**Conditioned Media Protocol**

**Step 1. L-WRN Preparation**

1. Thaw a cryotube of L-WRN or L-WR cells in the 37 °C water bath.
2. Transfer the cell solution to prewarmed medium (DMEM+10%FBS+PSG) immediately after the ice disappears.
3. Transfer the medium into a 175-cm2 cell culture flask.
4. Incubate the flask in the cell culture incubator.
5. Grow the cells until they become confluent (2 or 3 d).
6. Passage the cells by washing the cells with 10ml PBS and add 2.5ml trypsin-EDTA. (Tap the flask several times to coat the plate surface with the trypsin-containing solution.)
7. Expand the cells into a number of 175-cm2 cell culture flasks that needed. Starting from the 2nd passage, add G418 (500 μg ml−1) and hygromycin (500 μg ml−1) every time.

**Step 2. Media Collection**

1. When the cells become overconfluent (until some cell aggregates may come off), wash the cells with 10ml culture media (Advanced DMEM/F-12 with 20%FBS and PSG) and aspirate.
2. Add 25ml of culture media to each flask.
3. Incubate the flasks for 24hrs.
4. Collect the media from the flask into 50ml centrifuge tubes and add new culture media to the flask. Put the flasks back to the incubator.
5. Centrifuge the tubes at 2,000g for 5 minutes at room temperature and carefully collect the supernatant into a 500-ml bottle. (Try to avoid touching the cell pellet at the bottom of the tube) and the store the bottle at 4°C.
6. Every 24hr, collect the 2nd, 3rd and 4th conditioned media in the same bottle. (If 5 flasks of media are collected, then the final volume will be 500ml)
7. Add 250ml collected conditioned media to a new 500-ml bottle and add an equal volume of culture media (Advanced DMEM/F-12 with 20%FBS) to the bottle. (1:1 diluted). Mix it well and aliquot the medium into 50-ml centrifuge tubes. Store the medium at -80°C.
8. Collect the 5th to 8th and the 9th to 12th media by repeating step6- step7.

Note: Whether filter the conditioned medium and which kind of filter to use depend on the organoids you use. For MCR (Mist1-cre, *CDH1-/-*, RHOAL47V), 0.45 um filter should be used.