

When referring to this protocol, please cite: Przanowska RK*, Labban N*, Przanowski P, Hawes RB, Atkins KA, Showalter SL†, Janes KA†. (2024) Patient-derived response estimates from zero-passage organoids of luminal breast cancer. *Breast Cancer Res*, 26, 192.

Before starting:

- See “Clinical communication and collection of information for surgical cases” protocol for coordinating sample pickup and transfer
1. Use a scalpel to scrape tumor cells from the resected tissue and smear on six uncharged glass slides (Fisher Scientific 12550433). Place up to two slides (back-to-back) in a 50-ml conical tube containing 35 ml of addMEM/F12+++ with 70 µl of Primocin (final concentration = 100 µg/ml) prechilled on wet ice and transfer to the lab.
 - *This step is done with the help of a technician in surgical pathology (they know to avoid dye-marked regions for scrapes).*
 - *For tissue acquisition, see protocol titled “Clinical communication and collection of information for surgical cases.”*
 - *Scrapes for DNA/RNA isolation are collected on four additional charged slides (SuperFrost or equivalent; two slides for DNA, two slides for RNA) and transported in empty conical tubes without recovery medium. Dye contamination is not problematic for these samples.*
 - *A smear of tissue may also be collected directly on a charged slide for subsequent RNA FISH or immediate hematoxylin and eosin (or hemadiff) staining in surgical pathology.*
 2. Centrifuge tubes at 450 rcf for 5 minutes at 8°C.
 - *If a large amount of tissue remains on the slides, recover as much as possible by pipetting recovery medium on the slide to rinse off the cells then re-centrifuge. The rinsing will also increase the fat content of the recovered sample, so additional tube changes might be necessary.*
 3. Aspirate all but 0.3 ml (S = small) or 1 ml (L = large) of the recovery medium, use a 1-ml tip or 5-ml serological pipette pre-wetted with D-BSA to resuspend the tissue, and combine the contents of all tubes into a single 1.5-ml centrifuge tube (S) or 15-ml conical tube (L). Centrifuge at 300 rcf (S) / 450 rcf (L) for 5 minutes at RT (S) / 8°C (L).
 - *Choice of 1.5 ml or 15 ml tube depends on whether there is a small (S) or large (L) amount of material.*
 - *Pellets from scrapes tend to be small, so 1.5 ml tube is recommended to minimize material loss.*
 4. Aspirate all but 0.2 ml (S) / 2 ml (L) of the supernatant, add 1 ml (S) / 7 ml (L) of D-BSA, and resuspend the pellet. Centrifuge at 300 rcf (S) / 450 rcf (L) for 5 minutes at RT (S) / 8°C (L).
 5. Repeat Step 4 at least one more time to dilute out potential microorganisms, contaminating cell types, and dead cells/debris.
 - *If the supernatant is not clear, add additional washing steps until the supernatant is clear.*
 6. Aspirate all the supernatant carefully, so that only the sedimented tissue pellet remains, and resuspend in 1 ml (S) / 2 ml (L) of pre-warmed Type 1 medium.
 7. Add 100 µl of collagenase II (20 mg/ml stock, final concentration = 1 mg/ml) and 4 µl of ROCK inhibitor (5 mM stock, final concentration = 10 µM).
 8. (S) Wrap the 1.5-ml tube with parafilm and place in the bench-top thermoshaker set to 37°C and 350 rpm. (L) Wrap the 15-ml tube with parafilm and place at a slight angle (~15°) in an orbital shaker incubator set to 37°C and 140 rpm.
 9. Digest the tissue in increments of 15 minutes until clusters of 5–10 cells are observed. Pipette up and down 5–10 times every 15 minutes with a P1000 pipette pre-wetted with D-BSA to aid digestion.
 - *For most samples, one 15-minute incubation is sufficient.*
 - *If there is a very large amount of tissue, consider doubling the concentrations of collagenase II and ROCK inhibitor and digesting for longer than 15 minutes.*
 10. After incubation, add 200 µl of FBS directly to the tube to stop the digestion. Homogenize the digested tissue by pipetting it up and down vigorously with a P1000 pipette pre-wetted with D-BSA.

11. (*Optional*) For samples with a lot of debris post-digestion, pre-wet a 100- μ m strainer (VWR 76327-102) by pipetting 5 ml of D-BSA through the strainer into a new 50-ml conical tube. Swirl the tube to wet the sides of the tube and discard the D-BSA. Then, strain the digested tissue over the 100- μ m strainer into the 50-ml tube. Transfer the unfiltered tissue pieces into a second 50-ml tube (pre-wetted with D-BSA) by reversing the filter and flushing with 5 ml of D-BSA. Pipette the digested tissue up and down vigorously with a 5-ml sterile serological pipette pre-wetted with D-BSA. Repeat the straining process two more times, using the same filter to avoid wasting tissue. Centrifuge the flow-through at 300 rcf (S) / 450 rcf (L) for 5 minutes at RT (S) / 8°C (L). Aspirate the supernatant, leaving the last 800 μ l.
12. (*Optional, but recommended*) If the pellet is reddish, add 1 ml of red blood cell lysis buffer with a P1000 pipette, resuspend the pellet by pipetting up and down several times and incubate for 2 minutes at room temperature.
 - *1 ml of red blood cell lysis buffer is enough for pellets <100 μ l in size.*
13. Add 0.5 ml (S) / 2 ml (L) of D-BSA and pipette up and down several times. Centrifuge the mixture at 300 rcf (S) / 450 rcf (L) for 5 minutes at RT (S) / 8°C (L). Aspirate all of the supernatant carefully, so that only the sedimented tissue pellet remains.
14. Resuspend the pellet in an appropriate amount of Matrigel.
 - *As a guideline, 200 μ l is sufficient for a pellet of ~50 μ l. Samples typically yield enough cells for 30 μ l of matrigel.*
 - *The amount of Matrigel to add scales linearly with the pellet volume.*
15. Plate the cell-Matrigel suspension in 5 μ l drops, one per well of a 96-well plate or in a column of a 96-stripwell (Corning #9102). Turn the plate upside down and leave in the biosafety cabinet for 5 minutes. Transfer the plate, upside down, into a 37°C incubator and leave to solidify for 30 minutes.
16. Remove plate from incubator, flip right-side-up, and add 100 μ l of pre-warmed Type 2 medium to each well. Transfer to an incubator at 37°C and 5% CO₂.

Media and buffer recipes (reagent stock concentrations indicated in parentheses)

- adDMEM/F12+++ (for 500 ml total volume, store at 4°C for up to 6 months):
 - 500 ml adDMEM/F12
 - 5 ml penicillin-streptomycin
 - 5 ml Glutamax
 - 5 ml HEPES
- D-BSA (for 500 ml total volume, store at 4°C for up to 4 weeks):
 - 500 ml DMEM GlutaMAX
 - 5 ml penicillin-streptomycin (1% vol/vol)
 - 5 ml 10% BSA (fatty acid free, wt/vol in DPBS) solution
- Type 1 Medium (for 20 ml total volume, store at 4°C for up to 1 week):
 - 15.26 ml adDMEM/F12+++
 - 2 ml R-spondin conditioned medium
 - 2 ml Noggin conditioned medium
 - 400 µl B27 (50x)
 - 200 µl nicotinamide (1 M in DPBS)
 - 500 µl N-acetyl-L-cysteine (500 mM in water)
 - 40 µl Primocin (50 mg/ml)
 - 20 µl Y-27632 (5 mM in DMSO)
 - 10 µl Heregulin β1 (10 µM in DPBS-B) (DPBS-B = 0.1% wt/vol BSA (modified fraction V) in DPBS)
 - 10 µl human FGF-7 (10 µg/ml in DPBS-B)
 - 10 µl human FGF-10 (40 µg/ml in DPBS-B)
 - 2 µl A83-01 (5 mM in DMSO)
 - 1 µl human EGF (0.1 mg/ml in DPBS-B)
 - 0.68 µl SB202190 (30 mM in DMSO)
- Type 2 Medium (for 20 ml total volume, store at 4°C for up to 1 week):
 - 11.22 ml adDMEM/F12+++
 - 4 ml Wnt conditioned medium
 - 2 ml R-spondin conditioned medium
 - 2 ml Noggin conditioned medium
 - 400 µl B27 (50x)
 - 200 µl nicotinamide (1 M in DPBS)
 - 500 µl N-acetyl-L-cysteine (500 mM in water)
 - 10 µl hydrocortisone (1 mg/ml in ethanol)
 - 20 µl β-estradiol (100 µM in ethanol)
 - 20 µl forskolin (10 mM in DMSO)
 - 40 µl Primocin (50 mg/ml)
 - 20 µl Y-27632 (5 mM in DMSO)
 - 10 µl Heregulin β1 (10 µM in DPBS-B)
 - 10 µl human FGF-10 (40 µg/ml in DPBS-B)
 - 2 µl A83-01 (5 mM in DMSO)
 - 1 µl human EGF (0.1 mg/ml in DPBS-B)

Adapted from: Dekkers JF, et al. Long-term culture, genetic manipulation and xenotransplantation of human normal and breast cancer organoids. Nat Protoc. 2021;16(4):1936-65.