selected wells using Qubit HS dsDNA, adapted to a 96-well plate reader (May also be performed using standard Qubit protocol). Concentrations should be sampled both from wells containing single cells and multiplets in order to normalize separately.

i. Add 97 µl of 1X Qubit HS dsDNA solution to a flat-bottom, black plate
ii. Add 3 µl of cDNA sample
iii. Add Standards (NOTE: We make a 8-step ladder from 0ng/µl --> 10ng/µl Qubit Standard DNA in TE buffer)
iv. Read in plate reader using 485nM excitation/528nm emission
v. Calculate average well cDNA concentration for single cells and multiplets respectively

```
REAGENT name=1X Qubit HS dsDNA solution, vol=97ul
TRANSFER %? Container=flat-bottom, black plate
ADD %? %54
REAGENT name=Standards
ADD %? %?
PLATEREAD %? signal=485nm excitation/528nm emission
CALCULATE method=calculate average well cDNA
concentration for single cells and multiplets
respectively
```

6) (optional) cDNA quality control

Using Agilent HS 5000 DNA chips (or equivalent) to profile cDNA fragment lengths (See example cDNA profile in Fig. 2.).

8) Dilute cDNA

Dilute cDNA in water based on average concentration from Qubit measurements. Target concentration is 150pg/µl per well.

```
DILUTE %? solvent=H2O concentration=150pg/ul
```

9) Tn5 digestion

Tn5 is produced from psfTn5 (Addgene #79107), purified to ~3mg/ml and assembled with Illumina Tn5 adapters (see oligos in Reagents) as in Picelli et al, 2014 (Ref. 1).

Prepare Tn5 master mix:
NOTE: TAPS-PEG Buffer contains PEG, which is viscous. Buffer should equilibrate to room temperature before use to allow proper mixing.
Reagent - Reagent volume
Nuclease free H2O - 1.05µl
TAPS-PEG (50mM TAPS, 25mM MgCl2, 40% PEG-8000) - 0.5µl
psfTn5 (loaded with 50µM MEDS-A/B) - 0.25µl
Total - 1.8µl

```
REAGENT+ADD
```

Dispense 1.8 µl per well in a new plate (tagmentation plate).
Add 0.7 µl cDNA (normalized to 150pg/µl) to tagmentation plate
Cover with new lid, mix well by vortexing plate and spin down.
Incubate in thermocycler at 55°C for 10 min.
Remove (Cover?) immediately and stop the reaction by adding 1µl of 0.1% SDS per well.
Vortex, spin down and incubate 7 min. at Room temperature

```
DISPENSE %? vol=1.8ul
DISPENSE %? vol=0.7ul
COVER %? lid=new lid
MIX %? method=vortexing plate
SPIN %?
INCUBATE %? place=thermocycler, method=(55C:10min)
REMOVE %? substance=cover?
REAGENT name=0.1%SDS vol=?
DISPENSE %? vol=1ul
VORTEX %?
SPIN %?
INCUBATE (:7min)
```

10) cDNA library PCR and barcoding

Make PCR master-mix:
Reagents - Reagent volume:
H2O - 13.25µl
5x buffer - 5µl
dNTPs - 0.75µl
KAPA - 0.5µl
Total - 19.50µl

Dispense 19.5 µl per well to tagmentation plate (containing 3.5 µl sample after step 9)
Add primers/barcodes:
Dispense 2 µl per well (from 384-well index plates, with 3.75µM/each forward/reverse primers; see oligos in materials).
Total reaction volume is 25 µl (3.5 µl sample + 19.5 µl PCR mix and 2 µl primers).

```
DISPENSE %? %? vol=19.5ul
REAGENT name=primers/barcodes vol=?
DISPENSE %? Vol=2ul
```

Cover, vortex and spin down. Incubate in thermocycler as follows:
1 cycle:
72ºC 3 min.
95ºC 30 sec
12 cycles:
95ºC 15 sec
67ºC 30 sec
72ºC 45 sec
1 cycle:
72ºC 4 min
4ºC hold
Following barcoding, pool 2.5µl from each well to an 1.5ml Eppendorf tube.

```
COVER %?
VORTEX %?
SPIN %?
INCUBATE %? place=thermocycler, method=(72C:30min,
95C:30sec), (95C:15sec, 67C:30sec, 72C:45sec)x12,
(72C:4min, 4C:hold)
TRANSFER %? Container=1.5mL Eppendorf tube,
volume=2.5ul per well
```

11) Library cleanup

i. Add 0.9x pooled library volume of SPRI-bead solution. Incubate for 5 min. at room temperature.
ii. Place on magnetic rack for 3 min.
iii. Remove supernatant without disturbing magnetic beads.
iv. Add at least 1 mL 80% EtOH (fresh). Incubate for 30 sec.
v. Remove supernatant.
vi. Repeat EtOH wash.
vii. Air dry for 10 - 15 min.
viii. Re-suspend beads thoroughly in 100 µl EB or TE buffer.
ix. Place eppendorf on magnetic rack for 3 min.
x. Transfer supernatant to new 1.5ml Eppendorf tube.
xi. Repeat cleanup (from step 1-7) and elute in 30µl EB or TE buffer.
xii. (Optional) Place eppendorf on magnetic rack for 3 min. and transfer supernatant to new tube.

```
-
REAGENT name=SPRI-bead solution, vol=0.9x pooled
library volume
ADD %? %?
PLACE %? holder=magnetic rack, time=3min
REMOVE %? substance=supernatant
REAGENT name=80%EtOH(fresh) vol=at least 1mL
ADD %? %?
INCUBATE method=(:7min)
REMOVE %? substance=EtOH
LOOP begin=?, end=?, time=1
DRY %? dryer=air, time=10-15min
PLACE %? Holder=magnetic rack
REAGENT name=EB or TE, vol=100ul
SUSPEND %? %?
PLACE %? holder=magnetic rack, time=3min
TRANSFER %? Substance=supernatant, container=new 1.5mL
Eppendorf tube
-
LOOP begin=?, end=?, time=1
REAGENT name=EB or TE, vol=30ul
ELUTE %? %?
*PLACE %? holder=magnetic rack, time=3min
*TRANSFER %? Substance=supernatant, container=new 1.5mL
Eppendorf tube
```