**UTRICLE DISSOCIATION/FACS ISOLATION AND RNA-SEQUENCING FOR ADULT UTRICLE HAIR CELLS**

**Utricle dissociation**

1. Before starting the experiment, pre-warm the DNase-containing Papain (To make this solution, please follow the instruction for the Papain Dissociation system from Worthington Biochemical Corporation (Catalog Number LK003150) in 37-degree incubation chamber. Prepare CMF-PBS (calcium and magnesium-free PBS) with 2% FBS.
2. If using cultured utricles, remove the utricle-containing culture membrane to a slide. Use a transfer pipette with PBS to apply water pressure and then detach the utricle from the culture membrane. Then, use the transfer pipette to transfer the utricle to 1.5 mL Eppendorf tube. Note, pre-coat the transfer pipette with FBS or any type of serum to prevent utricle attach to the pipette during transfer.
3. After collecting all the utricles, wash them with CMF-PBS twice. Then, wash the utricles with 100 µl of papain. Depending on the quantity of the utricle, add 200-800 µl of papain (For example, add 500 µl to approximately 20 utricle). Rotate the Eppendorf tube for 10 times then, incubate the tube at 37°C with 200-500 rpm shaking for 50 minutes.
4. Put the reagents and the tubes on ice. Remove the papain solution and rinse the utricle with ice-cold CMF-PBS with 2% FBS twice. A brief centrifuge can help settle down the utricles.
5. Add 400 µl of the ice-cold CMF-PBS with 2% FBS into the Eppendorf tube. Place the Eppendorf on ice. Turn the 1000 µl pipette to 300 µl, gently triturate the utricle for as many times as needed, typically around 100-200 times. For the first couple of times, make sure to take a couple of microliters and check under the microscope for complete dissociation.

**FACS isolation of specific cell group**

1. Filter the cells through a 5 mL Polystyrene Round-Bottom Tube with Cell-strainer Cap (75 mm style) on ice
2. Add DAPI (with a final concentration of 0.1 µg/mL) to the cell-containing-tube to sort out dying cells
3. Place the cell containing tube into the BD FACS Aria cell sorting flow cytometer with a 100-µm nozzle. Remember to always bring a negative control (dissociated utricle without fluorescence). Try to sort the cells within one hour of dissociation.

**RNA isolation and cDNA amplification**

(Make sure everything is sterile and RNase/DNase free)

1. Before cell sorting, prepare the 10X reaction buffer as suggested in the user manual for SMART-Seq® v4 Ultra® Low Input RNA Kit.
2. Next, added 4 µl of nuclease-free water and 1 µl of 10X reaction buffer

into a 0.2-ml RNase-free PCR.

1. Sort approximately 2000 live cells directly into the reaction buffer mix. Measure the volume with a pipette and then bring the volume to 10.5 µl with nuclease-free water.
2. Pipette to mix the sample. Incubate at room temperature for 5 minutes. Thaw required solutions on ice.
3. (Most of the following steps are the same as the user manual) Place the samples on ice and add 2 μl of 3′ SMART-Seq CDS Primer II A (12 μM). Mix well by gently vortexing and then spin the tube(s) briefly to collect the contents at the bottom of the tube.
4. Incubate the tube(s) at 72°C in a preheated, hot-lid thermal cycler for 3 minutes
5. Prepare enough Master Mix for all the reactions, plus 10% of the total reaction mix volume, by combining the following reagents in the order shown at room temperature:

4 μl 5X Ultra Low First-Strand Buffer

1 μl SMART-Seq v4 Oligonucleotide (48 µM)

0.5 μl RNase Inhibitor (40 U/µl)

* 1. μl Total volume added per reaction
1. Immediately after the 3 min incubation at 72°C, place the samples on ice for 2 minutes
2. Preheat the thermal cycler to 42°C.
3. Add 2 µl per reaction, plus 10%, of the SMARTScribe Reverse Transcriptase to the Master Mix. Add the reverse transcriptase to the Master Mix just prior to use, making sure to gently mix the reverse transcriptase tube without vortexing before adding it
4. Add 7.5 μl of the Master Mix to each reaction tube. Mix the contents of the tubes by gently pipetting and spin them briefly to collect the contents at the bottom of the tubes.
5. Place the tubes in a thermal cycler with a heated lid, preheated to 42°C. Run the following program:

42°C 90 min

70°C 10 min

4°C forever

1. Thaw SeqAmp PCR Buffer and PCR Primer II A on ice. Do not thaw SeqAmp DNA Polymerase. Gently vortex each reagent tube to mix and spin down briefly. Store on ice. Prepare enough PCR Master Mix for all the reactions, plus 10% of the total reaction mix volume. Combine the following reagents in the order shown:

PCR Master Mix:

25 µl SeqAmp PCR Buffer

1 µl PCR Primer II A

3 µl Nuclease-Free Water

1 µl SeqAmp DNA Polymerase

30 µl Total volume per reaction

1. Add 30 μl of PCR Master Mix to each tube containing 20 µl of first-strand cDNA product from Section V.A. Mix well by gently vortexing, and briefly spin to collect the contents at the bottom of the tube
2. Place the tubes in a preheated thermal cycler with a heated lid and run the following program:

95°C 1 min

*8-12 cycles*\*:

98°C 10 sec

65°C 30 sec

68°C 3 min

72°C 10 min

4°C forever

**Purification of amplified cDNA**

1. For purification, add 1 μl of 10X Lysis Buffer (SMART-Seq® v4 Ultra® Low Input RNA Kit) to each PCR product.
2. Add 50 μl of AMPure XP beads to each sample.
3. Mix thoroughly by vortexing for 3–5 sec or pipetting the entire volume up and down at least 10 times.
4. Incubate the beads-cDNA mixture at room temperature for 8 min.
5. Briefly spin the samples to collect the liquid from the side of the tubes or plate wells. Place the samples on the any magnetic separation device for ~10 minutes or longer
6. While the samples are on the magnetic separation device, remove and discard the supernatant. Take care not to disturb the beads.
7. Add 200 μl of freshly made 80% ethanol to each sample without disturbing the beads. Incubate for 30 sec.
8. Carefully remove and discard the supernatant, taking care not to disturb the beads. (The cDNA should remain bound to the beads during the washing process)
9. Repeat the ethanol wash once more.
10. Briefly centrifuge the samples to collect the liquid from the side of the tubes or plate wells.
11. Place the samples on the magnetic separation device for 30 sec, then remove any residual ethanol with a pipette.
12. Incubate the samples at room temperature for ~15 min, until the pellet is no longer shiny, but before cracks appear.
13. Once the beads are dry, remove the samples from the magnetic separation device and add 17 μl of Elution Buffer to cover the bead pellet. Mix thoroughly by pipetting or gently vortexing to resuspend the beads.
14. Incubate at room temperature for 10 min to rehydrate.
15. Briefly spin the samples to collect the liquid from the side of the tubes or plate wells. Place the samples back on the magnetic separation device for 2 minutes, until the solution is completely clear.
16. Transfer clear supernatant (~15 μl) containing purified cDNA from each tube/well to a new tube/plate. Proceed to validation immediately or store at –20°C
17. To determine cDNA quality and quantity, aliquot 1 μl of the amplified cDNA for validation using the Agilent 2100 Bioanalyzer and Agilent’s High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626).

**Library preparation for sequencing**

1. Use Nextera XT DNA Library Prep Kit to prepare DNA library for sequencing.
2. Dilute the amplified cDNA so that it is 0.2 ng per µl.
3. Add 10 µl Tagment DNA Buffer (TD) to 5 µl sample (0.2 ng/ul) in the PCR tube.
4. Next, Add 5 µl Amplicon Tagment Mix (ATM) to the mix. Pipette to mix.
5. Centrifuge at 280 x g at 20 °C for 1 minute.
6. Place on the thermal cycler and run the following program:
	* + - 1. 55°C 5 min
				2. 10°C hold
7. Immediately after the sample reaches 10 °C, add 5 µl Neutralize Tagment Buffer (NT) to each sample. Pipette to mix.
8. Centrifuge at 280 x g at 20 °C for 1 minute.
9. Incubate at room temperature (RT) for 5 minutes.
10. Thaw as many Index 1(i7) adapter and Index 2 (i5) adapter as needed depending on the number of samples.
11. Add 5 µl of Index 1 adapter and 5 µl Index 2 in each sample.
12. Add 15 µl Nextera PCR Master Mix (NPM) to the sample. Pipette to mix.
13. Centrifuge at 280 x g at 20 °C for 1 minute.
14. Place on the thermal cycler and run the following program:
	* + - 1. 72°C 3 min
				2. 95°C 30 sec
			1. *12 cycles*:

95°C 10 sec

55°C 30 sec

72°C 3 min

* + - * 1. 72°C 5 min
				2. 10°C forever
1. Clean up the library with 40 µl AMPure XP Beads (0.8x). Follow the purification protocol from Step 3 to Step 12.
2. Add 52.5 µl resuspension buffer (RSB) to each sample. Mix thoroughly by pipetting or gently vortexing to resuspend the beads.
3. Incubate at room temperature for 10 min to rehydrate.
4. Briefly spin the samples to collect the liquid from the side of the tubes or plate wells. Place the samples back on the magnetic separation device for 2 minutes, until the solution is completely clear.
5. Transfer 50 µl supernatant containing amplified library from each tube to a new tube. Proceed to validation immediately or store at –20°C
6. For determine cDNA quality and quantity, aliquot 1 μl of the amplified cDNA for validation using the Agilent 2100 Bioanalyzer.