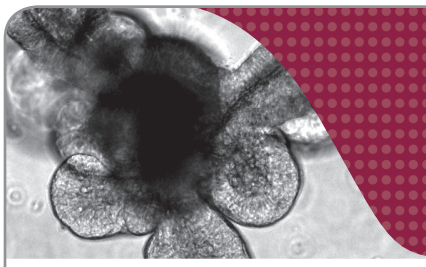

TECHNICAL BULLETIN

# Intestinal Epithelial Organoid Culture with IntestiCult™ Organoid Growth Medium (Mouse)

## Introduction

The use of three-dimensional (3D) culture systems continues to increase, due in large part to their potential to facilitate basic research into multicellular biological mechanisms and precision medicine, as well as to bridge the gap between traditional in vitro high-throughput screens and in vivo studies during drug development.[1,2] Adoption of 3D culture systems has been driven by growing recognition that organotypic cultures can provide a more physiologically relevant in vitro model than traditional monolayer culture techniques.[1,2] In the intestinal epithelial organoid culture system, intestinal stem cells recapitulate both the self-renewal capacity and differentiation hierarchy observed in the adult intestine in vivo (Figure 1). In addition to exhibiting all of the known cell types present in the adult intestinal epithelium, intestinal organoids are characterized by a crypt-villus organization, epithelial polarization and functional lumen,[3,4] which together make this culture system a powerful tool for investigating intestinal biology and the properties of intestinal stem cells.[3,4] Intestinal organoids are also being explored for potential contributions to regenerative medicine, as organoids have been demonstrated to incorporate into the colon of chemically injured mice to form segments of colon that are morphologically indistinguishable from the surrounding epithelium.[5] Intestinal organoids have been successfully co-cultured with both bacterial and immune cells, providing a model to study cell to cell interactions between epithelial tissues and the immune system.[6,7] As well, mouse intestinal organoids are amenable to genetic manipulations using the CRISPR-Cas system and infection with retroviruses and lentiviruses.[8,9]

This Technical Bulletin provides step-by-step instructions for the isolation, culturing, passaging and cryopreservation of mouse small intestinal and colonic crypts using IntestiCult™ Organoid Growth Medium (Mouse) and Corning® Matrigel® Matrix.

### Aliquoting & Storage of IntestiCult™ Organoid Growth Medium

Complete medium can be stored at 2 - 8°C for up to two weeks. To avoid repeated freeze-thaw cycles, aliquot complete medium into appropriate volumes and freeze at -20°C for up to three months. Do not re-freeze aliquots once thawed.

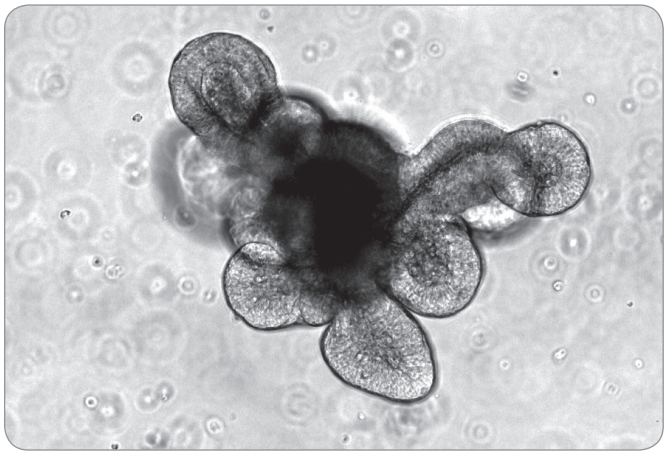

**Figure 1.** Light microscope visualization (10X) of a mouse intestinal epithelial organoid after five days of culture

## Protocol

### Section 1: Isolation of Mouse Intestinal Crypts

1. On the day of crypt isolation, remove the bottle of IntestiCult™ Basal Medium from the refrigerator and place on the bench-top to warm to room temperature (15 - 25°C). Remove the vials of IntestiCult™ Supplement 1 and Supplement 2 from the freezer and leave them to thaw at room temperature (15 - 25°C). Pipette up and down to mix Supplement 1 and 2 thoroughly.

   ***Note:*** *Once thawed, use immediately.*

2. Make the complete medium by adding 5 mL of Supplement 1 and 5 mL of Supplement 2 to the bottle of Basal Medium. Replace the cap and mix the medium well by inverting the bottle several times.

3. The medium must equilibrate to room temperature (15 - 25°C) before use. You will require 12 mL of complete medium to initiate organoid cultures in 12 individual culture wells (four wells for each of three plating densities) using crypts isolated from murine small intestine or colon following this protocol.

4. Immediately before use, add desired antibiotics to the complete IntestiCult™ Organoid Growth Medium. We recommend 50 µg/mL gentamicin or 100 units/100 µg per mL penicillin/streptomycin.

5. Prepare the additional media and reagents required for this procedure. Thaw 500 µL Corning® Matrigel® Matrix by placing the vial on ice. We recommend Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free (Corning® Product #356231). Place 500 mL PBS without magnesium or calcium (Catalog #37350), 100 mL PBS supplemented with 0.1% bovine serum albumen (BSA), and 25 mL DMEM/F-12 with 15 mM HEPES (Catalog #36254) on ice. Place Gentle Cell Dissociation Reagent (Catalog #07174) at room temperature (15 - 25°C). Pre-warm a tissue culture (TC)-treated 24-well culture plate (Corning® Product #3526) in an incubator at 37°C for at least 30 minutes.

   ***Note:*** *To ensure ideal Matrigel® dome architecture for organoid culture use tissue culture-treated plates.*


**Figure 2.** Isolation of mouse intestine: (A) removal of external membrane and fat from mouse intestine; (B) harvested mouse intestinal segment after removal of external membrane.

6. Sacrifice a mouse according to applicable ethical regulations and harvest 20 cm of small intestine proximal to the stomach. Use pointed forceps to remove any membrane, blood vessels and fat from the exterior of the intestine (Figure 2). Place the intestinal segment into a 10 cm dish containing 5 mL cold (2 - 8°C) PBS.

   ***For Colon:*** *The following protocol can be performed using the colon from one mouse; however, due to the high degree of variability between new users, it is suggested to use the colon from two or three mice to ensure a greater seeding density when performing the protocol for the first time.*

   *Harvest 3 - 6 cm of colon per sacrificed mouse; be sure to cut the colon a few millimeters below the cecum and a few millimeters above the rectum to prevent the introduction of excessive toxins and waste into the culture. Note, stem cell density is greatest in the proximal colon; therefore excising more tissue near the rectum will not significantly increase the number of crypts.*

7. Gently flush the intestinal segment with 1 mL cold (2 - 8°C) PBS by inserting a 1 mL pipette tip into one of the open ends of the intestine.

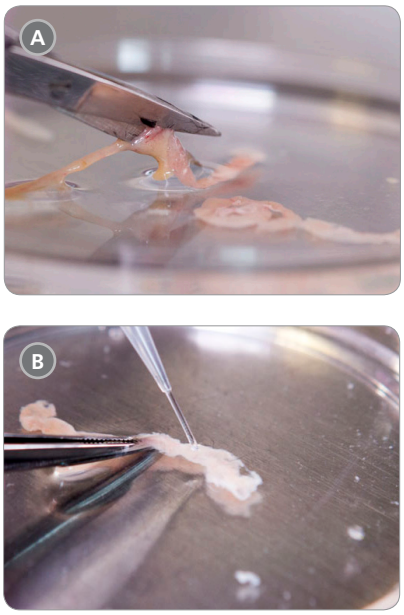


**Figure 3.** Preparatory steps for mouse intestinal segment: (A) cutting open intestinal segment; (B) washing intestinal segment with cold PBS.

## Attention: Pre-wet pipettes and pipette tips

Throughout the procedure you will need to pre-wet pipettes and pipette tips before manipulating intestinal pieces or crypts to prevent the tissue from sticking to the wall of the pipette.

## Attention: Be aware of centrifugation speeds

Throughout this procedure both 200 x *g* and 290 x *g* centrifugation speeds are used frequently.


**Figure 4.** Washing steps for mouse intestinal pieces

(A) intestinal pieces settled in bottom of tube; (B) intestinal pieces following aspiration of supernatant; (C) wash supernatants from first (left) and 20th (right) wash.

8. Using small scissors, cut the intestinal section open lengthwise (Figure 3A) and open such that the lumen of the intestine is facing up. Use a micropipette to gently wash the intestinal sheet with 1 mL cold (2 - 8°C) PBS three times (Figure 3B).
9. Transfer the intestinal segment to a clean 10 cm dish containing 15 mL of fresh, cold (2 - 8°C) PBS. Using forceps, move the intestinal segment through the clean buffer to rinse thoroughly.
10. Add 15 mL cold (2 - 8°C) PBS to a 50 mL conical tube. Using forceps, hold the washed intestine by one end over the tube. Starting from the bottom of the intestine, use scissors to cut the intestine into 2 mm pieces, allowing these pieces to fall into the buffer in the tube.
11. Pre-wet a 10 mL serological pipette with PBS and use it to gently pipette the intestinal pieces up and down three times.
12. Let the pieces settle by gravity (approximately 30 seconds; Figure 4A) and then gently aspirate off the supernatant, leaving enough liquid to just cover the pieces of tissue (Figure 4B).
13. Add 15 mL fresh cold (2 - 8°C) PBS and repeat the rinsing procedure by pipetting the suspended tissue pieces up and down three times using a pre-wetted 10 mL serological pipette.
14. Using the same pipette, repeat steps 12 – 13 another fifteen to twenty times, or until the supernatant is clear (Figure 4C).

    ***For Colon:*** *In contrast to the small intestine, when isolating colon crypts the supernatent typically becomes clear after only three to five washes with cold (2 - 8°C) PBS. Despite this appearance, colonic pieces should be washed fifteen times with cold PBS before proceeding to the next step.*
15. Remove the supernatent, resuspend the tissue pieces in 25 mL room temperature (15 - 25°C) Gentle Cell Dissociation Reagent and incubate at room temperature (15 - 25°C) for 15 minutes on a rocking platform at 20 rpm.

    ***For Colon:*** *Increase incubation time to 20 minutes.*
16. Let the tissue segments settle by gravity for approximately 30 seconds. Gently pipette off and discard the supernatant, leaving enough liquid to just cover the pieces of tissue.
17. Resuspend the tissue pieces in 10 mL cold (2 - 8°C) PBS containing 0.1% BSA and pipette up and down three times. Wait for the majority of the intestinal pieces to settle to the bottom (approximately 30 seconds).
18. Using the same pipette, gently remove the supernatant and filter it through a 70 µm filter, collecting the filtrate in a fresh 50 mL conical tube. Discard the filter and label the filtrate "Fraction 1". Place this fraction aside on ice.

19. Repeat steps 17 - 18 three times to generate fractions 2 - 4.

    ***For Colon:*** *Due to the persistence of debris and waste in the murine colon, an additional one or two fractions might be required. Generate the first four fractions, then plate 1 mL of each into a separate well in a 6-well plate and inspect under a bright field microscope at 4X. If fractions 3 and 4 have a high proportion of thin fibrous material, repeat steps 17 - 18 to generate fractions 5 and 6. If fractions 3 and 4 look clean, continue to centrifugation step 20. Proceed to step 20 after any additional fractions have been collected.*

20. Centrifuge the fractions at 290 x *g* for five minutes at 2 - 8°C. Carefully pour off and discard the supernatants, retaining the pellet in each tube.

21. Resuspend each of the pellets in 10 mL cold (2 - 8°C) PBS buffer containing 0.1% BSA. Transfer each suspension to a fresh 15 mL conical tube labeled with the appropriate fraction number.

22. Centrifuge the four fractions at 200 x *g* for three minutes at 2 - 8°C. Gently pour off the supernatants. The pelleted intestinal crypts will remain in the tubes.

## Section 2: Organoid Culture from Isolated Mouse Intestinal Crypts

1. Resuspend crypt fractions in 10 mL cold (2 - 8°C) DMEM/F-12.

2. Add 1 mL of each fraction to individual wells of a 6-well plate and assess the quality of the fractions using an inverted microscope (these samples can be added back to their respective fractions after evaluation). Select one or two fraction(s) that are enriched for intestinal crypts to carry forward for organoid culture. Crypts desirable for culture can be of various sizes, typically rectangular or circular in shape with relatively smooth edges, and resemble small, folded sections of an epithelial monolayer (Figure 5A and 5B). Fractions with higher concentrations of villi, single cells or debris are not suitable for organoid culture. Fractions 3 and 4 often exhibit the greatest enrichment for desirable crypts.

### Example: Crypt Fraction Volume Calculations

Crypts counted in 10 µL aliquot: 15
15 x 100 = approximately 1,500 crypts per mL in fraction
Volumes to centrifuge in step 4:
0.33 mL for 500 crypts
1.0 mL for 1500 crypts
2.0 mL for 3000 crypts

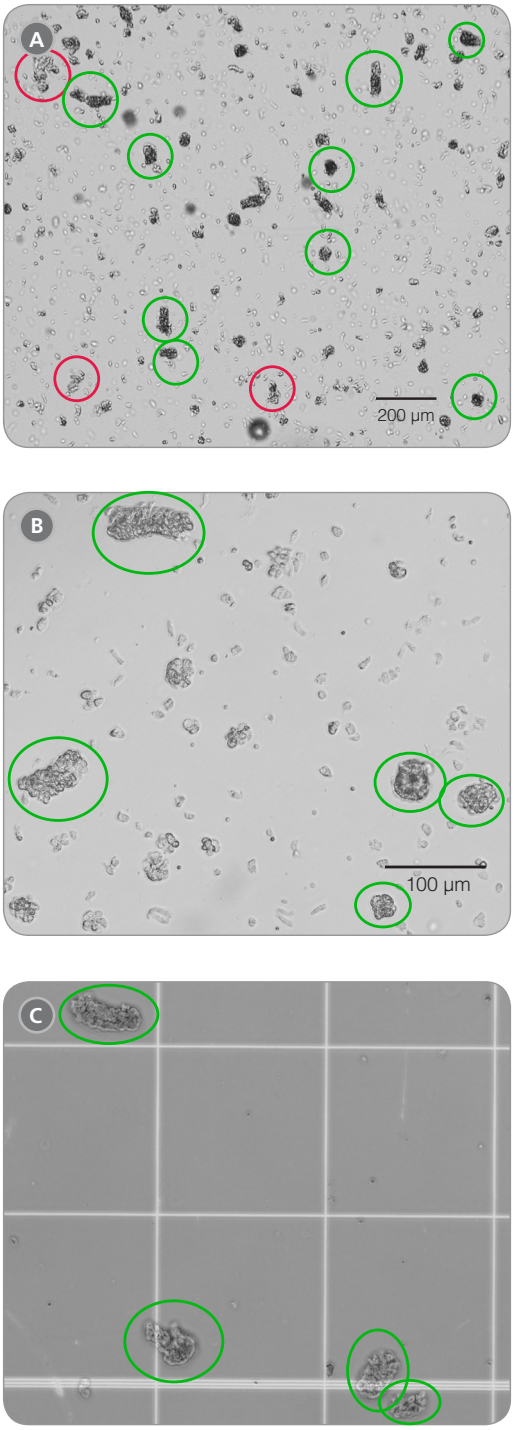


**Figure 5.** Assessing isolated intestinal crypt fractions prior to culture

Light microscope visualizations of intestinal crypt fractions (1 mL in well of 6-well plate) (A) Fraction 2 at 2X magnification; (B) Fractions 3 and 4 combined at 10X magnification; (C) light microscope visualization of intestinal crypt fraction (10 µL on hemacytometer) appropriate for organoid culture. Intestinal crypts circled in green are suitable for organoid culture and should be used to determine crypt concentration in the fraction. Red circles indicate villi, fragments of tissue or debris not suitable for organoid culture.

3. Using a pre-wetted pipette tip, take a 10 µL sample of the selected fraction(s) and deposit on a glass slide or hemacytometer. Using an inverted microscope, count the number of crypts in the aliquot (Figure 5C). Do not count single cells or large, multi-layered tissue fragments. Multiply by 100 to estimate the number of crypts per mL in that fraction.
4. Calculate the volume of the selected fraction(s) containing approximately 500, 1500 and 3000 crypts. Transfer the required volumes to three separate, labeled 15 mL conical tubes and centrifuge at 200 x *g* and 2 - 8°C for five minutes. Carefully pipette off and discard the supernatant.
5. Add 150 µL room temperature (15 - 25°C) complete IntestiCult™ Organoid Growth Medium to the pellet in each tube. Do not use cold medium.

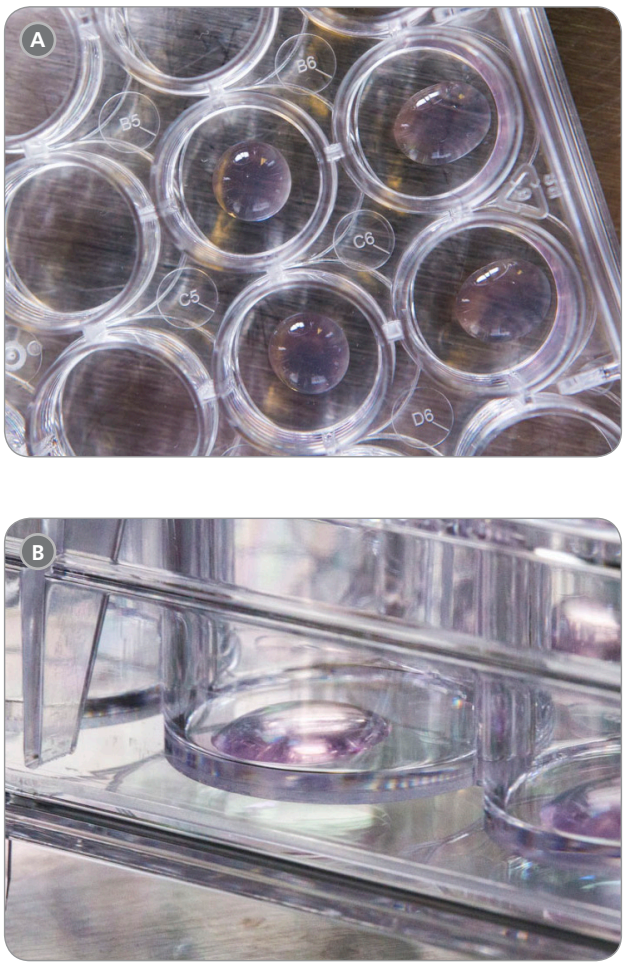


**Figure 6.** Matrigel® domes

Photos of domes containing crypts suspended in a 1:1 mixture of Matrigel® Matrix and IntestiCult™ Organoid Growth Medium taken from (A) above and (B) the side.

6. Add 150 µL undiluted Matrigel® Matrix to each tube. Using the same pipette tip, carefully pipette up and down ten times to resuspend the pellet. Avoid introducing bubbles.
7. Carefully pipette 50 µL of the 500-crypt suspension into the center of each of four wells of the pre-warmed 24-well plate. To prevent bubbles when plating, dispense to the first stop of the pipette. The samples should form domes in the center of each well (Figure 6). Repeat for the 1500-crypt and 3000-crypt suspensions for a total of 12 wells.

   ***Note:*** *Work quickly as the Matrigel® will begin to solidify.*
8. Place the plate at 37°C for 10 minutes to set the Matrigel®. Be careful not to disturb the domes when transferring the plate to the incubator.
9. Add 750 µL room temperature (15 - 25°C) complete IntestiCult™ Organoid Growth Medium to each well by pipetting the medium gently down the sidewall of the well. Do not pipette the medium directly onto the domed cultures.
10. Add sterile PBS to any unused wells to ensure proper hydration of the cultures.
11. Place the lid on the culture plate and incubate at 37°C and 5% $CO_2$.
12. Monitor cultures for organoid growth. Typically, crypts form spherical structures after about three hours of incubation (Figure 7A). After two to four days of incubation, small intestinal organoids typically begin to bud (Figure 7B), and complex, multi-lobed structures form at day five to seven (Figure 7C).

    ***For Colon:*** *Unlike the murine small intestinal system, organoid growth is slower when using colon crypts. By day two small cystic organoids will begin to appear (Figure 7D), and grow in size between days three and seven (Figure 7E). Between days seven and 10 colonic organoids can develop budding structures, though less defined than those of organoids from the small intestine (Figure 7F).*
13. Fully exchange the culture medium three times per week by carefully aspirating the existing liquid medium, keeping the pipette tip at the edge of the well bottom. Replace with 750 µL fresh, room temperature (15 - 25°C) complete IntestiCult™ Organoid Growth Medium.
14. Passage organoid cultures using a 1:6 split ratio after seven to 10 days of culture to avoid over-growth and excessive accumulation of debris within the organoid lumen. Proceed to Section 3 for the passaging protocol.

    ***For Colon:*** *Passage organoid cultures using a 1:2 split ratio seven to 10 days after plating, or when the density reaches 150 organoids per well.*

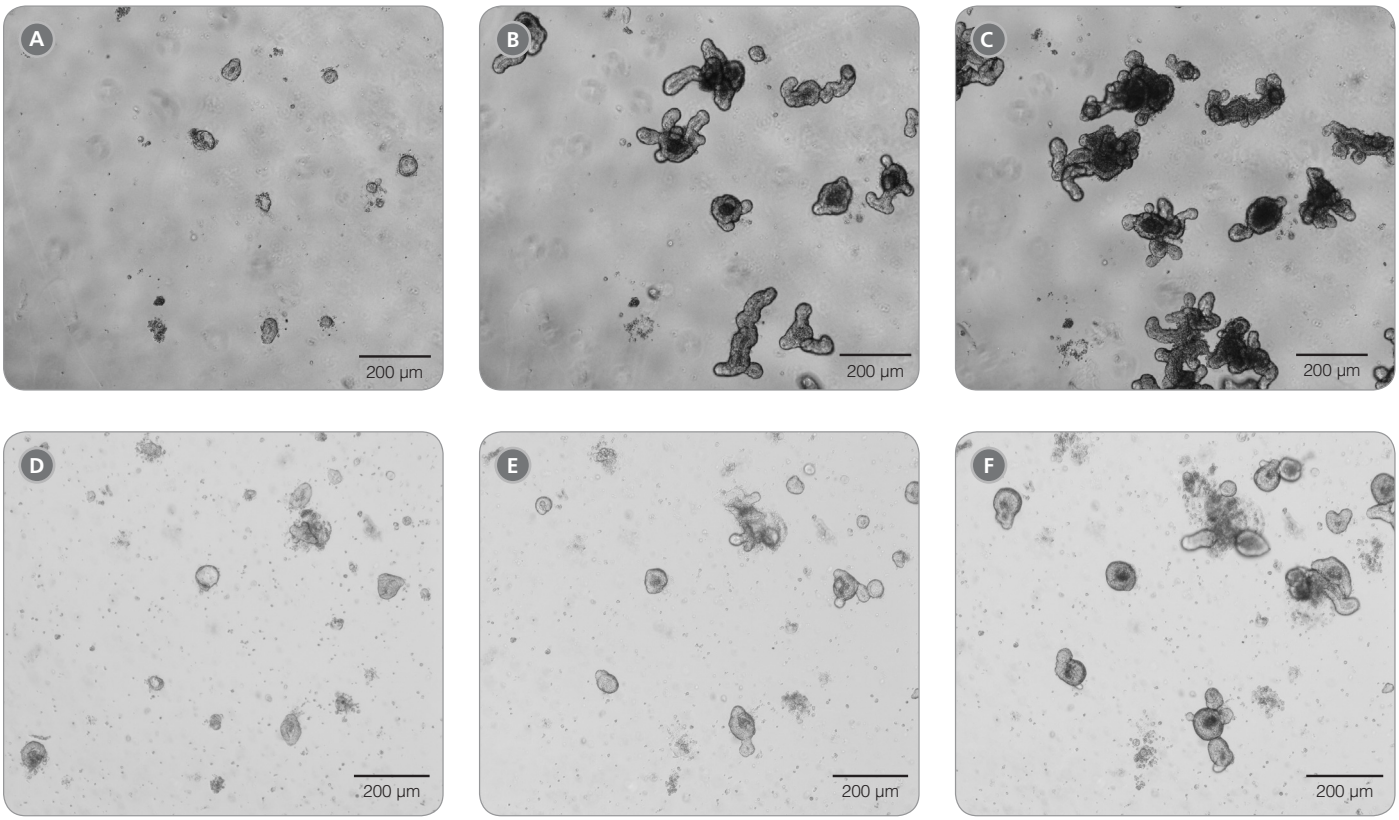


**Figure 7.** A timescale of organoid cultures from small intestine and colon after plating and incubation

Light microscope visualizations of intestinal crypts cultured in domes of 1:1 Matrigel® Matrix and IntestiCult™ Organoid Growth Medium are shown following incubation at 37°C and 5% $CO_2$ for small intestine at (A) Day 1 (B) Day 5 and (C) Day 7, and colon at (D) Day 1 (E) Day 3 and (F) Day 7.

## Section 3: Passaging Mouse Intestinal Organoids

1. On the day of passaging, remove the previously prepared complete IntestiCult™ Organoid Growth Medium from fridge or freezer and leave to warm to room temperature (15 - 25°C). The medium must be warmed to room temperature before use. You will require 4 mL of complete medium per well to be passaged. If you do not have complete medium prepared, please refer to Section 1, steps 1 and 2, of this protocol.
2. Add desired antibiotics to the IntestiCult™ Organoid Growth Medium (once thawed). We recommend 50 µg/mL gentamicin or 100 units/100 µg per mL penicillin/streptomycin.
3. Prepare the additional media, buffers and reagents that will be required during the procedure. Thaw 150 µL Matrigel® Matrix (Corning® Product #356231) per well to be passaged on ice. Also place Gentle Cell Dissociation Reagent (Catalog #07174) and 10 mL DMEM/F-12 with 15 mM HEPES (Catalog #36254) per well to be passaged on ice. Place the required number of TC-treated 24-well culture plates (Corning® Product #3526) at 37°C to pre-warm for 30 minutes.
4. Carefully remove the liquid culture medium from each of the wells to be passaged without disturbing the organoid-containing Matrigel® dome.

5. Add 1 mL Gentle Cell Dissociation Reagent on top of the exposed dome in each well and incubate at room temperature (15 - 25°C) for one minute.
6. Pre-wet a 1000 µL pipette tip with the Gentle Cell Dissociation Reagent in the well, and use it to break up the dome and organoids by pipetting up and down approximately twenty times.
7. Use the same pipette tip to transfer the suspension to a 15 mL conical tube. Rinse the culture well with an additional 1 mL Gentle Cell Dissociation Reagent and add this to the 15 mL tube.
8. Repeat steps 6 and 7 for each well to be passaged.
9. Incubate the 15 mL tubes at room temperature (15 - 25°C) on a rocking platform at 20 rpm for 10 minutes.
10. Centrifuge the tubes at 290 x *g* and 2 - 8°C for five minutes, then gently pour off and discard the supernatant.
11. Wash the pellets by resuspending in 10 mL cold (2 - 8°C) DMEM/F-12 using a pre-wetted 10 mL serological pipette, centrifuging at 200 x *g* at 2 - 8°C for five minutes, then gently pipetting off as much DMEM/F-12 as possible without disturbing the pellet and discarding the supernatant.
12. Add 150 µL room temperature (15 - 25°C) complete IntestiCult™ Organoid Growth Medium to the pellet in each tube. Add 150 µL undiluted Matrigel® Matrix to each tube and pipette up and down ten times to resuspend the pellet. Avoid introducing bubbles.
13. For each tube, pipette 50 µL of the medium/Matrigel® suspension into the center of each of four wells of a pre-warmed 24-well plate to form domes in the center of each well.
14. Place the lid on the culture plate and incubate at 37°C for 10 minutes to set the Matrigel®.
15. Gently add 750 µL room temperature (15 - 25°C) complete IntestiCult™ Organoid Growth Medium to each well by pipetting the medium gently down the sidewall of the well. Do not pipette the medium directly onto the domed cultures.
16. Add sterile PBS to any unused wells.
17. Place the lid on the culture plate and incubate at 37°C and 5% $CO_2$.
18. Exchange the culture medium three times per week by carefully aspirating the existing liquid medium, keeping the pipette tip at the edge of the well bottom. Replace with 750 µL fresh, room temperature (15 - 25°C) complete IntestiCult™ Organoid Growth Medium.

Using this culture system, organoids can be passaged indefinitely.

## Section 4: Cryopreservation of Mouse Intestinal Organoids

This protocol describes freezing and thawing the recommended 200 organoids per cryovial. For optimal results, cryopreservation is best performed when organoids are mature.

***Note:*** *Intestinal organoids are suitable for cryopreservation after two passages from primary culture or frozen; however, for best results organoids that are mature and expressing multiple buds should be used. Organoids from small intestine reach maturity between five to seven days of culture (Figure 8A). Colonic organoids mature more slowly, with less defined budding, reaching maturity between seven to 10 days of culture (Figure 8B).*

1. Prepare all media and reagents required for this protocol. Place PBS without magnesium or calcium (Catalog #37350), DMEM/F-12 with 15 mM HEPES (Catalog #36254) and CryoStor® CS10 (Catalog #07930) to cool on ice. Retrieve the plate containing the organoids to freeze.
2. Using an inverted microscope, count the number of mature organoids found in each well. Combine the contents of multiple wells as needed to achieve 200 organoids in each cryovial.
3. Remove the IntestiCult™ Organoid Growth Medium from each well containing organoids. Replace it with 1 mL of cold (2 - 8°C) PBS.
4. Break up the Matrigel® Matrix by pipetting up and down ten to twenty times with a PBS-wetted 1000 µL pipette tip. Transfer suspensions containing 200 organoids, combining wells if necessary, to a single 15 mL conical tube.
5. Wash each well with 1 mL of cold (2 - 8°C) PBS by pipetting up and down five times with a pre-wetted 1000 µL pipette tip and transfer to the 15 mL conical tube.
6. Pellet the organoids by centrifuging at 290 x *g* for five minutes at 2 - 8°C. Remove and discard the supernatent, being careful not to disturb the organoid pellet.
7. Wash the organoid pellet by resuspending in 7 to 10 mL of cold DMEM/F-12 with 15 mM HEPES. Gently flick the tube, or gently pipette the contents, to help break down the pellet if needed. Centrifuge the suspension at 200 x *g* for five minutes at 2 - 8°C then carefully remove and discard the supernatant.
8. Resuspend the organoid pellet in cold (2 - 8°C) CryoStor® CS10 freezing medium using 1 mL of freezing medium per cryovial of 200 organoids.
9. Using the same pipette tip, move the organoids suspended in CryoStor® CS10 to a labeled cryovial. Place the cryovial in a freezing container with 500 mL of isopropyl alcohol, or in an IPA-free freezing container.

10. Transfer the freezing container to a -80°C freezer for 24 hours then transfer the cryovial to liquid nitrogen (-135°C) for long-term storage. Intestinal organoids can be stored at -135°C for six months. Long-term storage at -80°C is not recommended.

    ***Note:*** *Work quickly to avoid prolonged exposure of non-frozen organoids to CryoStor® CS10.*

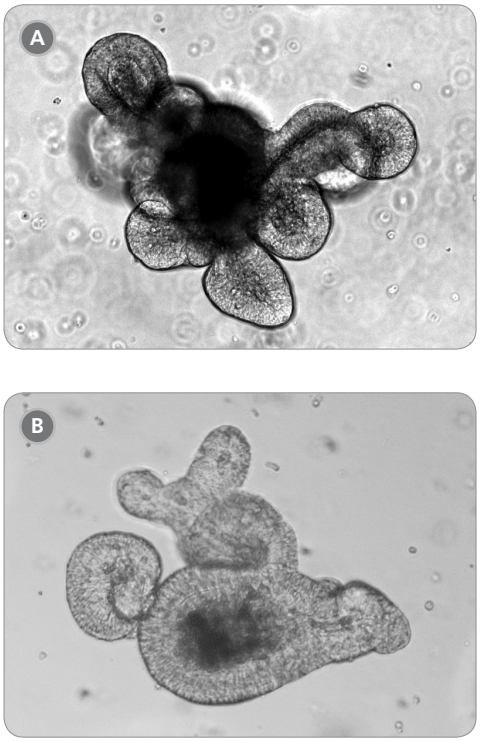


**Figure 8.** Light microscope visualization (10X) of mature intestinal organoids cultured in domes of 1:1 Matrigel® Matrix and IntestiCult™ Organoid Growth Medium and incubated at 37°C and 5% $CO_2$. (A) Small intestinal organoid after five days of culture and (B) colon organoids after 10 days of culture.

## Section 5: Thawing of Mouse Intestinal Organoids

1. Thaw 120 µL of Matrigel® Matrix (Corning® Product #356231) on ice and let previously prepared complete IntestiCult™ Organoid Growth Medium warm to room temperature (15 - 25°C). You will need 3.1 mL of complete media for four wells of a 24-well plate. If you do not have any prepared IntestiCult™ complete media refer to steps 1 - 4 in Section 1 of this protocol. Place a 24-well TC-treated plate (Corning® Product #3526) in a $CO_2$ incubator at 37°C to warm for 30 minutes.
2. Combine 2 mL of 25% BSA stock solution with 48 mL of DMEM/F-12 with 15 mM HEPES (Catalog #36254) in a 50 mL conical tube to generate a DMEM/F-12 washing solution containing 1% BSA. Leave at room temperature (15 - 25°C) for the duration of this protocol. The washing solution can be stored at 2 - 8°C for up to six months.
3. To a 15 mL conical tube, add 2 mL of DMEM/F-12 with 1% BSA solution at room temperature (15 - 25°C).

   ***Note:*** *Cells should be transferred to this tube immediately after thawing to avoid significant reduction in viability.*
4. Retrieve and thaw the frozen organoids by placing the cryovial in a 37°C water bath. Thawing is complete when the freezing medium becomes liquid, at which point the organoids will be visible at the bottom of the tube. Thawing at 37°C should take between 2 and 2.5 minutes; over-warming the medium may affect the growth efficacy of the organoids in culture.
5. Wipe the outside of the cryovial with 70% ethanol or isopropanol before opening. Add 1 mL of DMEM/F-12 with 1% BSA solution directly to the cryovial using a 1000 µL pipette. Mix the contents of the cryovial by pipetting up and down four times. Immediately transfer the contents of the cryovial to the 15 mL conical tube containing 2 mL of DMEM/F-12 with 1% BSA solution using a pre-wetted 1000 µL pipette tip.
6. Wash the cryovial two more times with 1 mL of DMEM/F-12 with 1% BSA solution and transfer to the conical tube. Be sure to wash the entire inner surface area of the cryovial, including the inside of the lid.
7. Wash the organoids to remove the CryoStor® CS-10 by centrifuging the organoid suspension at 200 x *g* for five minutes at 2 - 8°C. Carefully remove and discard the supernatent. Avoid introducing bubbles. If bubbles are present after centrifugation, carefully aspirate to remove the bubbles first, prior to aspirating the body of the supernatent.

8. Using a 200 µL pipette tip, resuspend the organoids by adding 100 µL of room temperature (15 - 25°C) complete IntestiCult™ Organoid Growth Medium.
9. Add 100 µL of Matrigel® using a 200 µL pipette tip. Mix the suspension by pipetting up and down five to ten times to ensure a consistent density and viscosity throughout the sample. Avoid introducing bubbles.
10. Using a pre-wetted 200 µL pipette tip, add 50 µL of the organoid suspension to four wells of the pre-warmed 24-well plate such that it forms a dome in the center of the each well. When plating, dispense to the first stop of the pipette to avoid introducing bubbles. Incubate the organoids at 37°C and 5% $CO_2$ for 10 minutes to allow the Matrigel® to solidify.
11. Add 750 µL of room temperature (15 - 25°C) complete IntestiCult™ Organoid Growth Medium to each well containing a Matrigel® dome by pipetting the medium gently down the wall of the well. Do not pipette directly onto the domed cultures.
12. Add sterile PBS to any unused wells.
13. Place the lid on the culture plate and incubate at 37°C and 5% $CO_2$. Exchange the media three times per week.
14. For the best results, passage the previously frozen organoids two times after thawing. After freezing, organoid growth will be slow. Organoids should be ready for passaging between five and seven days of culture after thawing, and five days after each passage. Typical organoid growth characteristics should be restored after one passage from frozen (Figure 9).

    ***Note:*** *It is not recommended to expose organoids to consecutive freeze-thaw cycles.*

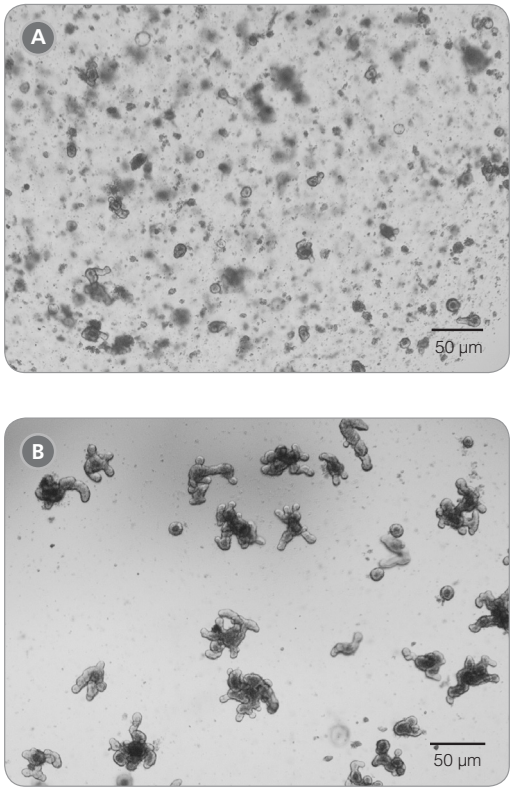


**Figure 9.** Intestinal organoid cultures from previously frozen organoids as visualized by light microscopy

Thawed intestinal organoids were cultured in domes of 1:1 Matrigel® Matrix and IntestiCult™ Organoid Growth Medium and incubated at 37°C and 5% $CO_2$. (A) Passage 0, Day 5; (B) Passage 1, Day 6.

# IntestiCult™ FAQs

Can the IntestiCult™ Organoid Growth Medium be thawed in a 37°C water bath rather than at room temperature (15 - 25°C)?

Aliquots of IntestiCult™ Organoid Growth Medium can be thawed in a 37°C water bath before use, however, we do not recommend refreezing medium that has been thawed using this method.

Why is the crypt isolation procedure performed at 4°C using cold DMEM/F-12?

The procedure for crypt isolation is performed at 4°C to ensure minimal damage to the intestinal crypts.

Can the segments of the intestine be larger than 2 mm? What is the range for the size?

Intestinal segments of 2 mm enable efficient washing and crypt dissociation using the method described in the protocol. Larger segments may require more rounds of rinsing and may result in lower crypt recovery.

Is it okay to centrifuge the pieces rather than wait for them to settle by gravity? If so, what speed should be used?

We recommend letting the pieces settle by gravity as centrifugation during washing may result in pelleting of additional impurities, while centrifugation during crypt isolation may result in pelleting of crypts and result in poor crypt recovery.

Why shouldn't multi-layered tissue fragments be counted when estimating the number of crypts per ml in the fraction? What are they if not aggregated crypts?

Large, multi-layered fragments are likely debris that were not separated out during the crypt isolation procedure. These pieces likely do not contain stem cells and will not develop into organoids. Such fragments are likely to be enriched in the earlier crypt fractions, and using the later fractions should eliminate most of these multi-layered structures.

Why can't cold IntestiCult™ medium be used in step 5 of the Intestinal Organoid Culture from Isolated Mouse Intestinal Crypts protocol? What should I do if I accidentally used cold IntestiCult?

Room temperature medium should be used to mix with the crypts and Matrigel® Matrix as cold medium will dissolve the matrix. If you accidentally use medium that has not warmed to room temperature, allow the mixture to warm room temperature before plating as domes in the pre-warmed 24-well plate. Note that once the medium and Matrigel® Matrix are combined, you will need to work quickly as it will start to solidify. The four wells should be plated within 30 - 60 seconds. Cooling the mixture briefly on ice will lower the viscosity again if the wells are not plated within this time.

What will happen if I use warm DMEM/F-12 in step 11 of Passaging of Mouse Intestinal Organoids protocol?

We recommend using cold DMEM/F-12 to wash the dissociated organoid pellets. Using warm medium could result in lower recovery due to cell damage during washing.

What will happen if organoid cultures are plated at a seeding density higher or lower than the recommended 200 organoids per well?

Organoids grow well when near other organoids, yet too high a seeding density can cause strain on the culture. It is not recommended to plate organoid cultures at a density higher than 200 organoids per well; however, we have shown that even at a density of 400 organoids per well, organoids recover within two passages (Figure 10). Organoids plated at a lower seeding density have been shown to recover after one passage.

Will organoids recover if they have been degraded before or after cryopreservation?

We have been able to show that as long as there are healthy, single, intestinal stem cells in the culture, previously degraded organoids recover after one passage (Figure 11). These organoids generally take an additional three to five days to establish, and will likely require multiple passages to generate an adequate population of organoids.

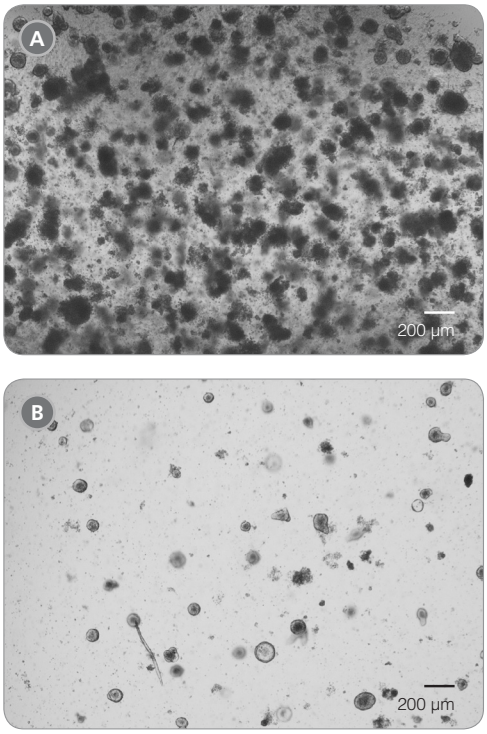


**Figure 10.** Intestinal organoid cultures from previously frozen organoids. Light microscope visualization of organoid cultures when plated at too high a seeding density

Four hundred cryopreserved organoids were thawed, plated in a single 50 µL dome of 1:1 Matrigel® Matrix and IntestiCult™ Organoid Growth Medium and incubated at 37°C and 5% $CO_2$. (A) Passage 0, Day 5 and (B) Passage 1, Day 6.

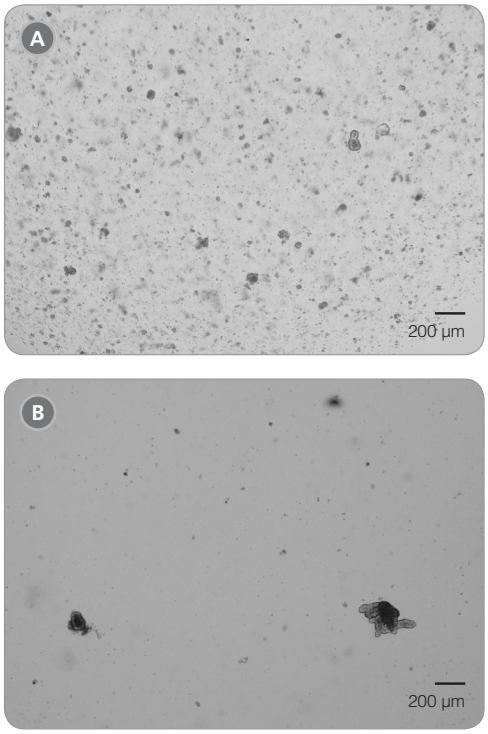


**Figure 11.** Light microscope visualizations of intestinal organoid cultures from frozen organoids that had been degraded either before or after cryopreservation

Organoids cultured in domes of 1:1 Matrigel® Matrix and IntestiCult™ Organoid Growth Medium are shown following incubation at 37°C and 5% $CO_2$ at (A) Passage 0, Day 5 and (B) Passage 1, Day 6.

# Intestinal Epithelial Organoid Culture with IntestiCult™ Organoid Growth Medium (Mouse)

## Product Information

| PRODUCT | QUANTITY | CATALOG # |
|---|---|---|
| IntestiCult™ Organoid Growth Medium (Mouse) | 1 Kit (100 mL Complete Medium) | 06005 |
| Gentle Cell Dissociation Reagent | 100 mL | 07174 |
| Dulbecco's Phosphate Buffered Saline (D-PBS) Without Calcium and Magnesium | 500 mL | 37350 |
| DMEM/F-12 with 15 mM HEPES | 500 mL | 36254 |
| Matrigel® GFR and Phenol Red-Free Basement Membrane Matrix | 10 mL | (Corning®) 356231 |
| CryoStor® CS10 | 100 mL | 07930 |
| Bovine Serum Albumin (BSA) | --- | --- |
| Costar® 24 Well Clear TC-Treated Multiple Well Plates, Individually Wrapped, Sterile | 50 plates | (Corning®) 3526 |
| Thermo Scientific™ Nunc™ Biobanking and Cell Culture Cryogenic 1.8 mL Tubes | 450 Tubes | (Thermo Fisher®) 12-565-167N |
| Falcon® 70µm Cell Strainer | 50 strainers | (Corning®) 352350 |

For more information, please visit **www.IntestiCult.com** or contact our Technical Support Department at **techsupport@STEMCELL.com**.

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