Protostelid Single Cell Protocol for mRNA reverse transcription

SSIII (Smart Seq version for SuperScript III)

1. Prepare reverse transcriptase master mix before beginning from the reagents listed below (Volumes are per reaction to be preformed).

Reagent(concentration) volume/reaction final concentration

SuperScript IV Reverse Transcriptase (200U/ul)	0.50ul	100U
Superase-IN (20U/ul)	0.50ul	10U
Superscript IV First-Strand Buffer (5X)	2.00ul	1X
DTT (100mM)	0.50ul	5mM
Betaine (5M)	2.00ul	1M
$MgCl_2$ (1M)	0.06ul	6mM
TSO (100uM)	0.10ul	1uM
Nuclease Free Water	0.04ul	_

2 . Make cell lysis master mix using the following reagents and volumes per reaction(*****If you are planning to transfer cells by mouth pipetting, leave water out of master mix and try to transfer cells in the corresponding volume (~0.30ul) of nuclease free water)**:

Superase-IN	0.20ul
0.2%-TritonX-100	1.8ul

Nuclease Free H_2O	0.30ul

3. Add 2.3ul of cell lysis master mix to each reaction tube.
4. Pick cells (For Protostelids: use a platinum needle. Drop needle directly into the tube containing lysis mix. Keep Lysis buffer on ice unless placing cells in tube. ******Watch that needle stays submerged in lysis buffer after closing tube. Quick spin down if it does not******)
5. After all cells are in lysis buffer, preform 6 rounds of freeze thaw using isopropanol from -80C with dry ice added and room temperature (~25C) water to insure cell lysis.
6. Add 1ul of (10uM) oligo-dT primer and 1ul of (10mM) dNTP mix to each reaction. (Alternatively a master mix can be made beforehand with 1ul of each reagent for the number of reactions that you plan to do and 2ul of this master mix can be added to each reaction)
7. Gently vortex each sample to mix and place immediately back on ice.
8. Incubate samples at 72C for 3min. Place immediately on ice.
9. Spin each tube down to collect liquid in the bottom and place immediately back on ice.
10. Add 5.7ul of reverse transcriptase master mix made in Step 1 to each sample.
11. Spin down samples to collect liquid at the bottom of each tube.
12. Place in thermocycler and run the following protocol using a heated lid (105C).

1 Cycle	50C	90min
10 Cycles	55C 50C	2min 2min
1 Cycle	80C	15min
Hold	4C	Forever

13. Prepare a PCR master mix using the following reagents (Volumes are per reaction to be preformed).

KAPA HiFi HotStart ReadyMIx (2X)	12.50ul
ISPCR primers (10uM)	0.25ul
Nuclease-free water	2.25ul

14. Add 15ul of the above PCR master mix to each sample for a total volume of 25ul.
15. Quickly spin down each tube, and then place samples in the thermocycler on the following setting:

1 Cycle	98C	3min
21 Cycles	98C 67C 72C	20s 15s 6min
1 Cycle	72C	5min

Hold	4C	Forever

******Bring Ampure XP beads to room temperature and vortex well before proceeding with purification steps*******

******Make up 80% EtOH to be used in the purification washes minutes before use*******

16. Add 25ul of Ampure XP beads to each sample for a 1:1 ratio. *****Mix solution well by pipetting up and down at least 10 times*****
17. Incubate for 8min at room temp.
18. Place samples on the magnet stand, and let stand for 5min.
19. After 5min carefully remove and discard liquid without disturbing the beads.
20. Wash beads with 200ul freshly prepared 80% EtOH. Let stand for 30s.
21. Remove and discard EtOH.
22. Repeat steps 20 and 21 once more.
23. Remove ALL EtOH from samples and let dry and room temperature with the lids open for 5min. *****It is critical that ALL ethanol be removed before moving to the next step.*****
24. Add 17.5ul of EB solution to each sample. *****Add EB in a way it runs over and hydrates beads on its way to the bottom of the tube.*****
25. Remove from magnet stand and homogenize beads and EB by pipetting up a down several times.
26. Incubate samples off of the magnet stand for 2min.

27. Place samples on the low-volume side of the magnet stand and incubate for 2min.
28. Transfer 15ul of the supernatant to a new well-labled 1.5ml tube without disturbing the beads. (2.5ul are left behind to help prevent bead carry-over).
29. Use 2ul of the 15ul to quantify using Qubit.
30. Use Qubit assays for template in another ISPCR reaction using GoTaq to be run on a 1.8% TBE gel for library visualization.

Proceed either to Covaris/NEB Next Ultra DNA or <u>Nextera XT</u>

<u>Nextera XT</u>

31. Use Qubit readings from step 28 to make 8ul of a 0.200ng/ul dilution of each sample.
32. Use 2ul of this dilution for quantification using Qubit.
33. If samples are all at ~0.200ng/ul proceed to next step.
34. Remove the ATM (Amplicon Tagment Mix), TD (Tagment DNA Buffer), and input DNA from -20C storage and thaw on ice.
35. Bring NT (Neutralize Tagment Buffer) to room temp. Visually inspect NT to ensure there is no precipitate. If there is precipitate, vortex until all particulates are resuspended.
36. Mix all reagents by inverting the tubes 3-5 times.
37. Add 10ul of TD Buffer to each PCR tube.
38. Add 5ul of input DNA at 0.200ng/ul (1ng total) to each tube.

39. Add 5ul of ATM. Mix by pipetting.
40. Give each sample a quick spin down.
41. 55C for 5min in thermocycler. Hold at 10C.
42. Proceed IMMEDIATELY to Neutralize NTA.
43. Add 5ul of NT Buffer to each tube. Mix by pipetting.
44. Quick spin down.
45. Incubate at room temp. for 5min.
46. Thaw NPM (Nextera PCR Master Mix) and index primers (about 20min on bench). ***Mix NPM by inverting tube. Spin index primers down before opening.***
47. Add 15ul of NPM to each PCR tube.
48. Add 5ul of index 2 primers (S-series) ***Do not recap index primers with old cap. Use new caps provided by Illumina***
49. Add 5ul of index 1 primers (N-series).
50. Mix by pipetting.
51. Quick Spin down.
52. Place samples in the thermocycler on the following setting:

1 Cycle	72C	3min
1 Cycle	95C	30s
12 Cycles	95C 55C 72C	10s 30s 30s

1 Cycle	72C	5min
Hold	10C	Forever

*****Bring AMPure XP beads to room temperature and vortex well before proceeding to the following steps******

*****Prepare 80% EtOH to be used in the following washes minutes before use*****

53. Give samples a quick spin down to collect all liquid at the bottom of the tube.
54. Add 25ul of AMPure XP beads to each sample (.50:1 ratio)
55. Mix samples well by pipetting up and down ~10 times.
56. Incubate at room temperature for 5min.
57. Place samples on the high-volume side of the magnet stand for 2min.
58. Discard the supernatant
59. Add 200ul freshly prepared 80% EtOH to each sample and let stand 30s.
60. Remove and discard EtOH.
61. Repeat steps 59 and 60 once more.*****It is critical to remove ALL EtOH after the second wash******
62. Air-dry for 8min with caps open.
63. Add 23ul of RSB (Resuspension Buffer) *****Add RSB so that it hydrates the beads on its way to the bottom of the tube.******
64. Homogenize solution by pipetting up and down at least 10 times.
65. Incubate off the magnet stand at room temp. for 2min.
66. Place back on the magnet stand for 2min or until solution is clear.

67. Transfer 21ul of supernatant to a new well-labled PCR tube.

HiSeq (PE 100-125) runs STOP HERE

For MiSeq v3 600 (PE 300) runs continue to step 68

68. Add 16.25ul of AMPure XP beads. Mix well.

69. Remove and discard supernatant.

70. Add 200ul fresh 80% EtOH to each sample.

71. Incubate 30s.

72. Remove and Discard EtOH.

73. Repeat steps 70 - 72 once more.

74. Make sure all EtOH has been removed.

75. Allow beads to dry with caps open for 5min.

76. Add 14ul of elution buffer.

77. Homogenize solution.

78. Incubate off mag stand for 5min

79. Place in mag stand and incubate for 5min or until solution is clear.

80. Remove 12ul of supernatant and place into a well-labled 1.5mL tube.

81. Use 1ul of your 12ul for quantification with Qubit.

82. Use 1ul of your 12ul for qPCR.

83. Keep 10ul to use for sequencing.