# Thawing cryopreserved human organoids

Read this protocol in its entirety before proceeding

### Thawing a vial of organoids

1. Place a 6-well culture plate in a 37°C incubator to warm for at least 1 hour.

2. Prepare a 10 mM working solution of ROCK Inhibitor Y-27632 (ROCK-I; ATCC® ACS-3030™) by adding 3 mL sterile water to a 10 mg vial of the ROCK-I.

3. Warm the appropriate growth medium at room temperature. Typically, you will need ~20 mL per vial to be thawed.

   Refer to model-specific information on the ATCC website or product sheet for the growth medium formulation.

4. Thaw the appropriate extracellular matrix on ice or at 2-8°C. Large volumes of extracellular matrix (ECM; multiple mL) may take 12-24 hours to thaw. Avoid multiple freeze/thaw cycles of the ECM.

5. Refer to lot-specific information on the COA for the number of cells contained within the cryovial. If specified in the model-specific product sheet, dilute the ECM to its 1X concentration. Once ECM is diluted keep on ice. Approximately 100 µL of 1X ECM is required per ~$5x10^5$ viable cells contained within the vial.

   Cryopreserved organoids from ATCC typically contain 1-$2x10^6$ viable cells per vial which and thus requires 200-400 µL of diluted ECM when seeding.

6. Transfer the cryovial from $LN_2$ storage and immediately place in a 37°C water bath and thaw rapidly. Be careful not to submerge the neck of vial. This process should take less than 2 minutes.

7. Decontaminate the vial with 70% ethanol and aseptically transfer to a biosafety cabinet (BSC).

8. Transfer the contents of the vial drop-wise to a 15 mL conical tube containing 10 mL of complete growth medium.

9. Centrifuge the conical tube at 300 x g for 5 minutes.

10. Carefully aspirate the supernatant without disturbing the pellet while removing as much liquid as possible.

11. Remove the pre-warmed 6-well culture plate from the incubator and place in the BSC.

12. Re-suspend the pellet in 1X ice-cold ECM by pipetting up and down 10-20X with a P200 pipettor.

13. Using a P200 pipette, aspirate 100 µL the ECM/cell suspension and dispense as small droplets in the surface of a single well in the 6-well plate. You should end up with approximately 8-12 droplets in the well.

14. Repeat for all the remaining ECM/cell suspension, dispensing 100 µL per well as small droplets.

15. After all of the suspension has been seeded, place the lid on the plate and invert (turn upside down). Place the plate, still inverted, in the cell culture incubator for 15-20 minutes to solidify the ECM.

16. While the ECM is solidifying, supplement the remaining 10 mL of pre-warmed complete growth medium with ROCKi to a final concentration of 10 µM. For example, add 10 µL of a 10 mM solution of ROCKi to 10 mL of growth medium.

17. After the ECM has solidified, return the plate to a BSC and flip right side up.

18. Add 2 mL per well of pre-warmed complete culture media containing 10 µM of ROCKi. Dispense the media along the wall of the well, not directly on the domes.

19. Return the 6-well place to the cell culture incubator.

For a more detailed protocol on thawing cryopreserved organoids, please see the following publication:

Clinton J, McWilliams-Koeppen P. Initiation, Expansion, and Cryopreservation of Human Primary Tissue-Derived Normal and Diseased Organoids in Embedded Three-Dimensional Culture. *Curr Protoc Cell Biol* (2018): e66. PubMed: 30265443 https://currentprotocols.onlinelibrary.wiley.com/doi/10.1002/cpcb.66