

1. Thaw one vial of cells into a single 10-cm tissue culture dish with 9 ml of appropriate growth medium.
  - *Locations in liquid nitrogen storage:*
    - 293T-Noggin: Rack 9A
      - *Obtained from Joan Brugge with permission from Hans Clevers*
      - *Handle and freeze like 293T cells (<https://www.atcc.org/products/crl-11268>)*
    - 293T-Rspo: Rack 9C
      - *Purchased from R&D Systems (#3710-001-01)*
      - *Handle and freeze like 293T cells (<https://www.atcc.org/products/crl-11268>)*
    - L-Wnt-3a: Rack 9A
      - *Obtained from Joan Brugge with permission from Hans Clevers*
      - *Handle and freeze like L cells (<https://www.atcc.org/products/crl-2648>)*
  - *Noggin cells will sometimes recover more slowly out of thaw than the others, so consider thawing these cells 1-2 days before the others to synchronize future steps.*
2. After 1-2 days, refeed each dish with 10 ml of the corresponding selection medium.
  - *Wash with 5 ml PBS before refeeding.*
  - *Noggin cells will sometimes grow more slowly out of thaw than the others, so wait closer to 48 hours before selection, as opposed to roughly 24 hours before selection for the others.*
  - *Selection medium should be made fresh before each use.*
3. Culture cells to ~85% confluency, refeeding with selection medium every 2–3 days.
  - *Typically, the cells reach ~85% confluency within 4 days after thawing, so they often do not require additional refeeds beyond Step 2.*
4. Expand the cells into three 10-cm dishes (1:3 split) with the appropriate selection medium, at a ratio informed by the amount of conditioned medium to be generated.
  - *Each 10-cm dish will be used to seed one 175 cm<sup>2</sup> flask, which yields 50 ml of conditioned medium.*
  - *150 ml of conditioned medium is adequate for organoid experimentation over the medium's lifetime. If more will be needed, adjust this step accordingly.*
5. Culture cells to ~85% confluency, refeeding with selection medium every 2–3 days.
  - *Cells often reach this confluency within 3 days after splitting, so a refeed is typically not necessary.*
6. Split the three 10-cm dishes into three 175 cm<sup>2</sup> flasks with 50 ml of the appropriate growth medium.
  - *These flasks are stored in the tissue culture room next to the 10-cm dishes.*
7. Culture cells to ~85% confluency, refeeding with growth medium every 2–3 days.
  - *Cells often reach this confluency within 3 days after splitting, so a refeed is typically not necessary.*
8. Refeed the flasks with 50 ml of the corresponding harvest medium.
  - *Do not perform a PBS wash when changing to harvest medium.*
9. Leave cultures undisturbed for 7 days.
10. Decant the conditioned medium from each flask into a 50 ml conical tube. Centrifuge the tubes at 300 g for 5 minutes.
11. Filter the supernatant through a sterile 0.2 µm filter unit (ThermoFisher 595-3320).
12. Store the conditioned medium in aliquots at –20°C (Noggin and R-spondin) or 4°C (L-Wnt-3a) for ~4 months.
  - *Lower organoid establishment rates and/or slower organoid growth than usual may be an indicator that the conditioned medium has gone bad and should be replaced.*

**Media recipes** (reagent stock concentrations indicated in parentheses)

- Noggin growth medium (for 500 ml total volume):
  - 500 ml DMEM, high glucose, pyruvate (Gibco 11995)
  - 5 ml penicillin-streptomycin
  - 50 ml FBS
  - 5 ml GlutaMax
- Noggin selection medium (for 50 ml total volume):
  - 50 ml Noggin growth medium
  - 833  $\mu$ l geneticin (30 mg/ml)
- Noggin harvest medium (for 500 ml total volume):
  - 500 ml adDMEM/F12
  - 5 ml penicillin-streptomycin
  - 5 ml HEPES
  - 5 ml GlutaMax
  
- R-spondin growth medium (for 500 ml total volume):
  - 500 ml DMEM, high glucose (Gibco 11965)
  - 5 ml penicillin-streptomycin
  - 50 ml FBS
  - 5 ml GlutaMax
- R-spondin selection medium (for 500 ml total volume):
  - 50 ml R-spondin growth medium
  - 150  $\mu$ l zeocin (100 mg/ml)
- R-spondin harvest medium (for 500 ml total volume):
  - 500 ml adDMEM/F12
  - 5 ml penicillin-streptomycin
  - 5 ml HEPES
  - 5 ml GlutaMax
  
- Wnt growth medium (for 500 ml total volume):
  - 500 ml DMEM , high glucose, GlutaMax, pyruvate (Gibco 10569)
  - 5 ml penicillin-streptomycin
  - 50 ml FBS
- Wnt selection medium (for 500 ml total volume):
  - 50 ml Wnt growth medium
  - 62.5  $\mu$ l zeocin (100 mg/ml)
- Wnt harvest medium (for 500 ml total volume):
  - Same as growth medium