Environmental RNA Barcoding through reverse transcription

SSIV (Smart Seq version for SuperScript IV)

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	_

3. Mix 2.3 ul of eRNA extracted with Zymobiomics RNA Miniprep kit with 1ul of (10uM) taged RiBbR primer and 1ul of (10mM) dNTP mix to each reaction.
4. Gently vortex each sample to mix and place immediately back on ice.
5. Incubate samples at 72C for 3min. Place immediately on ice for at least 1 min.
6. Spin each tube down to collect liquid in the bottom and place immediately back on ice.
7. Add 5.7ul of reverse transcriptase master mix made in Step 1 to each sample.

8. Spin down samples to collect liquid at the bottom of each tube.
9. Place in thermocycler and run the following protocol (eRNA-SS2) using a heated lid (105C).

1 Cycle	55C	10min
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
32 Cycles	98C 67C 72C	20s 15s 3min

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>