Totalscript RNA-seq Library Prep – from pools of 100 cells
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Cell collection and lysis:

96 well, U bottom plate

Ambion Single Cell Lysis Kit – good for 100 samples (basically one 96 well plate)
-although protocol says good for 1-10 cells, I've confirmed that I can use it for pools up to 100
http://www.lifetechnologies.com/order/catalog/product/4458235

Follow the directions for use of the lysis kit:
http://tools.lifetechnologies.com/content/sfs/manuals/cms_088421.pdf

1. Add 1µL DNase I to 9µL Single Cell Lysis Solution. Aliquot 10µL of lysis buffer into well that you will use for cell collection.

2. FACS-sort pools of 100 cells into wells. (See Isolation Protocols for FACS of specific cell populations). Incubate samples at RT for 5 min. No mixing is required.

3. Add 1 µL Single Cell Stop Solution, incubate at room temperature for 2 minutes, then place the samples on ice. No mixing is required.

4. Continue immediately with Totalscript library prep or seal plate thoroughly (or transfer samples to strip tubes), and store at -80C.

Library Prep:

Totalscript RNA-seq kit – Epicentre cat# TSRNA12924
http://www.epibio.com/applications/rna-sequencing/rna-library-prep/totalscript-rna-seq-kit

Anneal primers:

15.5 µL of Total RNA/cell sample/water	65°C for 2 min
2.5 µL Totalscript Optimized Buffer	Hold at 4°C
1 µL random hexamer primer	
= 18 µL total	

1st strand synthesis:

To 18 µL reaction, add:	25°C for 5 min
2.5 µL DTT	42°C for 25 min
0.5 µL dNTPs	70°C for 15 min
1 µL RiboGuard RNase Inhibitor	Hold at 4°C
1 µL Actinomycin D (250 ng/µL)	
1 µL EpiScript RT	
= 24 µL total	

2nd strand synthesis:

To: 24 μL reaction, add and mix on ice:
- 1 μL DTT
- 25 μL 2nd Strand Master Mix
- = 50 μL total

16°C for 1 hr
80°C for 15 min
Hold at 4°C

Safe stopping point. Reactions can be stored at -20°C.

Tagmentation:

(Note: One can use 25-39 μL of 2nd strand synthesis rxn for library prep. Adjust volume of tagmentation with water accordingly. For 100 cell samples, 39 μL were used.

In a fresh tube, add:
- 39 μL of sample, add:
- 14 μL water
- 10 μL Totalscript Tagment Buffer
- 1 μL Totalscript enzyme (Tn)

55°C for 5 min
Hold at 4°C

Add 5 μL of Totalscript Stop Solution, and incubate at RT for 5 min

Purify with AMPure XP Beads (at RT)

Add 65 μL well mixed beads
Incubate 5 min
Place sample on magnet 5 min
Remove SN, discard
Wash with 250 μL of 80% EtOH for 30 sec, discard
Wash again with 250 μL of 80% EtOH for 30 sec, discard
Air dry pellet 5 min
Resuspend pellet in 15 μL Elution Buffer (10 mM Tris-HCl, pH 8)
Incubate 2 min at RT
Place sample on magnet 5 min
Transfer 14 μL of gap-filled DNA to fresh tube

Oligo Replacement:

14 μL of sample, add:
- 4 μL Gap-fill Buffer
- 1 μL Index

45°C for 1 min
37°C for 30 min

Add: _____ 1 μL Gap-fill enzyme

37°C for 30 min
Hold at 4°C

Purify with AMPure XP Beads (at RT)

Add 25 μL well mixed beads
Incubate 5 min
Place sample on magnet 5 min
Remove SN, discard
Wash with 250 μL of 80% EtOH for 30 sec, discard
Wash again with 250 μL of 80% EtOH for 30 sec, discard
Air dry pellet 5 min
Resuspend pellet in 135 μL Elution Buffer (10 mM Tris-HCl, pH 8)
Incubate 2 min at RT
Place sample on magnet 5 min
Transfer 12 μL of gap-filled DNA to fresh tube

Safe stopping point. Gap-filled DNA can be stored at -20°C.

PCR Amplification:

To: 12 μL of sample, add:
0.5 μL Totalscript PCR Cocktail
12.5 μL 2X NEB Phusion HF PCR Master Mix
= 25 μL total

Run TOTALSCR program in the thermocycler 17 #cycles:
98°C for 10 sec
60°C for 30 sec
72°C for 1 min
Hold at 4°C

Purify with AMPure XP Beads (at RT)
Add ~0.8-09X AMPure XP beads (0.85X of 25 μL = 21.25 μL) well mixed beads
Incubate 5 min
Place sample on magnet 5 min
Remove SN, discard
Wash with 250 μL of 80% EtOH for 30 sec, discard
Wash again with 250 μL of 80% EtOH for 30 sec, discard
Air dry pellet 5 min
Resuspend pellet in 21 μL Elution Buffer (10 mM Tris-HCl, pH 8)
Incubate 2 min at RT
Place sample on magnet 5 min
Transfer 20 μL* of Totalscript library to fresh tube

Check size of library using the Agilent Bioanalyzer and quantitate using qPCR.