HEPATOPAC® Kit

MAINTENANCE INSTRUCTIONS

Basic Handling, Best Practices, and Troubleshooting Tips

TP-001 V2.0

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Product Description

HEPATOPAC kits contain the necessary materials to maintain HEPATOPAC cultures.

Materials and Storage

NOTE:
Read the Section entitled "Unpacking Instructions" before unpacking any boxes and complete Steps 1 – 6 before unpacking HEPATOPAC Box(es) C.

Upon receipt of boxes, refer to these instructions for unpacking and caring for the HEPATOPAC kit.

Kit Contents

Box(es) A: Store at -20°C

- HEPATOPAC Culture Media Components
- HEPATOPAC Maintenance Instructions and Application Protocol

Box(es) B: Store at 2-8°C

- HEPATOPAC Culture Media Components
- Additional Sterile Lids

Box(es) C: Store at 37°C/**10% CO_2** Incubator, humidified with full water pan ≥ 95%[1]

- HEPATOPAC Plate(s)
- Stromal Only Plate(s) as applicable

Additional Required Equipment and Materials:

- Laminar Flow Biological Safety Cabinet (BSC), Class II
- Cell Culture Incubator, 37°C, **10% CO_2**, ≥ 95% humidity
- 37°C Water Bath
- Phase Contrast Microscope with Digital Image Capture Accessories
- Refrigerator, 2-8°C Storage
- Freezer, -20°C Storage
- Pipette Aid
- Sterile Serological Pipettes (10 - 25 mL)
- Multichannel Pipette (Electronic or Manual)
- Micropipettes
- Sterile Micropipette Tips
- Sterile Reagent Reservoirs

[1] Refer to respective application protocols for experimental CO_2 requirements.

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Handling/Caution Statement

1. Always remove medium with a pipette.
2. Do not use a vacuum-powered aspiration device with HEPATOPAC plates.
3. Avoid scraping the monolayer with pipette tips. This may cause damage to the monolayer in that region of the well.
4. Ensure electronic pipettes are set to the lowest removal and application speeds.

Protocol

IMPORTANT NOTES:

1. At least two days prior to the arrival of HEPATOPAC kits, reset and calibrate the incubator to **37°C, 10% CO_2, ≥ 95% humidity**. The sodium bicarbonate content in the medium is buffered for 10% CO_2. High humidity is essential to minimize evaporation of medium and is achieved by using a full water pan in the incubator.

2. Upon arrival, ensure that Box C is in the upright position. After preparing the Biosafety Cabinet (as described below), open Boxes A and B first to unpack components required for medium preparation. Do not open Box(es) C until Maintenance Medium has been prepared and warmed (Steps 5-6 below).

3. Biosafety Level 2 (BSL-2) practices must be followed during all manipulations of HEPATOPAC cultures. Universal Precautions must be observed and all material should be treated as potentially infectious. Following these practices protects not only the user, but also helps maintain sterile conditions required for long-term culture of HEPATOPAC.

4. When working with HEPATOPAC or media components, always work in a biosafety cabinet and follow proper aseptic technique.

Unpacking Instructions

1. Wipe Biosafety Cabinet (BSC) with 70% ethanol.
2. Obtain the following supplies and place in the BSC:
   - Additional Sterile Culture Lids (provided)
   - Sterile Reagent Reservoirs
   - Pipette Aid
   - Sterile Serological Pipettes
   - Micropipettes
   - Sterile Micropipette Tips
   - Multichannel Pipette
3. Open Boxes A and B. Locate the bottles labeled:
   - Maintenance Base
   - Maintenance Component

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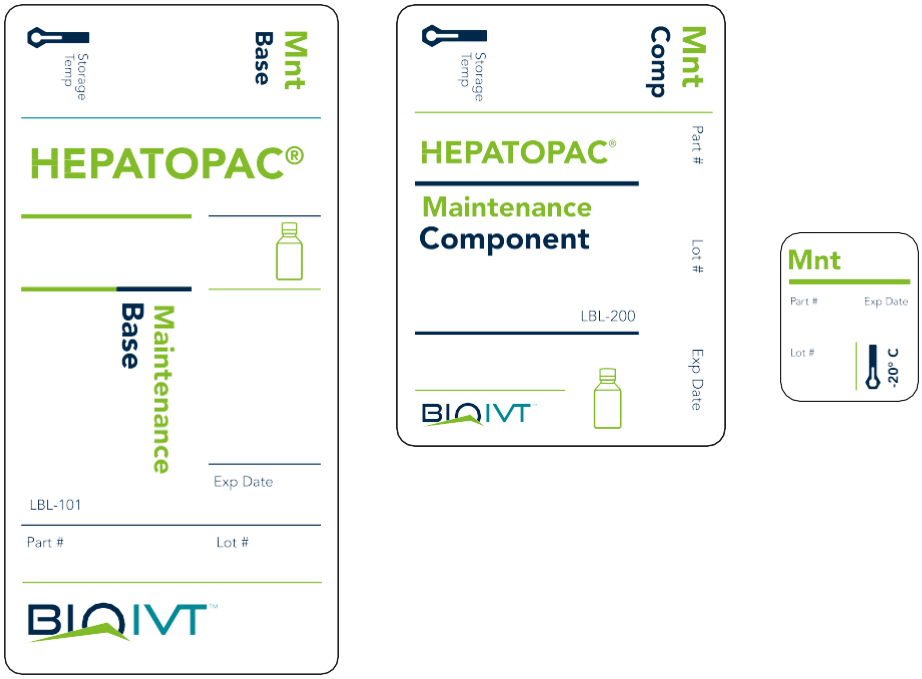
4. Locate the vial(s) labeled with a green line that say "Mnt".



5. Prepare the Maintenance Medium
   - Prior to preparing the medium, thaw Component Part #5030C, Serum Component Part #5029C or #5028C, and Component Part #5031C in a 37°C water bath. Component Part # 5010C is stored at 2-8°C and does not need to be thawed. Component Part #s 5011C, 5012C, 5025C and 5026C are stored at -20°C and must be thawed at **room temperature** (i.e. not in a 37°C water bath).
   - Add the indicated volume of each component in Tables 1 & 2 to the bottle(s) labeled "Maintenance Base".
     - Based upon species, reference the tables as listed below:
       - Table 1: Human, Monkey, or Dog HEPATOPAC Maintenance Medium
       - Table 2: Rat HEPATOPAC Maintenance Medium
     - Reference the appropriate column for the volume of each component required for either the 125 mL, 250 mL or 500 mL bottles.
   - After the Maintenance Base bottle(s) has been reconstituted with the components, it is hereafter referred to in this protocol as "**Maintenance Medium**." Label the bottle(s) appropriately. Store reconstituted medium at 2-8°C. Shelf life is 7 days.

NOTE:
- The final volume in the 125 mL bottle will be 50 mL of Maintenance Medium.
- The final volume in the 250 mL bottle will be 250 mL of Maintenance Medium.
- The final volume in the 500 mL bottle will be 500 mL of Maintenance Medium.

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Table 1: Human, Monkey, or Dog HEPATOPAC Maintenance Medium

Component Part Number	Thawing Conditions	Volume to Add to Maintenance Base in 125 mL Bottle	Volume to Add to Maintenance Base in 250 mL Bottle	Volume to Add to Maintenance Base in 500 mL Bottle
5030C	37°C water bath	860 µL	4.3 mL	8.6 mL
5029C	37°C water bath	5 mL	25 mL	50 mL
5010C	Room Temperature	1.8 mL	9 mL	18 mL
5011C	Room Temperature	0.5 µL	2.5 µL	5 µL
5012C	Room Temperature	0.5 µL	2.5 µL	5 µL

Table 2: Rat HEPATOPAC Maintenance Medium

Component Part Number	Thawing Conditions	Volume to Add to Maintenance Base in 125 mL Bottle	Volume to Add to Maintenance Base in 250 mL Bottle	Volume to Add to Maintenance Base in 500 mL Bottle
5030C	37°C water bath	888 µL	4.44 mL	8.88 mL
5028C	37°C water bath	5 mL	25 mL	50 mL
5031C	37°C water bath	0.5 mL	2.5 mL	5 mL
5026C	Room Temperature	60 µL	300 µL	600 µL
5025C	Room Temperature	15 µL	75 µL	150 µL
5012C	Room Temperature	0.5 µL	2.5 µL	5 µL

Table 3: Media Volumes

Species	24-Well Plate		96-Well Plate	
	Per Well	**Per Plate***	**Per Well**	**Per Plate***
Human	400 µL	10 mL	64 µL	7 mL
Monkey	400 µL	10 mL	64 µL	7 mL
Dog	400 µL	10 mL	64 µL	7 mL
Rat	300 µL	8 mL	50 µL	5 mL

*Total volume includes slight overage.

6. Place fully supplemented HEPATOPAC Maintenance Medium in the 37°C water bath and warm for ≥ 30 minutes.
7. Once HEPATOPAC Maintenance Medium is warm, unpack the HEPATOPAC plates in Box C per the following procedure (If there are multiple boxes, open one at a time):
    - First, open Box C and remove one inner box at a time.
    - Second, open the inner box and carefully remove the plates, keeping them upright.
    - Carefully place the HEPATOPAC plates in the BSC.

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NOTE:
Proceed with caution: Culture wells are filled to capacity.
24-well plates contain ~2.8 mL/well, 96-well plates contain ~280 µL/well.

8. Using a serological pipette, transfer HEPATOPAC Maintenance Medium to a sterile reservoir. Media volumes are listed in Table 3 above.

NOTE:
Medium must be added along the sidewall. Do not apply medium directly on the tissue layer. If working with multi-donor/multi-species plates, ensure that you use multiple reservoirs and add the appropriate amount of medium according to species.

9. Remove and dispose of the lid and foil seal from an individual HEPATOPAC plate. Complete a full medium change for one plate (Steps 10-13) and utilize a new sterile lid (provided) prior to handling additional plates.
10. Use a pipette to remove the medium from the HEPATOPAC plate.
    - Medium should be removed from a limited number of rows/columns to minimize the time that the wells are left dry. Replace the lid when the wells are without medium. Do not allow wells to sit without medium for longer than 30 seconds.

Important Handling Requirements:
1. Always remove medium with a pipette.
2. Do not use a vacuum-powered aspiration device with HEPATOPAC plates.
3. Avoid scraping the monolayer with pipette tips. This may cause damage to the monolayer in that region of the well.
4. Ensure electronic pipettes are set to the lowest removal and application speeds.

11. When medium is removed, the plate should be tilted as shown in Figure 1. Insert the tips at the lower edge of the well as shown in Figure 2. Gently guide the tips toward the bottom of the well and slowly remove the medium without disrupting the tissue layer. Check for complete medium removal.

12. To apply medium, tilt the plate forward, as shown in Figures 1 and 2, and position the tips at the top of the wells. Carefully dispense the medium, allowing it to slowly flow down the sidewalls. Medium volumes are provided in Table 3 and are listed according to well-format and species. Medium should always be applied to wells in the same order as it was removed.

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Figure 1: Position HEPATOPAC plate at a 45 degree angle when removing and replenishing media.

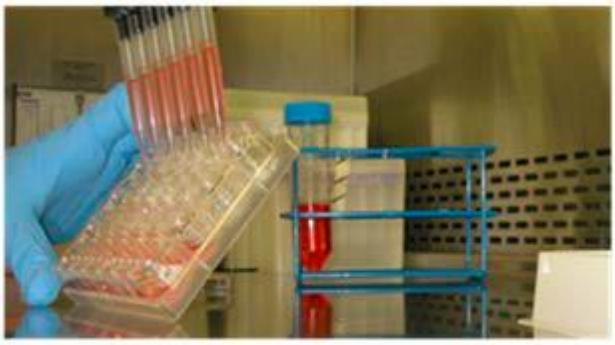
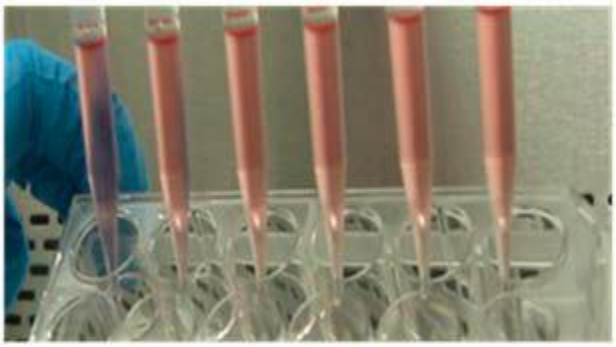


Figure 2: Position pipette tip at lower edge of well to reduce the chance of disrupting the cells.



13. Place plates in the 37°C, 10% CO_2 incubator, ≥ 95% humidity with a full water pan. Repeat Steps 9-13 with the remaining plates.

NOTE:
After the initial medium change, allow the HEPATOPAC plates to recover for two days at 37°C/10% CO_2 prior to initiating an application. For example, if the shipment is received on Tuesday, allow the cultures to recover until Thursday. The application may be started on Thursday. Prior to initiating an application, maintenance medium must be changed every two days (~48 hours). See Section entitled "Maintenance Instructions".

14. Observe a HEPATOPAC plate using a phase contrast microscope and note the following characteristics of the culture:
    - Cultures should have defined islands of hepatocytes surrounded by stromal cells, as shown in Figure 3.
    - Nuclei and nucleoli should be distinct.
    - Human and monkey hepatocyte islands are circular as shown in Figures 4 & 5.
    - Rat hepatocyte islands are "star shaped" as shown in Figure 6.
    - Dog hepatocyte islands, as shown in Figure 7 are larger than other species islands.

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Figure 3: Left - Islands of hepatocytes surrounded by stromal cells. Right - One-week old cultures that have a well-established bile network.

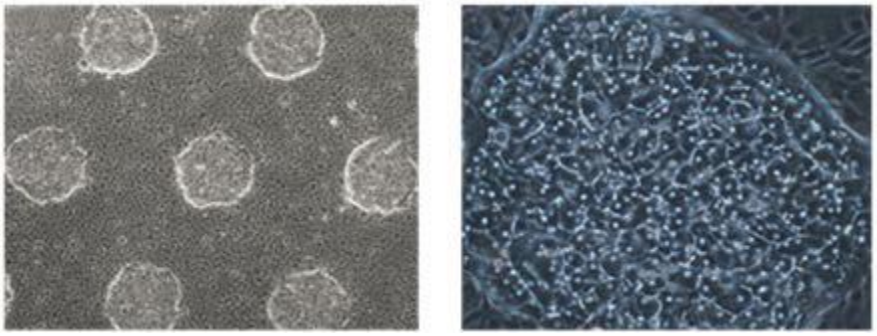

Figure 4: 10X image of a Human HEPATOPAC Island

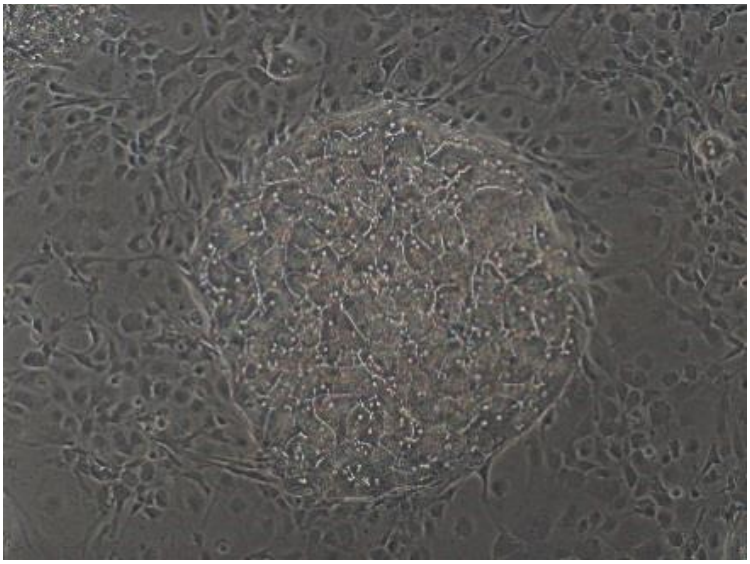

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Figure 5: 10X image of a Monkey HEPATOPAC Island

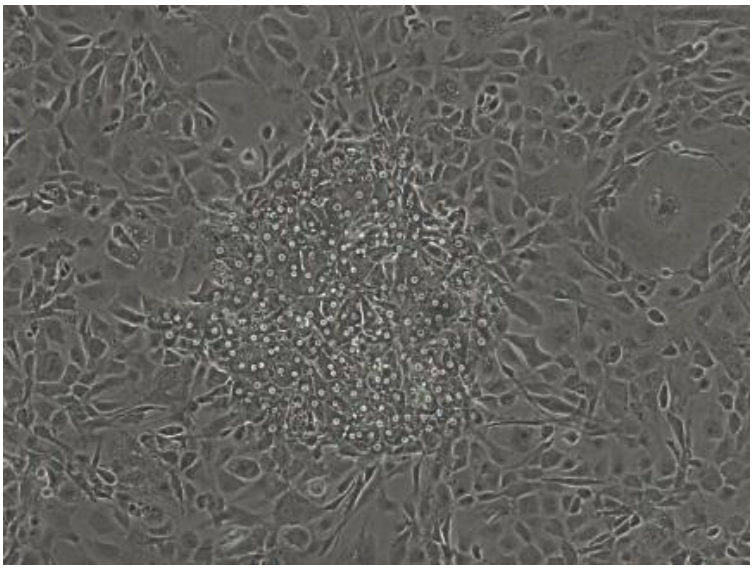

Figure 6: 10X image of a Rat HEPATOPAC Island

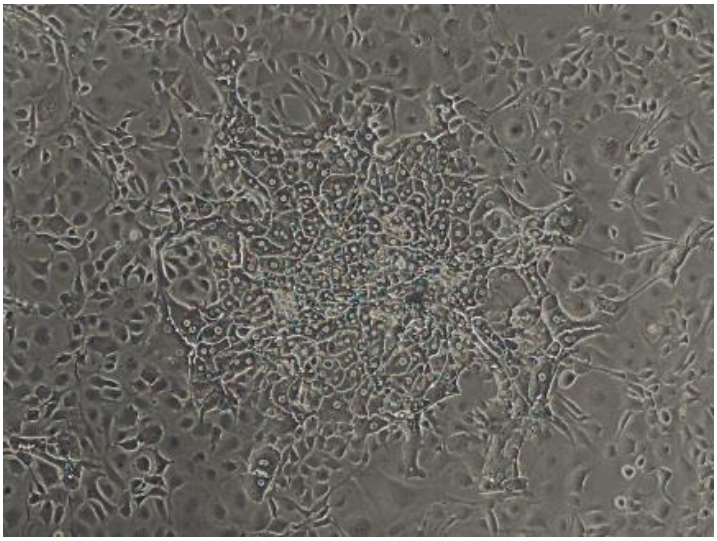

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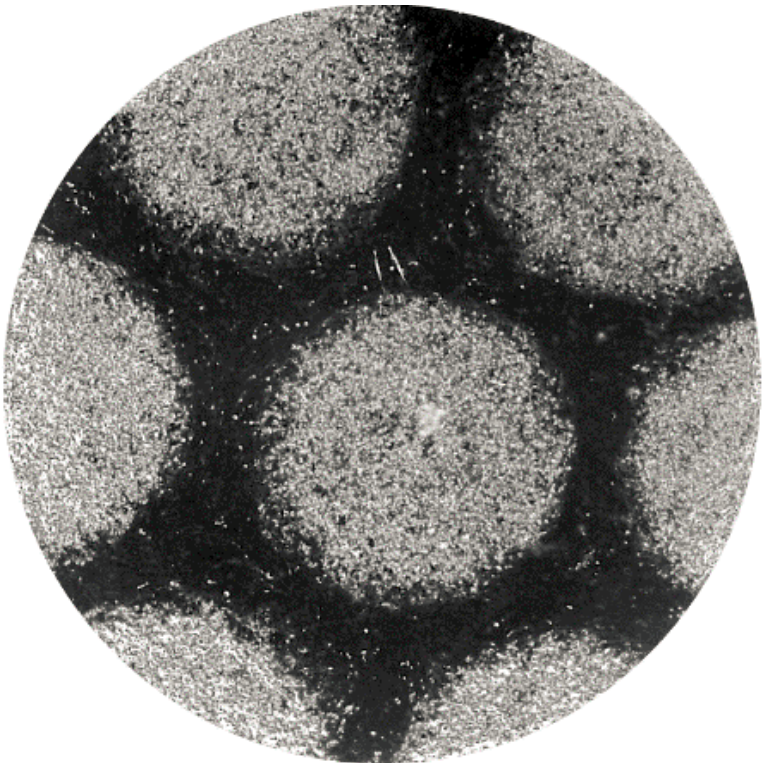
Figure 7: 4X image of a Dog HEPATOPAC Island



Maintenance Instructions

1. Medium must be changed every two days prior to beginning applications.
2. Warm species-specific maintenance medium in a 37°C water bath for ≥ 30 minutes prior to performing a medium change.
3. Observe cultures under a phase contrast microscope before each medium change to note the following characteristics:
    - Cultures should have defined islands of hepatocytes surrounded by stromal cells.
    - Nuclei and nucleoli should be distinct.
4. Carefully remove the HEPATOPAC plates from the incubator and place in the BSC.
5. Proceed with medium changes according to Steps 10-13 in the Section entitled "Unpacking Instructions".

NOTE:
Work with one or two plates at a time to minimize time out of the incubator and to ensure that the integrity of the culture is maintained.

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Best Practices

Note the following "Best Practices" when using HEPATOPAC kits.

1. Studies with HEPATOPAC cultures must be performed inside a Class II Biological Safety Cabinet using aseptic technique. Universal Precautions must be observed and all material treated as potentially infectious.
2. DO NOT aspirate medium from HEPATOPAC plates with a vacuum powered device. Medium must be removed with a micropipette; an electronic pipette is recommended. Vacuum-powered aspiration destroys cell to cell contacts between the primary hepatocytes and fibroblasts in the HEPATOPAC culture, resulting in peeling of the fibroblasts. Vacuum-powered aspiration forces also contribute to desiccation.
3. Because HEPATOPAC cultures are functional for several weeks, they can be sensitive to repeated injuries such as desiccation. To prevent this, replace the lid on the plate between the removal and subsequent addition of medium.
4. Avoid scraping the HEPATOPAC tissue layer with pipette tips. Medium should be removed and applied as seen in Figures 1 & 2.
5. When removing or applying medium, hold the plate at an angle of approximately 45° (Figure 1). This helps in complete removal of medium and allows medium to gently flow down the side of the well during medium application. It also helps prevent the pipette tips from disturbing the HEPATOPAC tissue layer.
6. Ensure that medium is completely removed from each well prior to the addition of fresh medium. After removing the medium, scan the plate to ensure medium is removed completely from each well. Any remaining medium in the well prior to fresh medium addition could cause the final volumes to differ across the plate. Hold the plate and pipette level to ensure that medium is completely removed from each well.
7. Work quickly but carefully with HEPATOPAC plates. Wells must not be without medium for longer than 30 seconds. Medium should always be applied to wells in the same order as it was removed.

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Troubleshooting

Problem	Likely Cause	Tips
Peeling of HEPATOPAC tissue layer around edge of wells	HEPATOPAC tissue layer was scraped with pipette tips	Take care to not disturb the tissue layer with the pipette tips when adding and removing medium. Please refer to Figures 1 & 2.
Peeling/sheeting of HEPATOPAC tissue layer throughout well	• Use of vacuum powered aspiration • Pipette speed is too fast	Ensure that vacuum-powered aspiration is **not** being used. If using an electronic pipette, ensure that the removal and application speed is at the lowest possible setting. Medium must be applied to the wall of the well rather than directly onto the tissue layer.
Necrotic or grainy looking islands (see Figure 8)	Desiccation	Minimize the amount of time the wells are left dry. Place the lid on the plate whenever wells are dry. Reduce the number of rows and columns from which media was removed prior to re-application.
"Stringy" looking fibroblasts that are contracted and no longer in contact with each other (see Figure 9)	Use of serum-free application medium	Serum-free application medium will result in "stringy" or contracted fibroblast morphology. If "stringy" fibroblasts are observed prior to the intentional use of serum-free medium, verify that the medium used for culture maintenance contained serum.
Uneven medium levels	• Incomplete medium removal • Uneven medium addition • Evaporation	• Refer to Steps 5-6 of Best Practices section. Ensure medium is completely removed from each well by visually scanning the plate prior to the addition of fresh medium. Tilting the plate at a 45° angle and holding the pipette level will aid in complete medium removal. • Ensure the pipette tips are secure and that equal amounts of medium are being taken up into all tips. • Ensure that the water pans in the incubator are full. This will reduce evaporation.

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Figure 9: Necrotic or "grainy" island (left) vs. a healthy island (right)

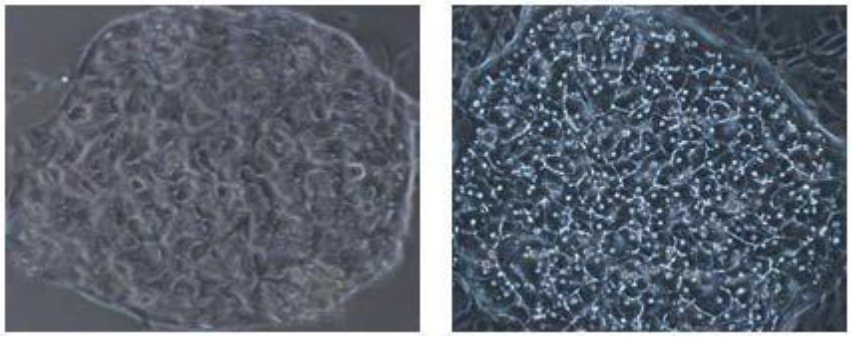
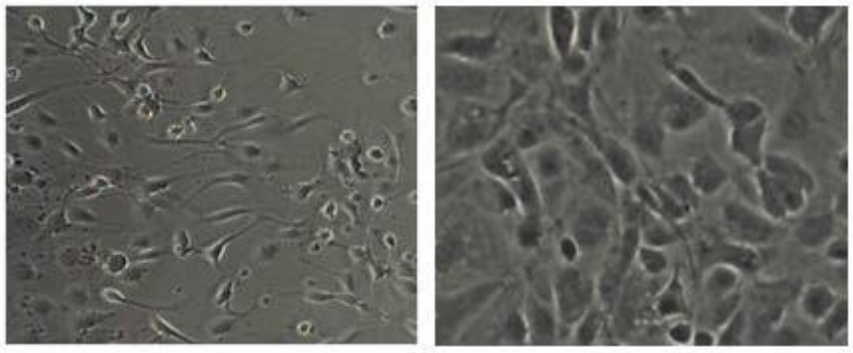


Figure 8: "Stringy" fibroblasts in the absence of serum (left) vs. fibroblasts maintained in serum supplemented medium (right).



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