IntestiCult™ Plus Organoid Growth Medium

Cell culture medium for establishment and expansion of human intestinal organoids

Catalog #100-1677 1 Kit

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Product Description

IntestiCult™ Plus Organoid Growth Medium (OGM) is a complete cell culture medium for the establishment and expansion of human intestinal organoids. Unlike other intestinal organoid culture media, IntestiCult™ Plus OGM facilitates the simultaneous expansion and differentiation of intestinal organoid cultures; this creates a balance between cell types (LGR5+ stem cells and proliferating cell populations, and terminally differentiated absorptive and secretory cells), which recapitulates the diversity of cell types found in the intestinal epithelium. IntestiCult™ Plus OGM divides each passage of the organoid cultures into distinct Start and Balance phases, which are supported by modulating the amount of IntestiCult™ Plus OGM Supplement added to the IntestiCult™ Plus OGM Basal Medium. The IntestiCult™ Plus OGM Supplement can also be removed entirely to create a Wnt-free medium to support the differentiation of small intestinal organoid cultures, as well as to favor Wnt-independent colorectal cancer organoids.

Organoids expanded with IntestiCult™ Plus OGM exhibit a distinct budded appearance, complete with goblet cells and secreted mucus. Proliferating cells are mostly found at the base of the crypt-like buds, and cultures can be passaged via either clump or single-cell passaging. IntestiCult™ Plus OGM is serum free and suitable for a wide variety of applications.

Should you intend to use this product for commercial purposes, please contact HUB at www.huborganoids.nl for a commercial use license or for clarification in relation to HUB licensing.

Product Information

The following components are sold as a complete kit (Catalog #100-1677) and are not available for individual sale.

COMPONENT NAME	COMPONENT #	SIZE	STORAGE	SHELF LIFE
IntestiCult™ Plus OGM Supplement	100-1676	0.5 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
IntestiCult™ Plus OGM Basal Medium	100-1675	100 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.

NOTE: Store components at -20°C upon receipt, or aliquot and store at -20°C. Thawed components can be re-frozen at -20°C with no change to stability. Do not exceed the component expiry date as indicated on the label.

Materials Required but Not Included

PRODUCT NAME	CATALOG #
Anti-Adherence Rinsing Solution	07010
Antibiotics (e.g. gentamicin or penicillin/streptomycin)	---
25% Bovine serum albumin (BSA) in phosphate-buffered saline (PBS)	---
CloneR™2	100-0691
Conical tubes, 15 mL and 50 mL	e.g. 38009 and 38010
Corning® Matrigel® Matrix, Growth Factor Reduced (GFR), Phenol Red-Free	Corning 356231
Costar® 24-Well Flat-Bottom Plate, Tissue Culture-Treated	38017
Costar® 6-Well Flat-Bottom Plate, Tissue Culture-Treated	38015
D-PBS (Without Ca++ and Mg++)	37350

PRODUCT NAME	CATALOG #
DMEM/F-12 with 15 mM HEPES	36254
Falcon® 96-Well Flat-Bottom Microplate, Tissue Culture-Treated	38022
Gentle Cell Dissociation Reagent	100-0485
Organoid Culture Plates, 24 Wells and 96 Wells	200-0561 and 200-0562
Reversible Strainers, Small, 37 µm and 100 µm	27215 and 27217
Serological pipettes, 5 mL or 10 mL	e.g. 38003 or 38004
Trypsin-EDTA (0.05%)	07910
Wide-bore disposable pipette tips, 1 mL and 200 µL	e.g. VWR CA15000-468
Y-27632 (Dihydrochloride)	72302

Preparation of Reagents

A. COMPLETE INTESTICULT™ PLUS START MEDIUM AND BALANCE MEDIUM

Use sterile technique to prepare complete IntestiCult™ Plus **Start** Medium or **Balance** Medium (IntestiCult™ Plus OGM Basal Medium + IntestiCult™ Plus OGM Supplement). The following example is for preparing 10 mL of complete medium. If preparing other volumes, adjust accordingly. Do not thaw or pre-warm components in a 37°C water bath.

1. Thaw IntestiCult™ Plus OGM Basal Medium at room temperature (15 - 25°C) or at 2 - 8°C overnight.
2. Thaw IntestiCult™ Plus OGM Supplement on ice.

NOTE: Once thawed, use each component immediately or aliquot and store at -20°C for up to 12 months. After thawing the aliquots, use immediately or store for up to 3 weeks at 2 - 8°C. Do not re-freeze.

3. Prepare IntestiCult™ Plus **Start** Medium or **Balance** Medium as follows:
   - **Start Medium:** Add 50 µL of IntestiCult™ Plus OGM Supplement to 10 mL of IntestiCult™ Plus OGM Basal Medium. Mix thoroughly.
   - **Balance Medium:** Add 10 µL of IntestiCult™ Plus OGM Supplement to 10 mL of IntestiCult™ Plus OGM Basal Medium. Mix thoroughly.

   NOTE: If not using immediately, store complete **Start** and **Balance** media at 2 - 8°C for up to 3 weeks.
4. Add desired antibiotics immediately before use (e.g. 50 µg/mL gentamicin or 100 units [100 µg/mL] penicillin/streptomycin).

B. DMEM + 1% BSA

Use sterile technique to prepare DMEM + 1% BSA. The following example is for preparing 50 mL of DMEM + 1% BSA. If preparing other volumes, adjust accordingly.

1. Add 2 mL of 25% BSA to 48 mL of DMEM/F-12 with 15 mM HEPES in a 50 mL conical tube.
2. Mix well by inversion. Place on ice.

   NOTE: If not using immediately, store at 2 - 8°C for up to 6 months.

Directions for Use

Please read the entire protocol before proceeding. Use sterile technique when performing the following protocols:

A. Isolation of Human Intestinal/Colonic Crypts from Tissue Biopsies
B. Organoid Culture in Matrigel® Domes
C. Clump Passaging of Human Intestinal/Colonic Organoids
D. Dissociation of Human Intestinal/Colonic Organoids
E. Single-Cell Passaging of Human Intestinal/Colonic Organoids
F. Plating Organoid Fragments for Monolayer Culture

A. ISOLATION OF HUMAN INTESTINAL/COLONIC CRYPTS FROM TISSUE BIOPSIES

The following steps are optimized for processing biopsy-sized samples of tissue. If processing larger amounts of tissue, adjust volumes as necessary.

1. Prepare a sufficient volume of IntestiCult™ Plus **Start** Medium (Preparation of Reagents, section A) and DMEM + 1% BSA (Preparation of Reagents, section B). Warm ~2.5 mL of IntestiCult™ Plus **Start** Medium to room temperature (15 - 25°C).
2. Thaw 100 µL of Matrigel® on ice.

   NOTE: This is sufficient Matrigel® for plating up to 4 x 50 µL dome cultures. Depending on the crypt count (step 14), a different amount of Matrigel® may be required.
3. Place a sufficient volume of D-PBS (without Ca++ and Mg++) and DMEM + 1% BSA (Preparation of Reagents, section B) on ice.
4. Warm a tissue culture-treated 24-well plate in a 37°C incubator overnight or for at least 2 hours. (Overnight warming at 37°C is recommended.)
5. In a 15 mL conical tube, wash the tissue sample with 10 mL of ice-cold D-PBS. Allow the tissue to settle by gravity (~5 seconds), then aspirate the supernatant.
6. Repeat step 5, leaving ~1 mL of supernatant in the tube.
7. Using a 1 mL wide-bore pipette tip, transfer the tissue and remaining supernatant to a 1.5 mL microcentrifuge tube.
8. Using sterile scissors, thoroughly mince the tissue into ~5 mm pieces. Using a 1 mL wide-bore pipette tip, transfer the tissue fragments to a new 15 mL conical tube. Rinse the microcentrifuge tube with ice-cold D-PBS and add the rinse to the tissue fragments.
9. Allow the tissue fragments to settle by gravity (~5 seconds), then aspirate the supernatant.
10. Add 10 mL of Gentle Cell Dissociation Reagent to the 15 mL tube containing the fragments. Incubate the tube on ice on a rocking platform set at medium speed (~40 RPM) for 30 minutes.
11. Centrifuge at 300 x g for 5 minutes. Aspirate the supernatant.

NOTE: For the remainder of this section, pre-wet pipette tips with DMEM + 1% BSA before manipulating the tissue sample. This prevents crypts from sticking to the wall of the pipette tip.

12. Add 1 mL of ice-cold DMEM + 1% BSA to the tube with the fragments. Vigorously pipette up and down 20 times with a 1 mL pipettor to remove crypts from tissue.

    NOTE: Avoid touching the side/bottom of the tube with the pipette tip.
13. Using a 1 mL pipettor, pass the contents of the tube through a 100 µm cell strainer (tilted on its side) into a new 15 mL conical tube. Rinse the original tube with 1 mL of DMEM + 1% BSA and pass through the strainer into the new tube.
14. Determine the total number of crypts in the sample as follows:
    a. Place 3 x 10 µL aliquots of the sample on an appropriate counting surface (e.g. a glass slide or 1 well of a 6-well plate).
    b. Using an inverted microscope, count the crypts in each aliquot.
    c. Determine the average number of crypts in the 3 aliquots, then multiply by 200 to determine the total number of crypts in the 2 mL sample.
    d. Determine how many culture domes can be plated at 1000 crypts per dome.

    Example: *Aliquot 1: 18 crypts*
    Aliquot 2: 23 crypts
    Aliquot 3: 19 crypts
    Average: 20 crypts
    Total number of crypts in 2 mL sample: 20 crypts x 200 = 4000 crypts total
    This is sufficient for 4 culture domes containing 1000 crypts each.

    NOTE: 1000 crypts per dome will result in 150 - 200 mature organoids; however, numbers may vary significantly.
15. Centrifuge the sample at 300 x g for 5 minutes. Aspirate all except 100 µL of supernatant. Proceed to section B for setting up Matrigel®-crypt dome cultures.

B. ORGANOID CULTURE IN MATRIGEL® DOMES

The following steps are for plating 4 x 50 µL culture domes containing ~1000 crypts each. If fewer or additional culture domes are required based on the crypt counts in section A, adjust the volume of Matrigel® and DMEM + 1% BSA to give a 1:1 final mixture (e.g. for 8 x 50 µL culture domes, add 200 µL of Matrigel® and 100 µL DMEM + 1% BSA to the 100 µL of supernatant in the sample tube).

1. Retrieve the warm 24-well plate from the 37°C incubator. Pre-wet a 200 µL pipette tip with DMEM + 1% BSA.
2. Add 100 µL of Matrigel® to the sample tube. Pipette up and down 10 times to thoroughly resuspend the pellet. Avoid introducing bubbles.

3. Using a pre-wetted 200 µL pipette tip, draw up 50 µL of the Matrigel®-crypt suspension and add to 1 of the 8 central wells of a 24-well tissue culture-treated plate as follows:
   a. Hold the pipette vertically over the center of the well. Bring the pipette tip near to (but not in contact with) the floor of the well.
   b. Slightly depress the plunger until a droplet is visible on the end of the pipette tip.
   c. Slowly lower the pipette until the droplet touches the floor of the well.
   d. Gently dispense (only to the first stop on the pipette) the remaining volume while lifting the pipette away from the well.

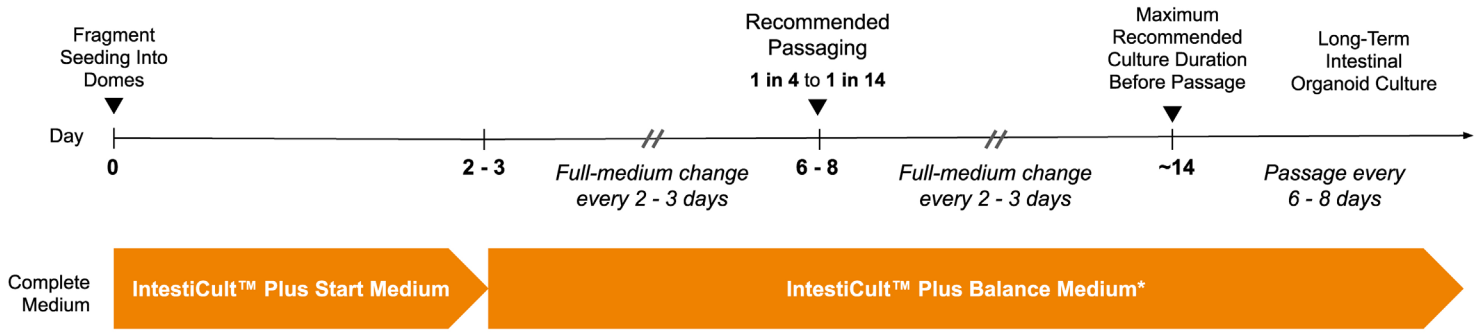
   NOTE: Work quickly to plate the Matrigel®-crypt suspension within ~60 seconds of removing it from ice.
4. Repeat step 3 until all of the Matrigel®-crypt suspension is dispensed.
5. Carefully transfer the plate to a 37°C incubator. Incubate at 37°C for 10 minutes to allow domes to solidify. Do not disturb the domes.
6. Add 2 µL of 10 mM Y-27632 (Dihydrochloride; 10 µM final concentration) to 2 mL of complete IntestiCult™ Plus Start Medium (Preparation of Reagents, section A) at room temperature. Mix thoroughly.

   NOTE: Each culture dome requires 500 µL of medium; 2 mL of medium is sufficient for 4 culture domes. If preparing a different number of culture domes, adjust the volume of the medium accordingly.
7. Add 500 µL of complete IntestiCult™ Plus Start Medium (+ Y-27632 [Dihydrochloride]) to each well by pipetting the medium gently down the wall of the well. Do not pipette directly onto the domes.

   NOTE: Y-27632 (Dihydrochloride) should remain in the medium for at least 2 days following crypt isolation to improve cell survival and organoid formation, after which it should no longer be included.

   NOTE: The addition of antibiotics is highly recommended for primary culture from tissue biopsies.
8. Add sterile D-PBS to unused wells.
9. Place the lid on the culture plate and incubate at 37°C and 5% CO_2.
10. Every 2 - 3 days, perform a full-medium change with complete IntestiCult™ Plus Start Medium (Y-27632 [Dihydrochloride] is no longer required).
11. Maintain the cultures in complete IntestiCult™ Plus Start Medium for the duration of the initial P0 passage of the culture.
12. Allow the culture in each well to grow to full size before passaging. This may take 1 - 3 weeks, depending on the quality of the initial crypt isolation.
13. Proceed to section C for clump passaging or section E for single-cell passaging.

C. CLUMP PASSAGING OF HUMAN INTESTINAL/COLONIC ORGANOIDS



*If a Wnt-free medium is desired (e.g. if performing optional differentiation of small intestinal organoids or expansion of colorectal cancer organoids), this can be replaced entirely by IntestiCult™ Plus OGM Basal Medium.

Figure 1. Expansion of Human Intestinal Organoids Using IntestiCult™ Plus Start Medium and IntestiCult™ Plus Balance Medium

It is recommended to passage every 6 - 8 days, although culture can be maintained for up to 14 days without passaging. Larger cystic or budded organoids will result in a higher yield of viable fragments than smaller, dark, or collapsed organoids.

1. Warm a 24-well tissue culture-treated plate in a 37°C incubator for at least 2 hours or overnight. (Overnight warming at 37°C is recommended.)
2. Prepare IntestiCult™ Plus Start Medium and warm to room temperature (15 - 25°C). Do not warm medium in a 37°C water bath.

   NOTE: For each well to be plated, 500 µL of medium will be required.
3. Thaw Matrigel® on ice; for each dome to be plated, 25 µL of Matrigel® will be required.
4. Place DMEM + 1% BSA on ice.

5. Assign the domes(s) to be passaged a split ratio. Depending on the cell line, organoids in IntestiCult™ Plus can be split anywhere from 1 in 4 to 1 in 14. If performing fragment counts, skip this step.
6. Carefully remove and discard medium from each well to be passaged, without disturbing the Matrigel® dome.
7. Add 1 mL of room temperature Gentle Cell Dissociation Reagent (GCDR) on top of the exposed dome in each well. Incubate for 1 minute at room temperature.
8. Pre-wet a 1 mL pipette tip with GCDR; use this pipette tip to thoroughly scrape the Matrigel® dome free of the well floor. Pipette the GCDR in the well up and down 2 - 3 times to break up the dome and the organoids. Ensure all pieces of Matrigel® have been rinsed free of the plate.
9. Using the same pipette tip, transfer the organoid mixture to a 15 mL conical tube.
10. Add 1 mL of GCDR to the newly emptied well. Using a pipette tip pre-wetted with GCDR, pipette the GCDR up and down 2 - 3 times to rinse the well. Transfer the contents of the well to the 15 mL conical tube from step 9.
11. Repeat steps 8 - 10 for each well to be passaged.
12. Incubate the tubes at room temperature upright for 10 minutes.
13. Centrifuge the tubes at 300 x g for 5 minutes at 2 - 8°C. Aspirate and discard the supernatant.
14. Add 1 mL of ice-cold DMEM + 1% BSA to each tube. Using a pre-wetted 1 mL pipette tip, resuspend organoids by pipetting up and down vigorously 15 - 25 times.

    NOTE: Organoid cultures with thicker epithelia will require more triturations than those with thinner epithelia.
15. OPTIONAL: Perform a fragment count of the cell suspension:
    a. Plate 2 x 10 µL domes of cell suspension from step 14 onto a 24-well plate (can be at room temperature).
    b. Count fragments within each 10 µL dome on a benchtop microscope at 4X. A clump of 3 or more cells is considered a fragment.
    c. Determine the sample volume of cell suspension required for the desired number of fragments per 50 µL dome.

    NOTE: For IntestiCult™ Plus cultures, it is recommended to seed 150 fragments per 50 µL dome.

    Example: *If seeding 4 x 50 µL domes, 600 fragments are required.*
    Dome 1: 35 fragments
    Dome 2: 45 fragments
    Average: 40 fragments in 10 µL dome
    Therefore, if seeding 600 fragments, a 150 µL sample of cell suspension is required.

    d. Transfer the sample volume determined in step c (e.g. 150 µL) from 1 mL cell suspension to a new 15 mL tube. Top up volume to 1 mL with DMEM + 1% BSA and continue to step 16.
16. Centrifuge the samples at 300 x g for 5 minutes at 2 - 8°C. Aspirate all except 100 µL of supernatant per sample.

NOTE: The following steps are for plating 4 x 50 µL culture domes from one well at a 1 in 4 split. If fewer or additional culture domes are required based on the split ratio in step 5, adjust the volume of Matrigel® and DMEM + 1% BSA to give a 1:1 final mixture (e.g. for 8 x 50 µL culture domes, add 200 µL Matrigel® and 100 µL DMEM + 1% BSA to the 100 µL of supernatant in the sample tube).

17. Remove the 24-well plate from the 37°C incubator.
18. Add 100 µL of Matrigel® to the sample tube. Pipette up and down 10 times to thoroughly resuspend the pellet. Avoid introducing bubbles.
19. Using a pre-wetted 200 µL pipette tip, draw up 50 µL of the Matrigel®-crypt suspension and add to 1 of the 8 central wells of a 24-well tissue culture-treated plate as follows:
    a. Hold the pipette vertically over the center of the well. Bring the pipette tip near to (but not in contact with) the floor of the well.
    b. Slightly depress the plunger until a droplet is visible on the end of the pipette tip.
    c. Slowly lower the pipette until the droplet touches the floor of the well.
    d. Gently dispense (only to the first stop on the pipette) the remaining volume while lifting the pipette away from the well.

    NOTE: Work quickly to plate the Matrigel®-fragment suspension within ~60 seconds of removing it from ice.
20. Repeat step 19 until all of the Matrigel®-crypt suspension is dispensed.
21. Carefully transfer the plate to a 37°C incubator. Incubate at 37°C for 10 minutes to allow domes to solidify. Do not disturb the domes.
22. Prepare 2 mL of IntestiCult™ Plus Start Medium (Preparation of Reagents, section A) at room temperature.

    NOTE: Each culture dome requires 500 µL of IntestiCult™ Plus Organoid Medium; 2 mL of medium is sufficient for 4 culture domes. If preparing a different number of culture domes, adjust the volume of medium accordingly.
23. Add 500 µL of IntestiCult™Plus Start Medium to each well by pipetting the medium gently down the wall of the well. Do not pipette directly onto the domes.

24. Add sterile D-PBS to unused wells.
25. Place the lid on the culture plate and incubate at 37°C and 5% CO_2.
26. Every 2 - 3 days, perform a full-medium change with IntestiCult™ Plus **Balance** Medium.
27. Allow the organoids to grow and mature, passaging every 6 - 8 days.

    NOTE: For increased maturation, organoids can be maintained for up to 14 days in IntestiCult™ Plus **Balance** Medium before passaging. To maintain organoids for increased maturation, reduce the seeding density to allow more room for each individual organoid to grow.

    NOTE: If transitioning the organoid cultures from an alternate organoid growth medium to IntestiCult™ Plus, we do not recommend switching the growth medium at the same time as passaging. Instead, maintain the organoids in the previous growth medium for the first 3 - 4 days of the culture; then, replace the growth medium with IntestiCult™ Plus **Start** Medium for the remainder of the passage, replacing the medium every 2 - 3 days. After passaging the organoids again, maintain the organoids as described in this protocol. This gradual transition reduces stress on the organoids while changing the medium and improves organoid survival.

D. DISSOCIATION OF HUMAN INTESTINAL/COLONIC ORGANOIDS

1. Aspirate all media from the organoid cultures without disturbing the organoids within the Matrigel® dome.
2. Add 1 mL of GCDR to each well of organoids to be harvested.
3. Incubate at room temperature (15 - 25°C) for 1 minute.
4. Using a 1 mL pipettor, vigorously pipette up and down to disrupt the Matrigel® dome and resuspend the organoids.
5. Pool the harvested wells in a 15 mL conical tube. Incubate at room temperature for 10 minutes with optional gentle agitation or rocking.
6. Centrifuge at 300 x g for 5 minutes at 2 - 8°C.
7. Remove and discard the supernatant. Add 5 mL ice-cold DMEM + 1% BSA to resuspend organoids. Centrifuge at 300 x g for 5 minutes at 2 - 8°C.
8. Aspirate the supernatant, removing as much as possible, being careful not to disturb the pellet. Add 1 mL of warm (37°C) Trypsin-EDTA (0.05%) to resuspend organoids.

    NOTE: If pooling larger numbers of organoid wells, increase the volume of Trypsin-EDTA (0.05%) to ensure efficient dissociation of the organoids.
9. Using a 1 mL pipettor, pipette up and down to mix thoroughly. Incubate at 37°C for 5 - 10 minutes.
10. Mix thoroughly by vigorous pipetting or vortexing to disrupt the organoids as much as possible. Use a microscope to check the organoids for sufficient disruption. Organoids should be dissociated into either individual cells or small fragments. If many large fragments or whole organoids remain, repeat pipetting/vortexing until fragments are sufficiently disrupted.

    NOTE: Perform the remaining steps as quickly as possible, as cells will start to clump together.
11. Add an equal volume of DMEM + 1% BSA (e.g. 1 mL DMEM + 1% BSA per 1 mL Trypsin-EDTA [0.05%]) and pipette up and down to mix thoroughly. Centrifuge fragments at 300 x g for 5 minutes at 2 - 8°C.
12. Carefully remove and discard the supernatant.

    NOTE: If a buoyant mucus layer is present, steps 11 and 12 may need to be repeated to properly pellet the organoid fragments.
13. If single-cell passaging, proceed to section E.
14. If seeding monolayer cultures, proceed to section F.

E. SINGLE-CELL PASSAGING OF HUMAN INTESTINAL/COLONIC ORGANOIDS

1. Prepare a new 15 mL conical tube. Pre-wet a 37 µm reversible cell strainer with 1 mL of DMEM + 1% BSA and place it on top of the new tube.
2. Pass the cell suspension through the 37 µm strainer and then keep the tube containing filtered suspension on ice.
3. Perform a cell count to determine live cells/mL (e.g. with NucleoCounter® NC-250).
4. Determine the volume of suspension needed to seed the required amount of Matrigel® domes, and transfer the suspension to a new conical tube. Centrifuge cells at 300 x g for 5 minutes.

    NOTE: A recommended starting seeding concentration is 50 live cells/µL, but this may need to be adjusted according to the donor or segment.
5. Centrifuge cells at 300 x g for 5 minutes. Aspirate all except 100 µL of supernatant.

NOTE: The following steps are for plating 4 x 50 µL culture domes containing 2500 cells each. If fewer or additional culture domes are required based on the counts in step 4, adjust the volume of Matrigel® and DMEM + 1% BSA to give a 1:1 final mixture (e.g. for 8 x 50 µL culture domes, add 200 µL Matrigel® and 100 µL DMEM + 1% BSA to the sample tube).

6. Remove the 24-well plate from the 37°C incubator. Pre-wet a 200 µL pipette tip with DMEM + 1% BSA.

7. Add 100 µL of Matrigel® to the sample tube. Pipette up and down 10 times to thoroughly resuspend the pellet. Avoid introducing bubbles.
8. Using a pre-wetted 200 µL pipette tip, draw up 50 µL of the Matrigel®-crypt suspension and add to 1 of the 8 central wells of a 24-well tissue culture-treated plate as follows:
   a. Hold the pipette vertically over the center of the well. Bring the pipette tip near to (but not in contact with) the floor of the well.
   b. Slightly depress the plunger until a droplet is visible on the end of the pipette tip.
   c. Slowly lower the pipette until the droplet touches the floor of the well.
   d. Gently dispense (only to the first stop on the pipette) the remaining volume while lifting the pipette away from the well.

   NOTE: Work quickly to plate the Matrigel®-cell suspension within ~60 seconds of removing it from ice.
9. Repeat step 8 until all of the Matrigel®-cell suspension is dispensed.
10. Carefully transfer the plate to a 37°C incubator. Incubate at 37°C for 10 minutes to allow domes to solidify. Do not disturb the domes.
11. Prepare 2 mL of IntestiCult™ Plus **Start** Medium (Preparation of Reagents, section A) at room temperature (15 - 25°C). Add CloneR™2 to IntestiCult™ Plus **Start** Medium at a 1 in 10 dilution (e.g. 1 mL CloneR™2 to 9 mL IntestiCult™ Plus **Start** Medium). Mix thoroughly.

    NOTE: Each culture dome requires 500 µL of IntestiCult™ Plus Organoid Medium plus CloneR™2; 2 mL of medium is sufficient for 4 culture domes. If preparing a different number of culture domes, adjust the volume of medium accordingly.
12. Add 500 µL of IntestiCult™ Plus **Start** Medium plus CloneR™2 to each well by pipetting the medium gently down the wall of the well. Do not pipette directly onto the domes.
13. Add sterile D-PBS to unused wells.
14. Place the lid on the culture plate and incubate at 37°C and 5% CO_2.
15. Every 2 - 3 days, perform a full medium change: for the first 4 - 5 days, use IntestiCult™ Plus **Start** Medium; for the remainder of the passage, use IntestiCult™ Plus **Balance** Medium.
16. Allow the organoids to grow and mature, passaging every 8 -12 days.

F. PLATING ORGANOID FRAGMENTS FOR MONOLAYER CULTURE

For establishing organoid monolayer cultures, either on membrane inserts or in standard culture plates, we recommend using IntestiCult™ Organoid Differentiation Medium (Catalog #100-0214). Use the cells isolated in section C of this protocol to proceed to section B of the Monolayer Culture protocol of the IntestiCult™ Organoid Differentiation Medium (Human) Product Information Sheet (PIS) (Document #10000007635).

Refer to Table 1 to estimate the number of wells/cells used to seed the referenced size of each membrane insert. Corning® Transwell® permeable supports are used for reference. Adjust accordingly for alternate manufacturers or well sizes.

NOTE: The number of cells per well can vary significantly. Please use this table as a guideline only.

Table 1. Transwell Seeding Guide

MONOLAYER CULTUREWARE	NUMBER OF WELLS OF INTESTINAL ORGANOIDS TO HARVEST (50 µL DOME) (per well to be seeded)	ESTIMATED NUMBER OF VIABLE CELLS (per well to be seeded)
HTS Transwell®-96 Permeable Support	0.25 - 0.33 wells	50 - 75,000 cells
6.5 mm Transwell® insert	1 - 2 wells	150 - 250,000 cells
12 mm Transwell® insert	2 - 3 wells	250 - 500,000 cells

Notes and Tips

Matrigel® is temperature-sensitive and will polymerize at room temperature within a few minutes; keep Matrigel® on ice at all times to prevent polymerization prior to plating.