TECHNICAL BULLETIN

Supplementary Protocols for IntestiCult™ Organoid Growth Medium (Human)

Introduction

The following are supplementary protocols for culturing intestinal organoids with IntestiCult™ Organoid Growth Medium (Human) (IntestiCult™ OGMH; Catalog #06010). These protocols include: differentiating intestinal organoids, culturing intestinal epithelial monolayers, as well as cryopreserving and thawing intestinal organoids. For complete culturing methods, use this document in coordination with the Product Information Sheet (Document #DX21423), which includes a materials list and instructions for isolating human colonic crypts from biopsy samples, establishing human intestinal organoids from the isolated crypts, as well as expanding and maintaining organoid cultures via passaging.

Required Materials

PRODUCT	CATALOG #
IntestiCult™ Organoid Growth Medium (Human)	06010
DMEM/F-12 with 15 mM HEPES	36254
Corning® Matrigel® Matrix, Growth Factor Reduced (GFR), Phenol Red-Free	Corning® 356231
D-PBS (Without Ca^{++} and Mg^{++})	37350
Y-27632	72302
Gentle Cell Dissociation Reagent	07174
Trypsin-EDTA (0.05%)	07910
CryoStor® CS10	07930
Bovine serum albumin (BSA)	---

Differentiating Intestinal Organoids

IntestiCult™ OGMH has been optimized to establish human colonic and intestinal epithelial organoid cultures, and to efficiently maintain and expand these cultures. The passaged organoid cultures are typically composed of cells that are more stem or progenitor in nature, compared with epithelial cells in adult intestinal tissue. This protocol describes a method for achieving more advanced differentiation of the cells within the organoid cultures, such that a thicker, more columnar epithelial monolayer is evident and features of the terminally differentiated cell types of the intestinal tract are represented. It should be noted that after following this protocol, the resulting differentiated organoids do not contain a significant adult stem cell population and cannot be further expanded or passaged.

Medium Preparation

The following example is for preparing 100 mL of Intestinal Organoid Differentiation Medium (IntestiCult™ OGMH Component A + DMEM/F-12 with 15 mM HEPES). If preparing other volumes, adjust accordingly.

1. Thaw IntestiCult™ OGMH Component A at room temperature (15 - 25°C) or at 2 - 8°C overnight. Mix thoroughly. Once thawed, use immediately or aliquot and store at -20°C for up to 3 months. After thawing the aliquots, use immediately. Do not re-freeze.
2. Add 50 mL IntestiCult™ OGMH Component A to 50 mL DMEM/F-12 with 15 mM HEPES. Mix thoroughly. If not used immediately, store at 2 - 8°C for up to 1 week.
3. Add desired antibiotics immediately before use (e.g. 50 µg/mL gentamicin).

For preparing IntestiCult™ OGMH, refer to the Product Information Sheet (Document #DX21423).

Differentiation of Intestinal Organoids

1. Establish organoid cultures as described in the Product Information Sheet (Document #DX21423). Passage organoids a minimum of one time before beginning the differentiation protocol. If organoids were generated using a different culture system, passage in IntestiCult™ OGMH for three passages prior to beginning the differentiation protocol.
2. At the start of the passage in which differentiation will be performed (Day 0), seed cultures as per the standard protocol using IntestiCult™ OGMH (1:1 mixture of Component A and Component B). Incubate at 37°C and 5% CO_2.
3. Perform a full medium change with IntestiCult™ OGMH on Day 2 and Day 4.
4. On Day 5, induce differentiation by performing a full medium change with Intestinal Organoid Differentiation Medium. Incubate at 37°C and 5% CO_2.

   NOTE: The growth phase can be continued for up to 9 days before inducing differentiation, depending on the size and quality of the organoids (Fig 1). If the organoids are not ready for differentiation by Day 9, continue performing full medium changes with IntestiCult™ OGMH every 2 days. Following differentiation, perform a full medium change with Intestinal Organoid Differentiation Medium every 2 days.

5. Organoids can remain in culture for 5 days after switching to Intestinal Organoid Differentiation Medium. It is typical to see characteristics of differentiation in the organoids after 2 - 3 days (Fig 2). This includes thickening of the organoid epithelial monolayer as cells take on a more columnar morphology, appearance of goblet cells and other specialized cell types, and accumulation of dead, apoptotic cells over time.

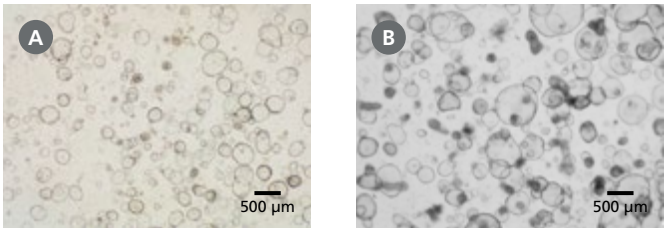

Figure 1. Size and Quality of Intestinal Organoids Prior to Differentiation Determines the Success of the Differentiation

A. Organoids that are too small and not yet ready for differentiation.

B. Organoids that are the correct size and quality for initiating differentiation.

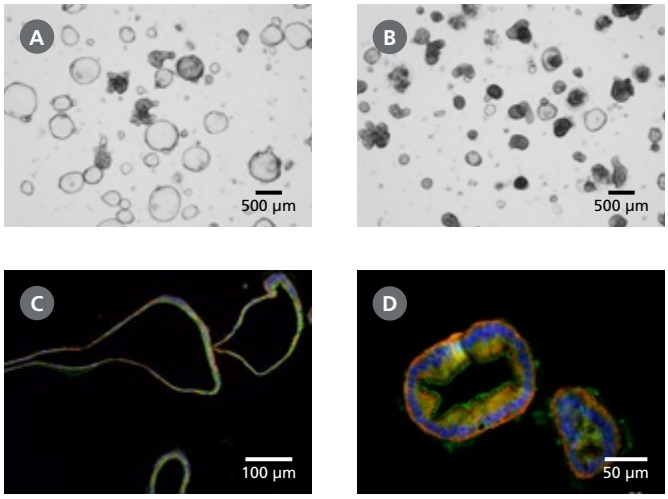

Figure 2. Organoid Morphology Changes as They Differentiate

As organoids differentiate, they begin to exhibit a morphology with increased epithelial layer thickness and more differentiated cell types. Undifferentiated organoids (A, C) have thinner walls, are less polarized, and show minimal staining for mature cell types. Once differentiated (B, D), the organoids show thickening of the epithelium as the cells begin to exhibit a columnar morphology. Specialized cells, including goblet cells, appear and there may be some dead cells in the culture.

A. Undifferentiated organoids after 9 days in IntestiCult™ OGMH. Organoids are mostly thin-walled with vacuous centers and are the correct size and quality to initiate differentiation.

B. Differentiated intestinal organoids. Most organoids in the culture have thickened walls and less vacuous centers.

C. Stained undifferentiated organoids after 9 days of growth in IntestiCult™ OGMH. Organoid walls are thin and lacking extensive polarization. Shown are Villin (green), E-cadherin (red), and DAPI (blue).

D. Stained, differentiated organoids after 4 days in Intestinal Organoid Differentiation Medium. Shown are Villin (green), E-cadherin (red), and DAPI (blue).

Growing Intestinal Organoids as a Monolayer

Intestinal organoids have provided researchers with a more physiologically-relevant cell model, which is being used across a range of research disciplines and can increase flexibility and precision in experimental design. However, intestinal organoid cultures do have the limitation of a closed luminal compartment, a physical characteristic that presents challenges for specific experiments. This protocol describes a method for using cells expanded in intestinal organoid cultures to generate intestinal epithelial monolayer cultures. These monolayers enable easy access to the apical side of the epithelial monolayer, facilitating studies such as those involving apical cell surface receptors, interaction with commensal or pathogenic microorganisms, or modeling the effect of potentially beneficial or harmful compounds in intestinal contents. Intestinal epithelial monolayer cultures can be established on glass coverslips that enable high-quality imaging, or on Transwell® permeable supports that enable measurement of active and passive transport of substances across the epithelial layer, as well as electrical barrier function assays. Monolayers can be generated using a range of cultureware, giving flexibility in experimental throughput.

In the protocol below, cells that have been expanded as organoid cultures are seeded onto a dilute culture matrix where they form a confluent monolayer and differentiate to model the intestinal epithelium within 7 days. These monolayers can be maintained with regular medium changes for up to 3 weeks.

Coating Cultureware

For growing intestinal organoids as a monolayer, coat cultureware with a dilute Matrigel® solution. The following instructions are for coating cultureware with Corning® Matrigel®; defined matrices such as Collagen I, Collagen IV, and Vitronectin XF (Catalog #07180) can also be used (see manufacturer's instructions).

1. Thaw one aliquot of Corning® Matrigel® on ice.
2. Dispense an appropriate amount of cold (2 - 8°C) D-PBS into a conical tube and keep on ice. See Table 1 for recommended volumes.
3. Add thawed Matrigel® to the cold D-PBS at a ratio of 1 µL of Matrigel® to 49 µL D-PBS. Mix well and keep on ice.
4. Immediately use the diluted Matrigel® solution to coat tissue culture-treated cultureware. See Table 1 for recommended coating volumes.

   NOTE: Ensure the solution evenly coats the entire bottom surface of the cultureware; insufficient coating results in poor cell attachment.

5. Incubate at 37°C for at least 1 hour before use. Do not let the Matrigel® solution evaporate.

   Note: If not used immediately, seal the cultureware to prevent evaporation of the Matrigel® solution and store at 2 - 8°C for up to 1 week. Allow stored coated cultureware to warm to room temperature for 30 minutes before proceeding to the next step.

6. Gently tilt the cultureware and allow the excess Matrigel® solution to collect at the edge. Remove the excess Matrigel® solution using a serological pipette or by aspiration, ensuring that the coated surface is not scratched.

Table 1. Recommended Volume of Dilute Matrigel®

CULTUREWARE	VOLUME OF DILUTE MATRIGEL® PER WELL
6-Well Plate	1000 µL
24-Well Transwell® Plate	100 µL
24-Well Plate	200 µL
96-Well Plate	100 µL

Medium Preparation

The following example is for preparing 100 mL of Monolayer Growth Medium (IntestiCult™ OGMH Component A + IntestiCult™ OGMH Component B + Y-27632). If preparing other volumes, adjust accordingly.

1. Thaw IntestiCult™ OGMH Component A and Component B at room temperature (15 - 25°C) or at 2 - 8°C overnight. Mix thoroughly.

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

2. Add 50 mL of Component B to 50 mL of Component A. Mix thoroughly.
3. Add 10 µM Y-27632. Mix thoroughly.

   NOTE: If not used immediately, store at 2 - 8°C for up to 1 week.

4. Add desired antibiotics immediately before use (e.g. 50 µg/mL gentamicin).

Plating Intestinal Organoids as a Monolayer

1. Harvest 3 - 4 Matrigel® domes of intestinal organoids (approximately 100 - 150 organoids) as described below. See Table 2 for the recommended number of domes to harvest for various cultureware.
2. Add 1 mL of Gentle Cell Dissociation Reagent to each well containing a dome to be harvested.
3. Incubate at room temperature (15 - 25°C) for 1 minute.
4. Using a 1000 µL pipettor, pipette vigorously to disrupt the Matrigel® dome and resuspend the organoids.
5. Incubate the suspension at room temperature for 10 minutes with gentle agitation or rocking.
6. Pool the harvested wells and centrifuge at 200 x *g* for 5 minutes at 2 - 8°C.
7. Discard the supernatant and add 3 mL ice-cold DMEM/F-12. Centrifuge at 200 x *g* for 5 minutes at 2 - 8°C.
8. Aspirate to remove as much of the supernatant as possible, being careful not to disturb the pellet. Resuspend the organoids in 1 mL of warm (37°C) Trypsin-EDTA (0.05%).
9. Using a 1000 µL pipettor, pipette up and down to mix thoroughly. Incubate the organoids at 37°C for 5 - 10 minutes.
10. Mix organoids thoroughly by vigorous pipetting or vortexing to disrupt the organoids as much as possible. Check the organoids using a microscope to ensure 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 (Fig 3).

    NOTE: Perform the remaining steps as quickly and efficiently as possible, as cells will start to clump together.

11. Add 1 mL DMEM/F-12 and mix thoroughly by pipetting. Centrifuge fragments at 200 x *g* for 5 minutes at 2 - 8°C.
12. Remove the supernatant and resuspend organoid fragments in Monolayer Growth Medium as indicated in Table 3.

Table 2. Recommended Number of Domes to Harvest When Plating Intestinal Organoids as a Monolayer

CULTUREWARE TO BE SEEDED	NUMBER OF DOMES TO HARVEST PER WELL TO BE SEEDED*
24-Well Plate	3 - 4 domes
24-Well Transwell® Plate	2 - 3 domes
96-Well Plate	1 dome

*Assuming 50 µL domes being harvested

NOTE: Seeding efficiency may vary depending on passage number of the organoids.

Table 3. Volume of Monolayer Growth Medium for Resuspending Organoids.

CULTUREWARE TO BE SEEDED	VOLUME OF MONOLAYER GROWTH MEDIUM PER WELL
6-Well Plate	1.5 mL
24-Well Plate	500 µL
24-Well Transwell® Plate	100 µL upper
96-Well Plate	100 µL

13. Slowly and gently add the cell suspension to each well. Incubate at 37°C and 5% CO_2. Monitor growth daily. Perform a full medium change every 2 - 3 days.

    Note: Monolayers generally reach 100% confluency within 2 - 3 days; in the event of poor attachment, slow proliferaton may be observed, but confluency is usually reached if given enough time.

Once the cultures are established, the cells remain viable and monolayer conflueny is maintained for at least 3 weeks, assuming the medium continues to be changed regularly. If needed for specific experiments, the Monolayer Growth Medium can be replaced with DMEM/F-12 for at least 24 hours without a significant reduction in cell viability or monolayer integrity. The monolayer can be maintained with Intestinal Organoid Differentiation Medium for at least 1 week to promote further cell differentiation.

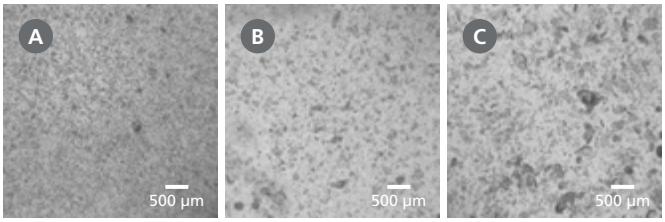

Figure 3. Organoids Must be Disrupted to Single Cells or Small Clumps for Seeding into Monolayers

A. Single cells are ideal for seeding into monolayers.

B. Small clumps are acceptable for seeding into monolayers.

C. Large clumps are not acceptable for seeding into monolayers. If large clumps remain after disruption, repeat pipetting and vortexing (Step 10) to achieve small clumps or single cells.

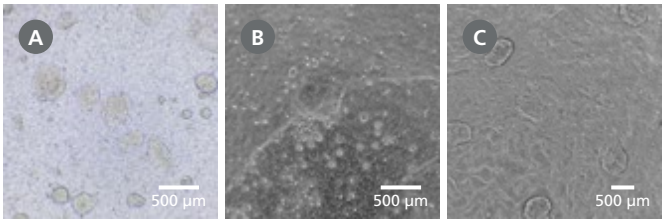

Figure 4. Growth of Intestinal Monolayers in 24-Well Transwell® Plates

Over time, the monolayer reaches 100% confluency. Time to 100% confluence is generally 2 - 3 days but may vary depending on the seeding efficiency, which is partly dependent on the passage number of the organoids.

A. Human intestinal monolayers at low confluency (less than 25%).

B. Human intestinal monolayers 2 days post-seeding (50% confluent).

C. Human intestinal monolayers after 7 days in culture (100% confluent).

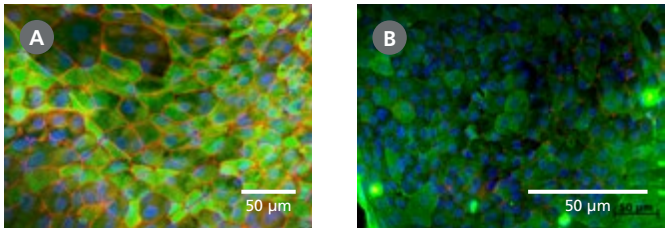

Figure 5. Intestinal Monolayer After 7 Days in Culture

Intestinal monolayer cultures were grown on glass coverslips in the bottom of a well and stained and imaged using fluorescent microscopy.
A. Strong staining for E-cadherin (red), Villin (green), and DAPI (blue).
B. Cytokeratin 18 (green) and Tricellulin (red) indicating the presence of tricellular tight junctions within the monolayer. Cell nuclei are stained with DAPI (blue).

Cryopreserving Intestinal Organoids

This protocol describes cryopreservation of 200 organoids per cryovial. For optimal results, cryopreservation should be performed when organoids are mature (~7 - 10 days post-passage).

NOTE: Intestinal organoids are suitable for cryopreservation after two passages from either primary culture or from organoids that have been cryopreserved, thawed, and cultured. Larger cystic or budded organoids result in a higher yield of viable fragments than smaller, dark, or collapsed organoids (Fig 6).

1. Place D-PBS (Without Ca^{++} and Mg^{++}), DMEM/F-12 with 15 mM HEPES, and CryoStor® CS10 on ice.
2. Count the number of mature organoids in each well. Combine the contents of multiple wells as needed to have 200 organoids in each cryovial.
3. Remove medium from each well to be cryopreserved and replace it with 1 mL of cold D-PBS.
4. Pre-wet a 1000 µL pipette tip with cold D-PBS and disrupt the Matrigel® dome by vigorously pipetting up and down 10 - 20 times. 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 D-PBS and transfer to the 15 mL conical tube.
6. Centrifuge organoids at 290 x *g* for 5 minutes at 2 - 8°C. Remove and discard supernatant, being careful not to disturb the organoid pellet.
7. Add 7 - 10 mL of cold DMEM/F-12 with 15 mM HEPES. Gently flick the tube, or gently pipette up and down, to help resuspend the pellet if needed. Centrifuge the suspension at 200 x *g* for 5 minutes at 2 - 8°C. Carefully remove and discard the supernatant.
8. Resuspend the organoid pellet in 1 mL of cold CryoStor® CS10 per cryovial of 200 organoids.

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

9. Using the same pipette tip, transfer the organoid suspension 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. Long-term storage at -80°C is not recommended.

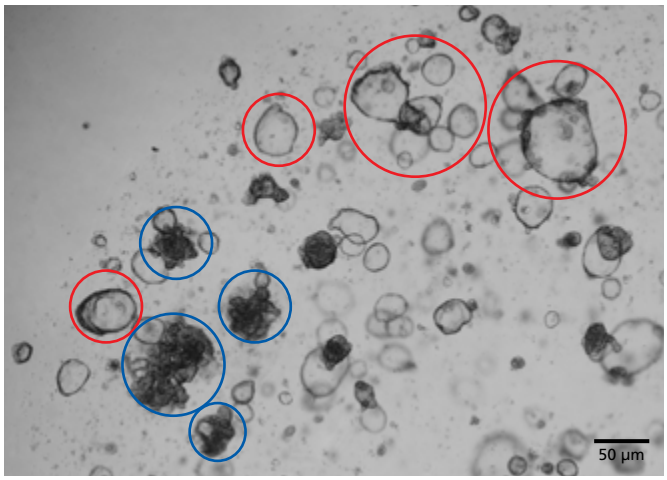

Figure 6. Human Intestinal Organoids for Cryopreservation

Human intestinal organoids should be cryopreserved when they are mature, approximately 7 - 10 days post-passage. For best results, larger cystic or budded organoids should be used (red circles). Smaller, dark, or collapsed organoids (blue circles) may not provide acceptable viability post-thaw.

Thawing Cryopreserved Intestinal Organoids

1. Thaw 120 µL of Matrigel® on ice. Prepare complete IntestiCult™ OGMH and warm to room temperature (15 - 25°C). For 4 wells of a 24-well plate, 3.1 mL of medium is required. Place a 24-well tissue culture-treated plate (Catalog #38017) in a 37°C incubator for 30 minutes.
2. Prepare Wash Solution by adding 2 mL of 25% bovine serum albumin (BSA) stock solution to 48 mL of DMEM/F-12 with 15 mM HEPES in a 50 mL conical tube. Store at room temperature for the duration of this protocol, or at 2 - 8°C for up to 6 months.
3. Add 2 mL of Wash Solution (prepared in step 2) at room temperature to a 15 mL conical tube.
4. Thaw 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 are visible at the bottom of the tube. Thawing at 37°C should take 2 - 2.5 minutes; over-warming the medium may affect organoid growth.

   NOTE: Proceed to the next step immediately after thawing organoids to avoid a significant reduction in viability. It is not recommended to expose organoids to consecutive freeze-thaw cycles.
5. Using a 1000 µL pipettor, add 1mL Wash Solution directly to the thawed organoids. Using a pre-wetted pipette tip, mix the contents of the cryovial by pipetting up and down 4 times. Immediately transfer the contents of the cryovial to the 15 mL conical tube containing 2 mL of Wash Solution.
6. Wash the cryovial with 2 x 1 mL of Wash Solution, transferring each wash to the conical tube. Be sure to wash the entire inner surface area of the cryovial, including the inside of the lid.
7. Centrifuge the organoid suspension at 200 x *g* for 5 minutes at 2 - 8°C. Carefully remove and discard the supernatant. Avoid introducing bubbles; if bubbles are present, carefully aspirate to remove the bubbles prior to removing the remainder of the supernatant.
8. Using a 200 µL pipette tip, add 100 µL of IntestiCult™ OGMH to resuspend organoids.
9. Using a 200 µL pipette tip, add 100 µL of Matrigel®. Mix the suspension by pipetting up and down 5 - 10 times, ensuring a consistent density and viscosity throughout the sample. Pipette only to the first stop to avoid introducing bubbles.
10. Using a pre-wetted 200 µL pipette tip, add 50 µL of the organoid suspension to each of four wells of the warm 24-well plate (prepared in step 1) such that it forms a dome in the center of each well. When plating, dispense to the first stop of the pipettor 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 IntestiCult™ OGMH 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 D-PBS to any unused wells. Place the lid on the culture plate and incubate at 37°C and 5% CO_2. Perform a full medium change 3 times per week.
13. For best results, passage previously frozen organoids two times after thawing before beginning experiments. Organoid growth may be slow in the first passage after thawing. Organoids should be ready for passaging at 7 - 14 days of culture after thawing, and 7 - 10 days after each passage.

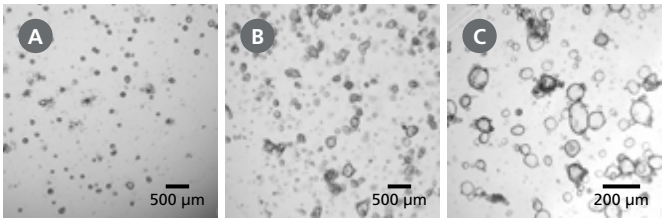

Figure 7. Intestinal Organoids Recover Quickly After Thawing

A. One day post-thaw, organoids are small but bright and healthy-looking.

B. After 4 - 5 days, organoids have begun to form buds.

C. Healthy human intestinal organoids after 2 passages post-thaw are ready for downstream culture, differentiation, or other experiments.

Related Products for Culturing Intestinal Organoids

PRODUCT	QUANTITY	CATALOG #
IntestiCult™ Organoid Growth Medium (Mouse)	100 mL	06005
STEMdiff™ Intestinal Organoid Kit	1 Kit	05140
STEMdiff™ Intestinal Organoid Growth Medium	100 mL	05145
Mouse Intestinal Organoids	200 Organoids	70931

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