User Manual

3dGRO® Pancreatic Organoids

SCC700-SCC720

Pack Size >1500 viable organoids/vial

Store in liquid nitrogen

FOR RESEARCH USE ONLY

Not for use in diagnostic procedures. Not for Human or Animal Consumption

Product Overview

Patient derived organoids (PDOs) are novel *in vitro* 3D cell models that have tissue-like functionality and are an alternative to traditional 2D cell lines and PDX animal models. PDOs can be derived from adult patient biopsies or resected tissues containing native LGR5+ stem cell populations and cultured within an ECM-rich substrate using specialized organoid media. Adult tissue derived organoids are thought to have more mature phenotypes than iPSC derived organoids and have been shown to predict patient responses to chemotherapeutics.

The 3dGRO® pancreatic organoid biobank is a collection of tissue-derived human pancreatic organoids for disease modeling research. The pancreatic organoid biobank contains highly characterized organoid lines from pancreatic ductal adenocarcinoma diseased patients, representing primary tumors containing wild-type and *KRAS* driver mutations.

Table 1: Pancreatic Organoid Biobank

ID	Age	Sex	KRAS Mutation	Split ratio	Days between Passaging	Media	Catalog Number
PPTO.02	62	M	G12D	1:4	8-9	A	SCC700
PPTO.13	73	M	G12R	1:4	8-10	A	SCC702
PPTO.26	81	M	G12V	1:6	7-9	A +C	SCC703
PPTO.30	73	M	G12D	1:8	8-9	A	SCC704
PPTO.36	76	F	G12D	1:3	10-12	B	SCC705
PPTO.46	66	F	G12R	1:4	6-8	A	SCC706
PPTO.49	55	M	G12V	1:6	7-9	A +C	SCC707
PPTO.61	60	F	Wild-Type	1:4	6-8	A+E+C	SCC708
PPTO.64	58	M	G12D	1:3	7-8	A+C	SCC709
PPTO.65	69	F	G12D	1:3	7-9	A+C	SCC710
PPTO.76	57	F	G12D	1:4	6-8	A+C	SCC711
PPTO.80	78	F	G12R	1:8	8-10	A+C	SCC712
PPTO.90	57	F	G12V	1:8	7-8	A+C	SCC713
PPTO.93	66	M	Q61H	1:3	7-9	A+C	SCC714
OCIP.174	59	F	Q61H	1:4	8-9	A	SCC716
OCIP.180	64	M	Wild-Type	1:4	10-12	A+E	SCC717

ID	Age	Sex	KRAS Mutation	Split ratio	Days between Passaging	Media	Catalog Number
OCIP.255	66	F	Unknown	1:6	6-7	A+E	SCC718
OCIP.285	65	F	Q61L	1:4	9-10	A	SCC719
OCIP.335	74	M	Wild-Type	1:6	6-7	A+E	SCC720

Note: PPTO lines are derived from patient primary tumors. OCIP lines are derived from xenografts.

Materials Provided

3dGRO® Pancreatic Organoids (Component No. SCC700-720): One (1) vial containing ≥ 1500 viable organoids/vial. Store in liquid nitrogen.

Materials Required (Not provided)

Materials are available at SigmaAldrich.com unless otherwise noted.

Growth-factor reduced (GFR) Matrigel (CLS356231), TrpLE Express Enzyme (1X) (ThermoFisher 12604013) culture ware and media components (see Protocols).

Quality Control Testing

- Viability: ≥ 1500 viable organoids/vial
- Organoid Growth: Pass
- Negative for infectious diseases by a Human Essential CLEAR panel by Charles River Animal Diagnostic Services.
- Verified to be of human origin and negative for mouse, rat, Chinese hamster, Golden Syrian hamster, and non-human primate interspecies contamination, as assessed by a Contamination Clear panel by Charles River Animal Diagnostic Services.
- Mycoplasma Contamination: Negative
- STR Profile: Pass

Storage and Stability

Store in liquid nitrogen. The organoids can be cultured for at least 10 passages after initial thawing without significantly affecting the cell marker expression and functionality.

Protocols

All protocols are performed within a Class II laminar flow biohood and with an aspirator unless otherwise specified. Incubators are humidified and are set to 37 °C and 5% CO_2. PPE should be worn such as gloves, lab coat, and safety glasses.

Preparing 1X Complete Medium for Human Pancreatic Organoids.

All components listed below are available at SigmaAldrich.com unless otherwise noted.

Pancreatic Organoid Medium A

Component	Volume for 100 mL	Volume for 1 L	Final Concentration	Catalog Number
DMEM/F12 Plus Basal Medium	44.39 mL	443.9 mL	1X	SCM162
B-27™ Supplement (50X)	2 mL	20 mL	1X	ThermoFisher 17504044
Ala-L-Glutamine Solution (100X), 200 mM	1 mL	10 mL	2 mM	G8541
HEPES Solution, 1M in water	1 mL	10 mL	10 mM	H3537
N-Acetyl-L-cysteine, prepared as 500 mM solution in water	250 µL	2.5 mL	1.25 mM	A9165
Nicotinamide, 1 M in water	100 µL	1 mL	1 mM	N0636
[Leu^{15}]-Gastrin I, reconstituted to 100 µM in PBS/ 0.1% BSA	10 µL	100 µL	10 nM	G9145
A-83-01, reconstituted to 1 mM in DMSO	50 µL	500 µL	0.5 µM	SML0788
Recombinant human Noggin, reconstituted to 100 µg/mL in PBS/ 0.1% BSA	100 µL	1 mL	100 ng/mL	GF173
Recombinant human FGF-10, reconstituted to 100 µg/mL in PBS/0.1% BSA	100 µL	1 mL	100 ng/mL	GF172
Wnt3A Conditioned Medium	20 mL	200 mL	20% v/v	SCM112
R-Spondin1 Conditioned Medium	30 mL	300 mL	30% v/v	SCM104
Penicillin-Streptomycin, 100x solution	1 mL	10 mL	1X	P4333

Pancreatic Organoid Medium B

Component	Volume for 100 mL	Volume for 1 L	Final Concentration	Catalog Number
DMEM/F12 Plus Basal Medium	34.39 mL	343.9 mL	1X	SCM162
B-27™ Supplement (50X)	2 mL	20 mL	1X	ThermoFisher 17504044
Ala-L-Glutamine Solution (100X), 200 mM	1 mL	10 mL	2 mM	G8541
HEPES Solution, 1M in water	1 mL	10 mL	10 mM	H3537
N-Acetyl-L-cysteine, prepared as 500 mM solution in water	250 µL	2.5 mL	1.25 mM	A9165
Nicotinamide, 1 M in water	100 µL	1 mL	1 mM	N0636
[Leu^{15}]-Gastrin I, reconstituted to 100 µM in PBS/ 0.1% BSA	10 µL	100 µL	10 nM	G9145
A-83-01, reconstituted to 1 mM in DMSO	50 µL	500 µL	0.5 µM	SML0788
Recombinant human Noggin, reconstituted to 100 µg/mL in PBS/ 0.1% BSA	100 µL	1 mL	100 ng/mL	GF173
Recombinant human FGF-10, reconstituted to 100 µg/mL in PBS/0.1% BSA	100 µL	1 mL	100 ng/mL	GF172
Wnt3A Conditioned Medium	20 mL	200 mL	20% v/v	SCM112
R-Spondin1 Conditioned Medium	40 mL	400 mL	40% v/v	SCM104

Penicillin-Streptomycin, 100x solution	1 mL	10 mL	1X	P4333

Specific organoid lines require additional supplementation of either EGF and/or CHIR99021. See Table 1 for media requirements of specific lines.

Component	Catalog Number	Volume for 100 mL	Volume for 1 L	Final Conc.
E: Recombinant human EGF, reconstituted to 100 μg/mL in PBS/0.1% BSA	Sigma E9644	50 μL	500 μL	50 ng/mL
C: CHIR99021, reconstituted to 10 mM in DMSO	Sigma SML1046	25 μL	250 μL	2.5 μM

1. Combine media components except EGF.
   Note: EGF is not stable long term and thus should be added fresh with each media change.
2. Filter combined components with 0.2 μM filter. Aliquots of complete Pancreatic Organoid Medium may be stored at least 6 months at -20 °C. Fresh or thawed media is stable for at least 2 weeks at 4 °C.
3. Y-27632 (ROCK inhibitor, SCM075) at 10 μM final concentration is recommended to be added to 1X Complete Medium during the first two days after passage. It is not necessary to add Y-27632 to the 1X Complete Medium during regular media exchanges.

Thawing Organoids

1. Prepare sufficient growth-factor-reduced Matrigel (CLS356231; diluted to 8 mg/mL) for six 25-μL domes. Set aside on ice.
2. Thaw a vial of human pancreatic organoids by submerging 3/5 of vial into a 37°C water bath until only a sliver of ice remains. Spray the outside of the vial with 70% ethanol or isopropanol.
3. In a sterile tissue culture hood, quickly and gently transfer the vial contents into a 15-mL conical tube containing 8 mL of **cold** DMEM/F12 PLUS (SCM162).
4. Centrifuge the 15-mL conical tube for 5 minutes at 320 *g*.
5. Carefully aspirate the media with a pipet, being cautious to avoid disturbing the organoid pellet. Keep at least 1 mL in the tube. Organoids should appear as an opaque layer at the bottom of the tube. A clear layer of residual Matrigel may be visible overlaying the organoid pellet.
6. Add 1 mL of **cold** Cell Recovery Solution (Corning 354253) to the tube and gently pipette to wash the residual Matrigel. Incubate the tube on ice for 30 minutes. Swirl the conical tube twice during the incubation.
7. Add 10 mL cold DMEM/F12 PLUS to the tube and centrifuge at 4 °C for 5 minutes at 320 *g*.
8. Carefully aspirate the media with a pipet, being careful not to disturb the organoid pellet.
9. Transfer 150 μL of thawed Matrigel to the organoid pellet. Quickly and gently resuspend the organoid pellet by pipetting up and down 5 times with a P-100 or P-200 micropipette set to 80 μL, being careful to avoid causing air bubbles.
10. Place the organoid suspension on ice for 5 minutes to cool the Matrigel + organoid suspension.
11. Remove the organoid suspension from ice and briefly swirl to mix. Dispense 25 μL domes into each well of a pre-warmed 24-well plate. Work quickly and minimize formation of air bubbles during pipetting.
12. Allow the domes to incubate for 10 minutes in a 37 °C humidified incubator with 5% CO_2.
13. Transfer the 24-well plate containing the organoid domes to a sterile tissue culture hood. Gently add 1 mL of 1X Complete Pancreatic Organoid Medium containing 10 μM ROCK inhibitor into each well containing the organoid domes.
14. Incubate plate in a 37 °C humidified incubator with 5% CO_2.
15. After 24 h, check for recovery by observation by bright field microscope. Live organoids should begin to show rounded or compact morphologies. Replace wells with 1 mL fresh 1X Complete Pancreatic Media containing 10 μM ROCK inhibitor per well.
16. Exchange media every other day with 1X Complete Pancreatic Organoid Media (No ROCKi added). Passage 4-5 days after thaw. Passage ratio should be kept at a conservative 1:1.25 to 1:2 for the first 1-2 passages. Once organoids have been established, they may be passaged using the optimal split ratio as specified in Table 1.

    Note: ROCK inhibitor is added only at the time of thawing and on the first 2 days after passaging. Subsequent media changes do not require ROCKi supplementation.

Passaging Organoids

1. Prepare enough GFR Matrigel for a volume of 25 µL per dome. To maintain approximate equal density, split organoids according to the ratio recommended in the table for each individual line (e.g. for a 1:4 ratio, passage 10 domes into 40 domes).
2. Carefully aspirate the media from each well without disturbing the domes. Add 500 µL TrpLE Express + 10 µM ROCK inhibitor to each well of 24-well plate and detach the Matrigel domes using a P-1000 micropipette.
3. Detach the Matrigel domes in each well using a P-1000 micropipette. Break the organoids by placing the pipette tip perpendicular to the bottom of the well and expel the organoids with a scraping motion. Repeat 10 times.
4. Incubate the plate for 5 minutes a 37 °C and use a P-1000 micropipette to resuspend up and down 3-6 times to break the organoids. Observe under the microscope and stop when the majority of the cell suspension is comprised of small clumps of 2-3 cells. If the suspension still contains large clumps of cells, repeat the incubation at 37 °C and resuspend again.

   NOTE: Do not allow incubation time to exceed 20 minutes as this may lower the cell viability of the organoids.
5. Once the suspension consists entirely of single cells and small clumps of 2-3 cells, transfer the cell suspension to a conical tube and add equal volume of cold DMEM/F12 PLUS medium. Centrifuge the conical tube for 5 minutes at 320g.
6. Carefully aspirate the media, being careful not to disturb the organoid cell pellet. If a clear layer of Matrigel is present overlaying the organoid cell pellet, remove as much Matrigel as possible. If the Matrigel layer is not clear but appears opaque, do not attempt to remove. An opaque Matrigel layer indicates that the smaller sized organoids that are difficult to pellet may be present in the layer.
7. Transfer the appropriate volume of thawed GFR Matrigel to the organoid cell pellet with a P-1000 micropipette. Quickly and gently resuspend the organoid cell pellet about 5 times with a P-1000 tip set to 20 µL below the volume transferred. Avoid causing formation of air bubbles.
8. Dispense 25 µL domes into wells of the culture plate. After each 1 minute of dispensing, place suspension on ice for 5 minutes to cool and prevent solidification of the Matrigel.
9. Allow the domes to incubate for 10 minutes in a 37 °C humidified incubator with 5% CO_2.
10. Remove plate from incubator and add 1X Complete Pancreatic Organoid Medium containing 10 µM ROCK inhibitor. Exchange media every 2 days with fresh media without ROCK inhibitor.

    Note: ROCK inhibitor is added only at the time of thawing and on the day of passaging. Subsequent media changes do not require ROCKi supplementation.

Cryopreservation of organoids

Important Notes Before Starting

- We recommend freezing 2 x 25 domes per cryovial. Each dome should contain at least 700-1000 organoids
- It is important **not** to dissociate the organoids prior to freezing (either by enzymatic dissociation or by mechanical methods). Do not use enzymatic dissociation to detach the organoids during banking. The Matrigel is minimally disrupted with a pipette tip only enough so that the pieces can be suspended in freeze medium. Organoids should be frozen a couple of days prior to the normal day of passage. For example, if you normally passage at day 7, then freezing should occur at Day 5
- Patient-derived organoids may be frozen in complete Pancreatic Organoid medium + 10% DMSO or in 3DGro® Organoid Freeze Medium (SCM301)
- Prepare a Mr. Frosty container and have it ready for storing organoids for freezing down at -80 °C

1. Chill sufficient 1X PBS + 10 µM ROCK inhibitor on ice for at least 30 minutes before starting.
2. Remove plate or dish containing organoid domes from 37 °C incubator and place in a tissue culture hood. Aspirate media.
3. Add sufficient volume of cold PBS + 10 µM ROCK inhibitor into each well containing the organoids.
4. Gently disrupt the organoid domes with a P-1000 micropipette set to 900 µL using chilled 1X PBS + 10 µM ROCK inhibitor.

   Important: Keep organoids as intact as possible. Use the expulsion of the cold PBS/ROCKi liquid from the p1000 pipette to dislodge the Matrigel domes. Transfer organoid suspension to a new 15 mL conical tube.
5. Add additional **cold** 1X PBS + 10 µM ROCK inhibitor to wash the plate or dish to collect residual organoids and transfer the organoid suspension to the conical tube.
6. Centrifuge the conical tube containing the organoid suspension for 5 minutes at 320 *g*.
7. Carefully aspirate the media, avoiding disruption of the organoid pellet. If a clear layer of Matrigel is present overlaying the organoid cell pellet, remove as much Matrigel as possible. If the Matrigel layer is not clear but appears opaque, do not attempt to remove. An opaque Matrigel layer indicates that smaller sized organoids that are difficult to pellet may be present in the layer. The residual Matrigel should not affect cryopreservation.
8. Resuspend the organoid pellet with freeze medium comprised of 1X Complete Pancreatic Organoid Medium containing 10% DMSO at 2 domes per mL.
9. Quickly transfer 1 mL into a cryovial and repeat until all suspension are transferred into cryovials.
10. Quickly transfer cryovials to a Mr. Frosty container and place in a -80°C freezer for 24 hours.
11. 24 hours later, transfer the cryovials to liquid nitrogen (-135 °C) for long-term storage.

Whole Mount Immunocytochemistry of Organoids

Important Notes Before Starting

The following protocol is meant to serve as a guidance for first time users and is based on organoids cultured in 24-well plates. The protocol may be modified and adapted once users are more familiar with the process.

- We recommend using a pair of scissors that have been sterilized with 70% ethanol or isopropanol to cut the ends of P-1000 tips to enlarge the opening. Modified P-1000 tips are used to transfer fixed organoids without shearing them. Do not use serological pipettes as they are too bulky to handle small volumes and organoids may stick to the side of the pipettes.
- During PBS washes, gravity is used to collect organoids. Do not use centrifugation as the centrifugal force will result in misshapen organoids.
- 4% paraformaldehyde performs the dual function of fixing the organoids and to help partially dissolve the Matrigel and release the organoids. It is important to remove as much of the Matrigel as possible from the organoids. Matrigel may result in increased background autofluorescence. The more confluent the organoids are inside the domes at the time of fixing, the more readily the Matrigel will dissolve.

1. Prepare a 4% paraformaldehyde (PFA) solution by diluting an 8% PFA Solution (Electron Microscopy Sciences, 157-8-100) 1:1 with 1X PBS.
2. Prepare modified P-1000 and P-200 pipette tips by cutting the ends with a sterilized scissor.
3. Aspirate the medium from each well containing an organoid dome. Wash each well twice with 1 mL 1X PBS. Aspirate between PBS washes.
4. Add 1 mL of the 4% PFA solution to each well. Incubate 45-60 minutes at room temperature on a gently rocking or shaking platform. The shaker/rocker will help expedite detaching the Matrigel domes and the release of the organoids from the Matrigel.

   Note: GFR Matrigel domes will partially dissolve when fixed in PFA. At the end of the incubation period, you will notice that many (but not all) the domes are dislodged and that some of the organoids (but not all) will have been released from the domes.
5. Using the modified P-1000 pipette tips, collect any released organoids along with the fixative solution and transfer the contents to a 50 mL conical tube. Allow the organoids to settle to the bottom of the conical tube by gravity (~10-15 minutes). DO NOT CENTRIFUGE.
6. In the meantime, add 1 mL 1X PBS per well to the 24-well plate containing the organoid domes. Incubate 10-15 minutes at room temperature. This is done to dilute the PFA in the dome.
7. Carefully aspirate the fixative from the conical tube containing the released organoids (from step 5) and leave a small amount of liquid behind. This will ensure that the organoid pellet will not be aspirated off.
8. Using modified P-1000 pipette tips, collect any released organoids along with the PBS solution from each well (from step 6) and transfer the contents to the 50 mL conical tube. Allow the organoids to settle to the bottom of the conical tube by gravity (~10-15 minutes).
9. Repeat steps 5-8 two more times.
10. Add 0.8 mL of 1X PBS into each well that contains residual organoid domes.
11. Carefully aspirate the supernatant from the conical tube containing released organoids. Leave a small amount of liquid behind. This will ensure that the organoid pellet will not be aspirated off.
12. Add 4.8 mL 1X PBS to the organoid pellet. Swirl the conical tube to resuspend the organoid pellet. Using a modified P-1000 tip, transfer 200 µL of the organoid suspension into each well containing the 0.8 mL volume of residual organoid domes (from step 10).

    Note: Some organoids may stick to the modified P-1000 tip.
13. If staining will not be performed immediately, seal the 24-well plate containing fixed organoids with parafilm and store in the fridge at 2-8 °C for up to 1 month.
14. When ready to perform ICC, transfer the 24-well plate containing the fixed organoids to a dissecting microscope.
15. Using modified P-200 tips (from step 2), pipette 1-4 organoids into each well of an 8-well chamber slide. Remove any residual PBS using an unmodified P-200 pipette tip. Avoid accidentally pipetting up the organoids and shearing them through the P-200 tip.
16. Add 0.4 mL Blocking Buffer (5% horse serum plus 0.5% Triton X-100 in 1X PBS) to each well of an 8-well chamber slide containing the fixed organoids. Block at 2-8 °C overnight or at room temperature for 2-4 hours.

    Note: Use the serum from the same species as the host secondary antibody.
17. Using an unmodified P-200 pipet, remove the blocking buffer while tilting the chamber slide. Avoid pipetting the organoids through the P-200 tip.
18. Prepare primary antibodies or directly conjugated antibodies (300-500 µL) in Blocking Buffer.
19. Add primary antibodies. Incubate overnight at 2-8 °C on a gently shaking or rotating platform.
20. Next day, wash 3X with 1X PBS for 10-15 minutes each on the shaking/rotating platform.

    Note: Do not use centrifugation. Remove the PBS with a P-200 pipet after each wash while tilting the chamber slide.
21. Prepare secondary antibodies (300-500 µL) in Blocking Buffer.
22. Add secondary antibodies. Incubate overnight at 2-8 °C on a gently shaking or rotating platform.
23. Next day, wash with 1X PBS for 10-15 minutes on the shaking/rotating platform.

    Note: Do not use centrifugation. Remove the PBS with a P-200 pipet after each wash while tilting the chamber slide.

24. Counterstain with DAPI (5 µg/mL in 1X PBS) for 15-20 minutes.
25. Wash 3X with 1X PBS for 10-15 minutes each on the shaking/rotating platform.

    Note: Do not use centrifugation. Remove the PBS with a P-200 pipet after each wash while tilting the chamber slide.
26. Add 300-400 µL of 1X PBS into each well. Samples are now ready to be imaged on a confocal microscope.

Representative Data

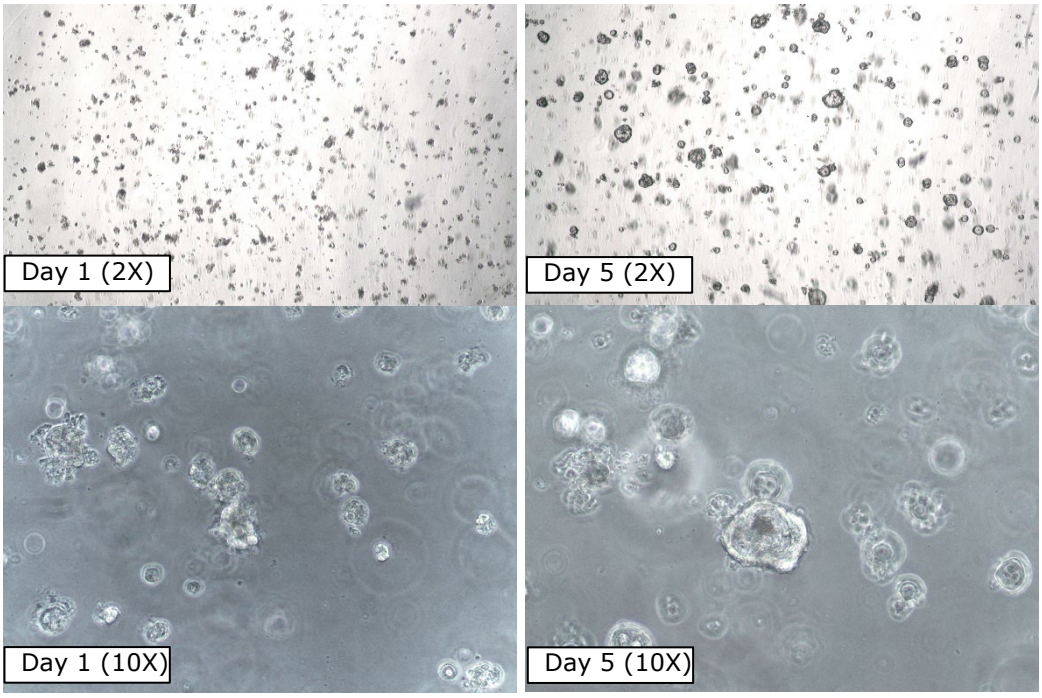

Figure 1. Time course of PDAC cancer organoid growth over a 5-day time period shows positive cell proliferation after thaw (SCC700, PPTO.02).

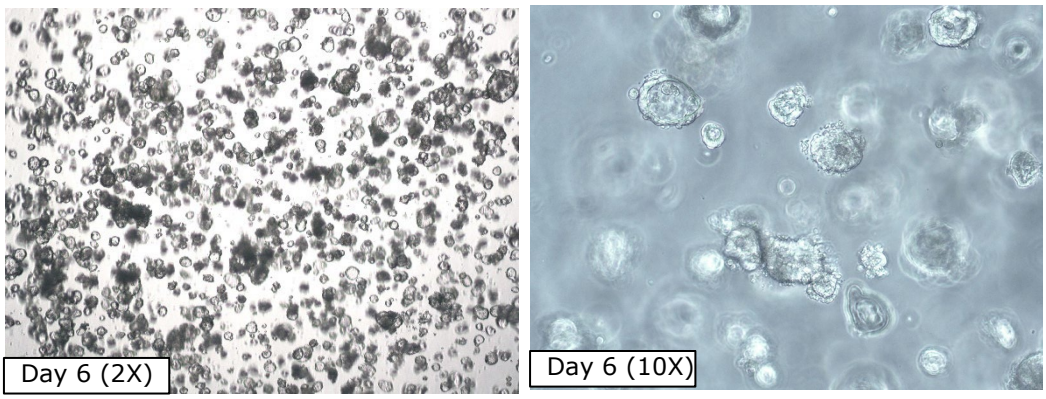

Figure 2. Human PDAC cancer organoid (SCC706, PPTO.46) morphology and confluency prior to cryopreservation on day 6 (2X, 10X).

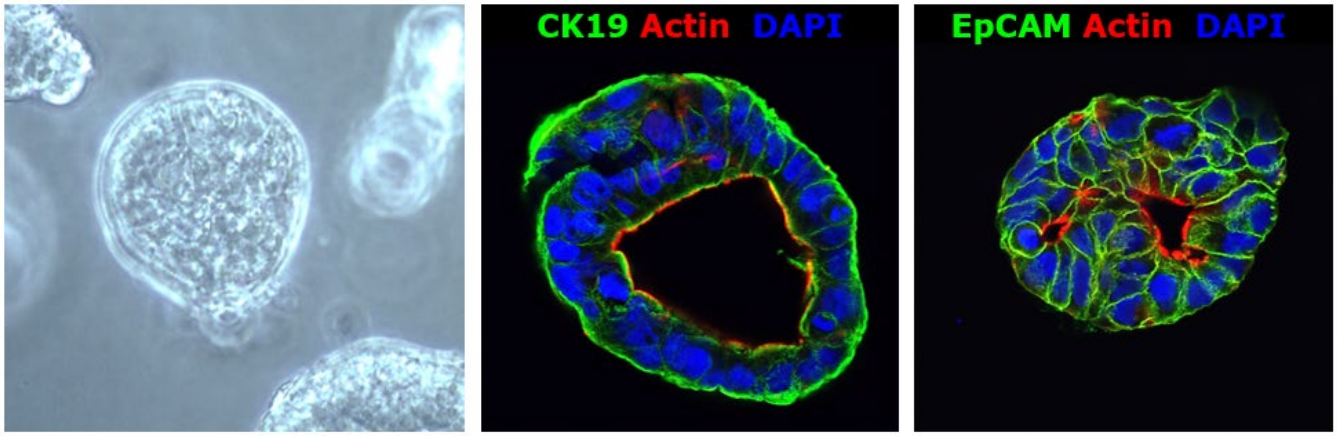

Figure 3. Immunocytochemical characterization of human pancreatic organoids (SCC700, PPTO.02). Human PDAC cancer organoids are positive for Cytokeratin 19 (CK19), epithelial cell adhesion molecule (EpCAM) and Actin. Cells counterstained with DAPI (blue).

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