

BIOPSY FREEZE PROTOCOL

(Human Intestinal Biopsy Cryopreservation and Subsequent Enteroid Production)

Materials:

PBS without Ca⁺ or Mg⁺

Matrigel, .5M EDTA, Y-27632

Biopsy Wash Solution: PBS, Fungizone (1:100), PenStrep (same concentration you use in medium)

Crypt Wash Solution: DMEM, Fungizone (1:100), PenStrep (same concentration you use in medium)

Biopsy Freezing Medium:

90% growth medium (CMGF+)

10% DMSO

Y-27632 10 μ M (final concentration)

Growth Medium:

CMGF+

To make 50mL

- 8 mL CMGF- (You may add Pen-Strep)
- 25 mL (50%) Wnt3a conditioned media
- 10 mL (20%) Spondin media
- 5 mL (10%) Noggin media
- 1mL B27 Supplement 50x
- 500 μ L N2 Supplement
- 500 μ L Nicotinamide (1M stock; final conc. 10 mM)
- 100 μ L n-acetylcysteine (500mM stock; final 1mM)
- 50 μ L Gastrin (10 μ M stock; final conc. 10 nM)
- 50 μ L A83 (TGF β type I receptor inhibitor) (500 μ M stock; final conc. 500 nM)
- 25 μ L EGF (final conc. 50 ng/ml)
- 16.6 μ L SB202 (P38 inhibitor) (stock 30mM; final conc. 10 μ M)

FREEZE:

1. Collect biopsies during colonoscopy/endoscopy in tubes of ice-cold PBS, and store on ice while in transit to lab. Get multiple "bites" per sample site if possible. The more starting material the better.
2. Prepare a series of wash tubes: 15mL conical tube per sampling site containing about 5mL of **wash solution** each. Label with biopsy site to avoid confusing samples. Keep on ice within cell culture hood.
3. Ethanol sample tubes and bring into cell culture hood (on ice).
4. WASH: Pre-wet 10mL serological pipette with any medium containing FBS to prevent biopsy sticking to pipette walls. Use that serological pipette to pipette biopsy up and down 10 times in sample tube then use that same pipette to transfer biopsy to wash tube 1 trying to carry as little liquid as possible with the biopsy. Repeat until biopsy has been pipetted in all three wash tubes

(four tubes total when you count initial sample tube) and leave biopsy in tube 3 (still on ice) while you wash any other samples.

5. Label Cryovial with
 - a. sample identification
 - b. date
 - c. your initials, Lab, Institution, protocol number
6. Add 500uL cold **Biopsy Freezing Medium** to each cryovial
7. Use p1000 to transfer biopsy without any liquid (use suction force of tip to grab biopsy) from wash tube 3 to appropriate cryovial
8. Swish vial and visually ensure biopsy is submerged then tighten lid and place in isopropanol-containing freezing container and move immediately to -80 freezer overnight.
 - a. If you wish to freeze another cell line simultaneously, leave biopsies in wash tube 3 while you split/prepare your other cell line. When other cell line is in cryovials, then transfer biopsies to cryovials. This prevents either line from hanging out in DMSO too long out of the freezer.

THAW:

1. Remove cryovial from storage and thaw in 37° waterbath; swish and look for ice frequently. Do not submerge. Remove from waterbath IMMEDIATELY once ice is melted, douse with ethanol, and bring to culture hood. Remove lid carefully and set down inside up, so you can use lid again.
2. Remove freezing medium with p1000
3. Slowly, add 1mL growth medium +Y compound dropwise to cryovial with biopsy
4. Loosely recap cryovial and let rest on ice for 10 minutes. Gently swish a few times.
While Biopsy is soaking in growth medium and releasing freezing medium, prepare
 - a. Rinse/Shake microcentrifuge tube with 500uL PBS
 - b. EDTA/Shake microcentrifuge tube #1 with 1mL PBS and 40uL .5M EDTA
 - c. EDTA/Shake microcentrifuge tube #2 with 1mL PBS and 40uL .5M EDTA
5. Remove medium with p1000 and add 1ml PBS to cryovial
6. Transfer biopsy using p1000 suction force or wide mouth p1000 tips to prepared microcentrifuge Rinse/Shake microcentrifuge tube with 500uL PBS
7. Shake vigorously about 10 shakes. Debris falls off. Check under microscope to see if any crypts fall off (this is unlikely). This will make your crypt prep cleaner, and serves as a PBS rinse before EDTA treatment (to remove Ca+ Mg+ ions from interfering with EDTA)
8. Transfer biopsy to EDTA/Shake Tube #1. Shake to mix. Leave on ice. Begin shaking and checking after 6 minutes
9. Keep shaking and checking for crypts by placing tube under microscope and looking for floating crypts.
10. Once about 30% of crypts have dissociated (often 6-12 minutes), transfer biopsy to EDTA tube #2 and top off EDTA tube #1 with any DMEM, growth medium, or HBSS containing Ca+ Mg+ to dampen EDTA reaction.
11. Invert to mix EDTA tube #1 then immediately pellet crypts using bench mini spinner, remove EDTA solution, and Resuspend crypts in 500uL DMEM + PenStrep + Fungizone. Leave on ice while finishing EDTA tube #2

12. Keep shaking and checking for crypts in EDTA tube #2. Stop when entire surface area of colon biopsy is "swiss cheese" appearance, indicating all crypts have fallen out (15-17 minutes).
13. Top off EDTA tube #2 with any DMEM, growth medium, or HBSS containing Ca⁺ Mg⁺ to dampen EDTA reaction.
14. Invert to mix EDTA tube #2 then immediately pellet crypts using bench mini spinner, and Resuspend crypts in 500uL DMEM + PenStrep + Fungizone.
15. Pellet both tubes, remove wash media, and resuspend each in Matrigel for plating
16. Spot out in four tiny dots per well of 24-well plate, and allow to harden in incubator with plate upside down so crypts sink to surface where they have best nutrient diffusion
17. Once Matrigel has solidified, add 500uL of growth medium (I don't know if adding Y compound matters, but I did it anyway)