PRECODE

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PRECODE SOPs For Pancreatic Tumor Organoid Culture and nucleic acid preparation

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1. Preparation Of Media For Pancreatic Tumor Organoid Culture

1.1. Purpose: To describe the procedures for preparation of media used in establishing, passaging and expansion of human pancreatic organoids from resected tumor tissue. This is a general procedure and certain details and reagents may vary depending on the particular tumor type and characteristics and site of model generation.

1.2. Scope: This SOP applies to all laboratory personnel at University of Verona, who are involved in establishing organoids from human pancreatic tumor tissue.

1.3. Responsibilities: As per institutional regulations, authorized personnel processing the pancreatic tumor samples must ensure that:

- All samples used are obtained lawfully and with appropriate consent;
- All procedures are followed correctly and are compliant with the SOP;
- All samples are adequately labeled during processing;
- All the applicable documentation and records are collected, updated and maintained for all samples.

Authorized personnel responsibilities also include:

- Preparation of stock and supplemented media, recognizing when additional stock medium needs to be prepared and when media and other reagents need to be ordered;
- Culturing, splitting, freezing and thawing the organoids as required;
- Recognizing when new cells need to be thawed and when cells have to be frozen down;
- Recognizing when organoid culture stocks are getting low and when more cells need to be frozen down.

1.4. Health and Safety: Authorized personnel carrying out this procedure should maintain safe working practices and observe all relevant environmental health and safety guidelines. This includes the appropriate use of personal protective equipment, and procedures for waste disposal, disinfection and biosafety. Gloves and lab coat are the minimal amount of protection that should be worn. Snug fitting non-permeable (waterproof) gloves are best. Eye protection is also recommended when exposure to splashing liquid nitrogen, solvents and biological active material is possible.

1.5. Definition and Acronyms

- SOP: Standard Operation Procedure
- Ad-DF: Advanced Dulbecco's Modified Eagles Medium with Nutrient Mixture F-12 Hams
- HSM+++: Human Splitting Medium
- NAC: N-acetyl cysteine
- DM: Digestion Medium
- hCPLT: Human Complete Medium
- RhoKi: Y-27632 – Rho kinase inhibitor

1.6. Quality Control

1. Monitor culture by microscopic examination regularly and examine for signs of microbial contamination or cell distress.
2. Regular mycoplasma testing of the organoid culture is recommended, since mycoplasma contamination cannot be detected easily by visual examination.
3. Record culturing, passaging and freezing of cells in notebook. Be sure to also include the date on which media was changed, cells were split or frozen. You should also update the liquid nitrogen logbook if removing a vial to start new batch of cells or when transferring cryogenic vials with frozen cells to the liquid nitrogen.

4. Each time you split the organoids, you increase the passage number by one. When you start cells from a freeze down, it is recommended that you start with the passage number at which the organoids were frozen and every time the organoids are passaged, you increase the passage number by one.
5. The personnel should also document any anomalies and/or deviations from the specified SOP;
6. All the tubes, bottles, cell culture well-plates and cryovials should be labelled properly and legibly. Labels should contain information regarding organoid identifier number, passage number as well as original isolation from tissue, passage date and initials.
7. All growth factors, stock media and supplemented media should be stored at the appropriate storage temperature.
8. When preparing media, make sure you date and put your initials on the media bottle.
9. During processing, it is recommended that tissue and organoids are kept on ice, except unless specified.

1.7. Procedure to prepare HSM+++

Note:

- HSM+++ is the base for the media used for culture of human pancreatic cancer, and this can be stored at 4ºC for up to 1 month.

1.7.1. Equipment, Supplies & Reagents

Equipment

- Biosafety Cabinet
- Vortex Mixer
- Pipet aid
- P200
- P1000

Supplies

- 15 ml falcon tubes (Falcon, # F2097)
- 50ml falcon tubes (Falcon, # F2070)
- 5ml plastic pipettes (Falcon, # F7543)
- 10ml plastic pipettes (Falcon, # F7551)
- 25ml plastic pipettes (Falcon, # F7525)
- Ice bucket with ice
- Waste container (for pipet tips, etc)

Reagents

- 70% ethanol
- Advanced Dulbecco's Modified Eagles Medium with Nutrient Mixture F-12 Hams – 500ml (Gibco, #12634-028)
- GlutaMax – 200mM (Gibco, #35050-061)
- Hepes – 1M (Gibco, #15630-080)
- Primocin – 50mg/ml (Invivogen # ant-pm-2)

1.7.2. Stock solution preparation

Glutamax, Hepes, and Primocin, specified above are ready to use. Avoid multiple freeze-thaws of Primocin.

1.7.3. Preparation of HSM+++

1. Start-up biosafety cabinet, disinfect the work area surface and P1000 and P200 pipets by spraying liberally with 70% ethanol and allowing to air dry.
2. Spray reagent containers with 70% ethanol and place them inside the Biosafety Cabinet. Allow to air dry.
3. Carefully open the Ad-DF medium.
4. Add 5ml each of GlutaMax and Hepes.
5. Add 1ml Primocin.
6. Close the bottle and label as follows: HSM+++**,** Initials**,** Date**,** Expiration date (4 weeks after the opening date).
7. Medium should be stored in the refrigerator at 4ºC.

1.8. Procedure to prepare hCPLT

Note:

- HSM+++ is the base for hCPLT. Refer to section 1.7 for HSM+++ recipe.

1.8.1. Equipment, Supplies & Reagents

Equipment

- Biosafety Cabinet
- Vortex Mixer
- Pipet aid
- P200
- P1000

Supplies

- 15 ml falcon tubes (Falcon, # F2097)
- 50ml falcon tubes (Falcon, # F2070)
- 5ml plastic pipettes (Falcon, # F7543)
- 10ml plastic pipettes (Falcon, # F7551)
- 25ml plastic pipettes (Falcon, # F7525)
- Ice bucket with ice
- Waste container (for pipet tips, etc)
- PETG media bottles – 250ml (Corning, # 431732)
- 0.22µm sterile filters (Millipore, # SLGV004SL)

Reagents

- 70% ethanol
- HSM+++ (see section 1.7 for preparation procedure)
- B-27 supplement – 50X concentrated) (Gibco, # 17504-044)
- N-Acetylcysteine (NAC) (Sigma-Aldrich, # A9165-5G)
- Nicotinamide (Sigma-Aldrich, # N0636)
- hEGF (Gibco, # PMG8043)
- TGFβ Receptor inhibitor A83-01 (Tocris, # 2939)
- FGF10 (Peprotech, # 100-26)
- mNoggin (Peprotech, # 250-38)
- R-spondin-1 conditioned medium (Section 1.12)
- Gastrin (Tocris, # 3006)
- Primocin – 50mg/ml (Invivogen, # ant-pm-2)

- Y-27632 Dihydrochloride (RhoKi), (Sigma, # Y0503)
- Wnt3a conditioned medium (Section 1.10) or Wnt3a-Afm conditioned medium (Section 1.11)
- DPBS (Gibco, # 14190-144)
- Ultrapure distilled water (Invitrogen, # 10977-035)
- DMSO (Sigma, # D26509)

1.8.2. Stock solution preparation

- B-27 and Primocin specified above are ready to use. Avoid multiple freeze-thaws of Primocin.
- Wnt3a conditioned media preparation is described in section 1.10. Wnt3a conditioned media could be substituted with Wnt3a-Afamin (Wnt-Afm) conditioned media if available. Wnt3a-Afm conditioned media preparation is described in section 1.11.
- R-spondin conditioned media preparation is described in section 1.12.
- See chart below for preparation of the other stock solutions.
- For solubilization of some reagents, use a 37°C water bath heat (H), as indicated in the chart below. For sterile filtration of some reagents, use 0.22µm syringe filters.

Reagent	Weight	Diluent	Volume	Heat/Vortex /Filter (H/V/F)	Stock Concentration	Final Concentration
NAC	5g	UltraPure dH20	61.2ml	V/F	500mM	1.25mM
Nicotinamide	4.88g	DPBS	40ml	V/F	1M	10mM
hEGF	1mg	UltraPure dH20+ 0.1% BSA	2ml	No	500µg/ml	50ng/mL
A83-01	10mg	DMSO	950µl	H/V	25mM	500nM-
FGF10	1mg	DPBS+ 0.1% BSA	1ml	No	1mg/ml	100ng/ml
mNoggin	100µg	UltraPure dH20+ 0.1% BSA	1ml	No	100µg/ml	100ng/ml
hGastrin	1mg	DPBS	4.8ml	V	100µM	10nM
RhoKi	5mg	UltraPure dH20	1480µl	No	10.5mM	10.5µM

1.8.3. Preparation of hCPLT

1. Thaw the required stock solutions (see table below) on ice in an ice bucket.
2. Place HSM+++ media bottle on ice.
3. Start-up biosafety cabinet, disinfect the work area surface and P1000 and P200 pipets by spraying liberally with 70% ethanol and allowing to air dry.
4. Spray reagent containers with 70% ethanol and place them inside the Biosafety Cabinet. Allow to air dry.
5. For making **100ml** hCPLT add the following into a 250ml sterile PETG bottle.

Reagent	Stock Concentration	Volume
HSM+++	NA	36.42ml
R-Spondin conditioned media	NA	10ml
Wnt3a/Wnt3a-Afm conditioned medium	NA	50ml
mNoggin	100µg/ml	100µl
B-27	50X	2ml
NAC	500mM	250µl
Nicotinamide	1M	1ml
hEGF	500µg/ml	10µl
FGF10	1mg/ml	10µl
A83-01	25mM	2µl
Gastrin	100µM	10µl
Primocin	50mg/ml	200µl
RhoKi	10.5µM	100µl

Note:

- RhoKi is added to the hCPLT only if it is used to passage organoids and to single cell suspensions.

6. Mix contents by inverting the bottle several times.
7. Label the flask as follows: hCPLT, Initials, Date.
8. hCPLT should be stored in the refrigerator at 4ºC or kept on ice during use.
9. hCPLT can be stored up to 2 weeks at 4ºC.

1.9. Procedure to prepare DM

Note:

- DM is used to digest primary human tissue.
- HSM+++ is the base for DM, and this can be stored at 4ºC for up to 1 month.
- 8ml of DM is required per tumor sample.

1.9.1. Equipment, Supplies & Reagents

Equipment

- Biosafety Cabinet
- Vortex Mixer

- Pipet aid
- P200
- P1000

Supplies
- 15 ml falcon tubes (Falcon, # F2097)
- 50ml falcon tubes (Falcon, # F2070)
- 5ml plastic pipettes (Falcon, # F7543)
- 10ml plastic pipettes (Falcon, # F7551)
- 25ml plastic pipettes (Falcon, # F7525)
- Ice bucket with ice
- Waste container (for pipet tips, etc)
- PETG media bottles – 250ml (Corning, # 431732)
- 0.22µm sterile filters (Millipore, # SLGV004SL)

Reagents
- 70% ethanol
- HSM+++ (see section 1.7 for preparation procedure)
- Collagenase II (Gibco, # 17101-015)
- Dispase I (Gibco, # 17105041)
- Y-27632 Dihydrochloride (RhoKi), (Sigma, # Y0503)
- DNAse I (Sigma, # D50250150 KU)

1.9.2. Stock solution preparation

- RhoKi (10.5mM stock) is prepared by resuspending 5mg in 1480µl ultrapure dH_2O. The final concentration to be used is 10.5µM.
- DNAse I stock solution (10mg/ml) is prepared by resuspending 10mg DNAse I in 1ml DPBS.

1.9.3. Preparation of DM

1. Start-up biosafety cabinet, disinfect the work area surface and P1000 and P200 pipets by spraying liberally with 70% ethanol and allowing to air dry.
2. Spray reagent containers with 70% ethanol and place them inside the Biosafety Cabinet. Allow to air dry.
3. Carefully open the HSM+++ medium and transfer 100ml into a PETG media bottle.
4. Add pre-weighed Collagenase II (500mg) and Dispase I (100mg). Mix by inverting.
5. Label as follows: DM, Initials, Date and store on ice until use.
6. Add 100µl RhoKi to 100ml DM just before use.

1.10. Procedure to prepare Wnt3a Conditioned Media

Notes:
- Corbo Laboratory uses Wnt3a-expressing cell line, with a G418-selectable marker, that can be purchased from ATCC (L Wnt-3A, ATCC CRL-2647).
- Cells can be used for around 10 passages to harvest conditioned medium.
- Note that Wnt3a requires the presence of lipids to be active, and therefore can only be produced in medium with FBS.
- Wnt activity in Wnt3a conditioned media can be tested using the Dual Luciferase Reporter assay.

1.10.1. Equipment, Supplies & Reagents

Equipment

- Biosafety Cabinet
- Vortex Mixer
- Pipet aid
- P200
- P1000

Supplies

- 15 ml falcon tubes (Falcon, # F2097)
- 50ml falcon tubes (Falcon, # F2070)
- 5ml plastic pipettes (Falcon, # F7543)
- 10ml plastic pipettes (Falcon, # F7551)
- 25ml plastic pipettes (Falcon, # F7525)
- Ice bucket with ice
- Waste container (for pipet tips, etc)
- 175 cm2 tissue culture flanks
- 15cm plates
- PETG media bottles – 250ml (Corning, # 431732)
- 0.22µm sterile filters (Millipore, # SLGV004SL)
- Sterile glass media bottles

Reagents

- 70% ethanol
- DMEM with Glutamax and Pyruvate (Gibco, # 41965-062)
- FBS (Gibco, #26140079)
- Penicillin/Streptomycin (Pen/Strep) - Pen: 10K U/ml; Strep: 10K µg/ml) (100x concentrated (Thermo Fisher Scientific, # 15140-122)
- Trypsin (Gibco, # 2530054)
- Geneticin (G418, Gibco, # 10131027)
- DPBS (Gibco, # 14190-144)
- A frozen vial of Wnt3a expressing cell line

1.10.2. Procedure to prepare culture media for Wnt3a expressing cells

1. Take an aliquot of Pen/Strep from -20ºC and thaw it at room temperature or in the 37ºC water bath.
2. Start-up biosafety cabinet, disinfect the work area surface and P1000 and P200 pipets by spraying liberally with 70% ethanol and allowing to air dry.
3. Spray reagent containers with 70% ethanol and place them inside the Biosafety Cabinet. Allow to air dry.
4. Carefully open the DMEM bottle. Add 60 ml of FBS and 5ml Pen/Strep solutions to the DMEM. Mix by inverting the bottle 2-4 times.
5. Close the bottle and label with Initials, Date, Expiration date (4 weeks after the opening date).
6. Medium should be stored in the refrigerator at 4ºC.

1.10.3. Procedure to establish a culture of Wnt3a expressing cells, using a cryofrozen Wnt3a cell line

1. Start-up biosafety cabinet, clean the work area surface and clean the P1000 and P200 pipets with 70% ethanol.
2. Wipe the exteriors of all tubes/containers with 70% ethanol and transfer them into the biosafety cabinet.
3. Wnt3a culture medium (1.10.2) should be out of the fridge for at least 30 min to reach room temperature and then warm up to 37ºC before starting the procedure.
4. Take a tweezer, cryo protection gloves and a box filled with sufficient amount of dry ice. Remove a vial of Wnt3a expressing cells from the liquid nitrogen tank and place it on the dry ice. Remember to be very careful as the vials are extremely cold and could cause skin damage if held for too long.
5. Thaw the vial rapidly by agitation in a 37ºC water bath until there is still little frozen material (the additional frozen material will thaw by the time you take the vial out of the water bath and proceed to culture). Thawing should be rapid (within 60-120 seconds). Remove the vial from water bath and wipe it down with 70% ethanol at room temperature and transfer to a biosafety cabinet. All following steps must be done under aseptic conditions.
6. Transfer cell suspension into a 15ml conical tube containing 9.5ml Wnt3a culture media.
7. Centrifuge cells at 200g for 5 min. Aspirate media.
8. Resuspend cells in 1ml culture media and seed cells in a 175 cm^2 culture flask containing 50ml culture medium.
9. Cells are confluent in typically 2-4 days and will need to be passaged.
10. To passage cells, aspirate out the spent media wash once with 25ml DPBS. Aspirate DPBS.
11. Incubate cells with 2ml trypsin at 37ºC for 2 min.
12. Quench with 18ml culture media.
13. Centrifuge cells at 133g for 5 min and remove media.
14. Resuspend cells in 6ml culture media and transfer 1ml each to 6 x 175 cm^2 culture flasks each containing 50ml culture media. Add 62.5 µl G418 to only one of the flasks (the “Selection Flask”), while leaving the other 5 flasks (the “Conditioning Flasks”) free of G418.

1.10.4. Procedure to prepare Wnt3a conditioned medium

1. When the “Conditioning Flasks” are confluent (after 2-4 days), carefully pour off the media from the cells and wash the cells with 25ml DPBS.
2. Trypsinize the cells using 2ml trypsin.
3. Quench the trypsin with 18ml culture media without G418.
4. Pool all the cells in a 1L sterile glass bottle. Add additional culture media without G418 to bring the total volume to 600ml.
5. Plate 20ml of cells onto 15cm culture dishes, making a total of 30 dishes.
6. Incubate cells for one week in the tissue culture incubator.
7. After one week, use a serological pipette to remove the media from the cells and transfer into 50ml conical tubes.
8. Centrifuge the media for 5 min at 300g to pellet floating cells.
9. Carefully pour the supernatants into a 0.2 µm sterile filter attached to a sterile bottle and filter the media.
10. Aliquot filtered media and either freeze and store at -20ºC or store at 4ºC.

1.11. Procedure to prepare Wnt3a-Afamin (Wnt3a-Afm) Conditioned Media

Notes:

- Wnt3a-Afamin conditioned medium is a special media where Wnt3a is conjugated to Afamin. Afamin is a serum glycoprotein that forms a complex with Wnt proteins and helps keep Wnt soluble. While serum must normally be present to solubilize Wnt3a in conditioned medium, co-expression of Wnt3a with afamin bypasses the requirement for serum in the conditioning medium since the presence of afamin alone will keep the Wnt soluble.
- Wnt activity in Wnt3a conditioned media can be tested using the Dual Luciferase Reporter assay.

1.11.1. Equipment, Supplies & Reagents

Equipment
- Biosafety Cabinet
- Vortex Mixer
- Pipet aid
- P200
- P1000

Supplies
- 15 ml falcon tubes (Falcon, # F2097)
- 50ml falcon tubes (Falcon, # F2070)
- 5ml plastic pipettes (Falcon, # F7543)
- 10ml plastic pipettes (Falcon, # F7551)
- 25ml plastic pipettes (Falcon, # F7525)
- Ice bucket with ice
- Waste container (for pipet tips, etc)
- 175 cm2 tissue culture flanks
- 15cm plates
- PETG media bottles – 250ml (Corning, # 431732)
- 0.22µm sterile filters (Millipore, # SLGV004SL)
- Sterile glass media bottles

Reagents
- 70% ethanol
- DMEM with Glutamax and Pyruvate (Gibco, # 41965-062)
- FBS (Gibco, # 26140079)
- Penicillin/Streptomycin (Pen/Strep) - Pen: 10K U/ml; Strep: 10K µg/ml) (100x concentrated (Gibco, # 15140-122)
- 100X MEM Non-Essential Amino Acids solution (Gibco, #11140050)
- Advanced Dulbecco's Modified Eagles Medium with Nutrient Mixture F-12 Hams – 500ml (Gibco, # 12634-028)
- GlutaMax – 200mM (Gibco, # 35050-061)
- Hepes – 1M (Gibco, # 15630-080)
- TrypLE Express (Gibco, # 12605-028)
- Zeocin (Invitrogen, R25001)
- Hygromycin B – 50mg/ml (Gibco, # 10687010)
- DPBS (Gibco, # 14190-144)

- A frozen vial of Wnt3a-Afm expressing cell line

1.11.2. Procedure to prepare culture media for Wnt3a-Afm expressing cells

1. Take an aliquot of Pen/Strep from -20ºC and thaw it at room temperature or in the 37ºC water bath.
2. Start-up biosafety cabinet, disinfect the work area surface and P1000 and P200 pipets by spraying liberally with 70% ethanol and allowing to air dry.
3. Spray reagent containers with 70% ethanol and place them inside the Biosafety Cabinet. Allow to air dry.
4. Carefully open the DMEM bottle. Add 50ml of FBS, 5ml 100X MEM non-essential amino acids solution and 2.5ml Pen/Strep to the DMEM. Mix by inverting the bottle 2-4 times.
5. Close the bottle and label with Initials, Date, Expiration date (4 weeks after the opening date).
6. Medium should be stored in the refrigerator at 4ºC.

1.11.3. Procedure to prepare conditioning media for Wnt3a-Afm expressing cells

1. Take an aliquot of Pen/Strep from -20ºC and thaw it at room temperature or in the 37ºC water bath.
2. Start-up biosafety cabinet, disinfect the work area surface and P1000 and P200 pipets by spraying liberally with 70% ethanol and allowing to air dry.
3. Spray reagent containers with 70% ethanol and place them inside the Biosafety Cabinet. Allow to air dry.
4. Carefully open the Ad-DF (500ml) bottle. Add 5ml of Pen/Strep, 5ml 1M HEPES and 5ml Glutamax to the Ad-DF. Mix by inverting the bottle 2-4 times.
5. Close the bottle and label with Initials, Date, Expiration date (4 weeks after the opening date).
6. Medium should be stored in the refrigerator at 4ºC.

1.11.4. Procedure to establish a culture of Wnt3a-Afm expressing cells, using a cryofrozen Wnt3a/Afm cell line

1. Start-up biosafety cabinet, clean the work area surface and clean the P1000 and P200 pipets with 70% ethanol.
2. Wipe the exteriors of all tubes/containers with 70% ethanol and transfer them into the biosafety cabinet.
3. Wnt3a-Afm culture medium (1.11.2) should be out of the fridge for at least 30 min to reach room temperature and then warm up to 37ºC before starting the procedure.
4. Take a tweezer, cryo protection gloves and a box filled with sufficient amount of dry ice. Remove a vial of Wnt3a expressing cells from the liquid nitrogen tank and place it on the dry ice. Remember to be very careful as the vials are extremely cold and could cause skin damage if held for too long.
5. Thaw the vial rapidly by agitation in a 37ºC water bath until there is still little frozen material (the additional frozen material will thaw by the time you take the vial out of the water bath and proceed to culture). Thawing should be rapid (within 60-120 seconds). Remove the vial from water bath and wipe it down with 70% ethanol at room temperature and transfer to a biosafety cabinet. All following steps must be done under aseptic conditions.
6. Transfer cell suspension into a 15ml conical tube containing 9.5ml Wnt3a-Afm culture media.
7. Centrifuge cells at 200g for 5 min. Aspirate media.
8. Resuspend cells in 1ml culture media and seed cells in a 15cm tissue culture dish containing 20ml culture medium.
9. Cells are confluent in typically 2-4 days and will need to be passaged.
10. To passage cells, aspirate out the spent media and wash once with 25ml DPBS. Aspirate DPBS.

11. Incubate cells with 3ml TrypLE at 37ºC for 2 min.
12. Quench with 6ml culture media and transfer to a 15ml Falcon tube.
13. Centrifuge cells at 200g for 5 min and remove media.
14. Resuspend cells in 1ml culture media and transfer 250µl each to 4 x 15 cm tissue culture dish, each containing 20ml culture media. Add 100µl Zeocin and 80µl Hygromycin B to the plates.

1.11.5. Procedure to prepare Wnt3a-Afm conditioned medium

1. When the cultures are confluent (after 2-4 days), prepare 2 sets of 15 cm dishes:
    a. 4 "Selection Plates" each containing 20 mL Culture Medium + 100 µL of Zeocin + 80 µL hygromycin B, to keep the cells in culture.
    b. 16 "Conditioning Plates" each containing 20 mL Culture Medium without Zeocin or hygromycin B, to generate conditioned medium.
2. Take the culture plates out of the incubator. Carefully pour off the media from the cells and wash the cells with 25ml DPBS.
3. Trypsinize the cells using 3ml TrypLE as indicated in 7.11.4 (steps 10-13).
4. Pool all the cells in a single 50ml Falcon tube and resuspend them in 5ml of culture media.
5. Seed 250µl of the cell suspension into each of the 20 culture dishes (from step 1).
6. After 2 days, change the medium of the selection plates, to fresh culture medium without Zeocin and hygromycin B. When these plates are confluent (typically 4 days after passaging), split the 4 selection plates into 20 new plates as indicated in steps 1-5).
7. Meanwhile, after 2 days of passaging, change the medium of the Conditioning plates and return to the incubator for 7 days.
8. After 7 days, collect the medium from the Conditioning Plates (as batch 1), and replace the medium with 20ml conditioning media. It is best to do these two plates at a time so that the cells don't dry out.
9. Centrifuge the collected conditioned medium at 300g for 6 min at 4ºC, and separate the conditioned medium from any pelleted cells.
10. Filter-sterilize the conditioned medium through a 0.2 µm filter, and store at 4ºC.
11. 5 days after the first conditioned medium collection, collect and filter a second batch of conditioned medium as described in steps 7-10, and then throw away the Conditioning Plates.
12. Store the batches of Afm/Wnt3a-conditioned medium at 4ºC. The Wnt3a activity is comparable between the two batches. However, we have noticed that Wnt3a activity in this medium decrease after freezing, so we recommend not to freeze. We have stored medium up to 2 months at 4ºC, and noticed no decrease in Wnt3a activity.

1.12. Procedure to prepare R-spondin-1 Conditioned Media

Note:
- Rspo1-expressing cell line have been purchased from Trevigen (Catalog no. 3710-001-K).
- Cells can be used for around 10 passages to harvest conditioned medium.

1.12.1. Equipment, Supplies & Reagents

Equipment
- Biosafety Cabinet
- Vortex Mixer
- Pipet aid
- P200
- P1000

Supplies
- 15 ml falcon tubes (Falcon, # F2097)

- 50ml falcon tubes (Falcon, # F2070)
- 5ml plastic pipettes (Falcon, # F7543)
- 10ml plastic pipettes (Falcon, # F7551)
- 25ml plastic pipettes (Falcon, # F7525)
- Ice bucket with ice
- Waste container (for pipet tips, etc)
- 175 cm2 tissue culture flanks
- 15cm plates
- PETG media bottles – 250ml (Corning, # 431732)
- 0.22µm sterile filters (Millipore, # SLGV004SL)
- Sterile glass media bottles

Reagents
- 70% ethanol
- Advanced Dulbecco's Modified Eagles Medium with Nutrient Mixture F-12 Hams – 500ml (Gibco, # 12634-028)
- FBS (Gibco, # 26140079)
- Penicillin/Streptomycin (Pen/Strep) - Pen: 10K U/ml; Strep: 10K µg/ml) (100x concentrated (Gibco, # 15140-122)
- Trypsin (Gibco, # 2530054)
- Zeocin (Invitrogen, # R25001)
- DPBS (Gibco, # 14190-144)
- A frozen vial of R-spondin-1 expressing cell line (from Trevigen # 3710-001-K)
- HSM+++ (section 1.7)

1.12.2. Procedure to prepare culture media for R-spondin-1 expressing cells

1. Take an aliquot of Pen/Strep from -20ºC and thaw it at room temperature or in the 37ºC water bath.
2. Start-up biosafety cabinet, disinfect the work area surface and P1000 and P200 pipets by spraying liberally with 70% ethanol and allowing to air dry.
3. Spray reagent containers with 70% ethanol and place them inside the Biosafety Cabinet. Allow to air dry.
4. Carefully open the DMEM F-12 bottle. Add 60 ml of FBS and 5ml Pen/Strep solutions to the DMEM. Mix by inverting the bottle 2-4 times.
5. Close the bottle and label with Initials, Date, Expiration date (4 weeks after the opening date).
6. Medium should be stored in the refrigerator at 4ºC.

1.12.3. Procedure to establish a culture of R-spondin-1 expressing cells, using a cryofrozen R-spondin-1 cell line

1. Start-up biosafety cabinet, clean the work area surface and clean the P1000 and P200 pipets with 70% ethanol.
2. Wipe the exteriors of all tubes/containers with 70% ethanol and transfer them into the biosafety cabinet.
3. R-spondin-1 culture medium (1.12.2) should be out of the fridge for at least 30 min to reach room temperature and then warm up at 37ºC before starting the procedure.
4. Take a tweezer, cryo protection gloves and a box filled with sufficient amount of dry ice. Remove a vial of R-spondin-1 expressing cells from the liquid nitrogen tank and place it on the dry ice. Remember to be very careful as the vials are extremely cold and could cause damage to skin if held for too long.

5. Thaw the vial rapidly by agitation in a 37ºC water bath until there is still little frozen material (the additional frozen material will thaw by the time you take the vial out of the water bath and proceed to culture). Thawing should be rapid (within 60-120 seconds). Remove the vial from water bath and wipe it down with 70% ethanol at room temperature and transfer to a biosafety cabinet. All following steps must be done under aseptic conditions.
6. Transfer cell suspension into a 15ml conical tube containing 9.5ml R-spondin-1 culture media.
7. Centrifuge cells at 133g for 5 min. Aspirate media.
8. Resuspend cells in 1ml culture media and seed cells in a 175 cm^2 culture flask containing 50ml culture medium and 150µl Zeocin (100µg/ml).
9. Cells are confluent in typically 2-4 days and will need to be passaged.
10. To passage cells, aspirate out the spent media wash once with 25ml DPBS. Aspirate DPBS.
11. Incubate cells with 2ml trypsin at 37ºC for 2 min.
12. Quench with 18ml culture media.
13. Centrifuge cells at 133g for 5 min and remove media.
14. Resuspend cells in 6ml culture media and transfer 1ml each to 6 x 175 cm^2 culture flasks each containing 50ml culture media. Add 62.5 µl Zeocin to only one of the flasks (the "Selection Flask"), while leaving the other 5 flasks (the "Conditioning Flasks") free of Zeocin.

1.12.4. Procedure to prepare R-spondin-1 conditioned medium

1. When the "Conditioning Flasks" are confluent (after 2-4 days), carefully pour off the media from the cells and wash the cells with 2 times with 25ml DPBS.
2. Add 50ml of HSM+++ to each flask.
3. Incubate cells for one week in the tissue culture incubator. After 1 week, carefully decant the media from the cells and transfer to 50ml Falcon tubes.
4. Centrifuge the media for 5 min at 300g to pellet floating cells.
5. Carefully pour the supernatants into a 0.2µm sterile filter attached to a sterile bottle and filter the media.
6. Aliquot filtered media and either freeze and store at -20ºC or store at 4ºC.

1.13. Procedure to perform Dual Luciferase Reporter assay to check Wnt3a activity in the Wnt3a conditioned media used for organoid culture

Note:

- To monitor the activity of WNT3a we use TCF/LEF Reporter kit that contains transfection-ready TCF/LEF luciferase reporter vector, which is a Wnt pathway-responsive reporter. This reporter contains a firefly luciferase gene under the control of multimerized TCF/LEF responsive element located upstream of a minimal promoter. The TCF/LEF reporter is premixed with constitutively-expressing Renilla luciferase vector as internal control for transfection efficiency. The kit also includes a non-inducible Firefly luciferase vector premixed with constitutively-expressing Renilla luciferase vector as negative control. The non-inducible luciferase vector contains a firefly luciferase gene under the control of a minimal promoter, without any additional response elements. The negative control is critical to determining pathway specific effects and background luciferase activity.
- Ratio between Firefly and Renilla luciferase is an indication for Wnt activity of the conditioned medium.
- **Good batch** of Wnt conditioned medium gives a Firefly/Renilla ratio > 30.

1.13.1. Equipment, Supplies & Reagents

Equipment

- Biosafety Cabinet
- Vortex Mixer
- Pipet aid
- P20
- P200
- P1000

Supplies
- 15 ml falcon tubes (Falcon, # F2097)
- 5ml plastic pipettes (Falcon, # F7543)
- 10ml plastic pipettes (Falcon, # F7551)
- 25ml plastic pipettes (Falcon, # F7525)
- Tips p1000 (Corning, #4140)
- Tips p200 (Corning, #4138)
- Tips p20 (Corning, #4136)
- Ice bucket with ice
- Waste container (for pipet tips, etc)
- 75 cm2 tissue culture flask
- 96-microwell plate (Corning, #3596)
- 96-micowell white plate (Corning, #277105)
- 12-well plate (Greiner, #662102)

Reagents
- 70% ethanol
- DMEM (Gibco, #41965-062)
- DMEM-F12 Advanced (Gibco, #12634-028)
- Optimem (Gibco, #31905070)
- Hepes (Gibco, #15630-080)
- Glutamax (Gibco, #35050-061)
- FBS (Gibco, # 26140079)
- Penicillin-Streptamycin (Gibco, #15140-122)
- Trypsin (Gibco, # 2530054)
- DPBS (Gibco, # 14190-144)
- A 75cm2 flask with 293T cells
- CCS-018L-Cignal RCF/LEF Reporter Assay Kit (Qiagen, #3368-41-CCS018L)
- Dual Luciferase reporter assay system (Promega, #E1940)
- Attractene (Qiagen, #301005)
- Wnt conditioned media batches to test, 2 ml from each batch

1.13.2 Procedure:

Day 1:

1. Thaw the constructs and attractene on ice.
2. Prepare cells

   Trypsinize a plate of 293T cells and stop reaction with DMEM + 10% FBS + 1% Penicillin-Streptamycin (P/S).

   Centrifuge and resuspend cells in Optimem (without FBS) and count cells.

   Plate in each well 100 µl of Optimem + 5% FBS + 1% P/S containing 30000 cells/well. (If you plate 10 well you need 300’000 cells in 1 ml of media).

   For each conditioned media, plate 3 constructs in triplicate (Negative/Inducible/Positive). Plate also an internal control of not transfected cells (NT), as described:

Negative	Negative	Negative	Inducible	Inducible	Inducible	Positive	Positive	Positive	NT	NT	NT

Incubate at 37ºC for 30 min.

3. Prepare transfection mix

Spin the constructs.

On ice, in a 12-well plate prepare the pre-transfection mix as follow:

Construct (multiply for the number of wells):

	OPTIMEM (no FBS)	CONSTRUCT
NEG	25 µl	1 µl
IND	25 µl	1 µl
POS	25 µl	1 µl

Attractene (multiply for the number of wells):

	OPTIMEM (no FBS)	ATTRACTENE
NEG	25 µl	0.6 µl
IND	25 µl	0.6 µl
POS	25 µl	0.6 µl

Pool both pre-mix together (Optimem-Construct + Optimem-Attractene).

Incubate mix for 20' at room temperature.

4. Transfect cells

In each well add 50 µl of transfection mix, as described:

Negative	Negative	Negative	Inducible	Inducible	Inducible	Positive	Positive	Positive	NT	NT	NT

Incubate at 37ºC for 24h.

Day 2:

5. Control transfection in 293T GFP^{+}-cells (positive control). If the GFP^{+} cells are more than 60-70% proceed with the assay.
6. Stimulate the cells with batches of Wnt conditioned medium (for each 96-well plate, 8 batches can be tested): For each batch, make a 50% Wnt CM (dilute 1:1 in DMEM-F12 Advanced + 1% Glutamax + 1% Hepes).
7. Leave to grow for another 24 hours.

Day 3:

8. Lysis cells

Prepare 1x passive lysis buffer (1x PLB) by diluting 5x passive lysis buffer from Promega kit with distilled H_2O.

Remove all medium from the wells and wash with 100 µl DPBS **(Be careful because cells can be detached)**. Add 20µl 1x PLB to each well.

Incubate in rotation at room temperature for 15 min.

9. Results

In the meantime, prepare 1x Stop&Glo® solution by diluting 50X Stop&Glo® substrate with Stop&Glo® buffer (as described in kit protocol).
Prepare Luciferase Assay Buffer II® (LARII) solution by diluting Luciferase Assay Substrate® in Luciferase Assay Buffer®.
After 20 min of incubation transfer 20 µl of lysate cells solution into a 96-well white plate (for luminescence).
Add 100 µl each well of LARII®.
Measure, without cover plate, the luminescence in 560 nm (Firefly luciferase).
Add 100 µl each well of Stop&Glo® 1X and measure the luminescence in 480 nm (Renilla luciferase).
Ratio between Luciferase and Renilla is and indicator for WNT activity of the conditioned medium.

2. Establishment Of Organoids From Human Pancreatic Tumor Tissues

2.1. Purpose: To describe the procedures for establishment of organoids from surgically resected human pancreatic cancer tissues, maintenance of these cultures, passaging them and procedures for cryofreezing them. This is a general procedure and certain details and reagents may vary depending on the particular tumor type and characteristics and site of model generation.

2.2. Scope: This SOP applies to all laboratory personnel at University of Verona, who are involved in establishing organoids from human pancreatic tumor tissue.

2.3. Responsibilities: As per institutional regulations, authorized personnel processing the pancreatic tumor samples must ensure that:

- All samples used are obtained lawfully and with appropriate consent;
- All procedures are followed correctly and are compliant with the SOP;
- All samples are adequately labeled during processing;
- All the applicable documentation and records are collected, updated and maintained for all samples.

Authorized personnel responsibilities also include:

- Preparation of stock and supplemented media, recognizing when additional stock medium needs to be prepared, and when media and other reagents need to be ordered;
- Culturing, splitting, freezing and thawing the organoids as required;
- Recognizing when new cells need to be thawed and when cells have to be frozen down;
- Recognizing when organoid culture stocks are getting low and when more cells need to be frozen down.

2.4. Health and Safety: Authorized personnel carrying out this procedure should maintain safe working practices and observe all relevant environmental health and safety guidelines. This includes the appropriate use of personal protective equipment, and procedures for waste disposal, disinfection, and biosafety. Gloves and lab coat are the minimal amount of protection that should be worn. Snug fitting non-permeable (waterproof) gloves are best. Eye protection is also recommended when exposure to splashing liquid nitrogen, solvents and biological active material is possible.

2.5. Definition and Acronyms

- SOP: Standard Operation Procedure
- HSM+++: Human Splitting Media
- DM: Digestion Medium
- hCPLT: Human Complete Medium
- RhoKi: Y-27632 – Rho kinase inhibitor
- CRS: Cell Recovery Solution

2.6. Equipment, Materials & Reagents

Equipment

- Biosafety Cabinet
- Suction system to aspirate liquids using a Pasteur pipet, set up inside the biosafety cabinet
- Light Microscope
- Centrifuge
- Orbital Shaker
- Vortex Mixer
- Gas Burner
- Pipet aid
- P200

- P1000
- Cell Culture Incubator (37°C & 5% CO_2)

Supplies
- Disposable scalpels (Swann Morton, # 0501)
- 15 ml falcon tubes (Falcon, # F2097)
- 50ml falcon tubes (Falcon, # F2070)
- 5ml plastic pipettes (Falcon, # F7543)
- 10ml plastic pipettes (Falcon, # F7551)
- 25ml plastic pipettes (Falcon, # F7525)
- Sterile glass Pasteur pipettes (VWR, # 14673-010)
- 10 cm Petri dish (Corning, # 430167)
- 24-well Suspension Plates (Greiner, # 662-102)
- 6-well Suspension Plates (Sarstedt, # 833-920-500)
- Cryovials (VWR, # 66008-706)
- Parafilm (VWR, # 82824-547)
- Cell Strainer - 70µm (VWR, # 10199-656)
- Ice bucket with ice
- Waste container (for pipet tips, etc)
- Dewar flask or ice bucket with sufficient liquid Nitrogen for snap-freezing cryovial
- Mr. Frosty freezing container (VWR, #55710-200)

Reagents
- 70% ethanol
- HSM+++ (section 1.7)
- hCPLT: Used for culture of organoids from PDAC.
- Matrigel, growth factor reduced phenol red free (Corning, # 356231)
- Digestion Medium (DM) (section 1.9)
- Y-27632 – Rho kinase inhibitor (Sigma, # Y0503)
- DNAseI (Sigma, # D5025-150KU)
- ACK blood cell lysis solution (Gibco, # A1049201)
- Recovery Cell Freezing Medium (Gibco, # 12648-010)
- FBS (Gibco, # 26140079)
- UltraPure DNase/RNase-Free Distilled Water (Thermo-Fisher, # 10977-015)
- Cell Recovery Solution (Corning, # 354253)

Note:

- Matrigel should be stored in a -20ºC freezer. Matrigel begins to harden and polymerize at temperatures above 4ºC. If polymerization has occurred, do not reuse the Matrigel. Allow enough time for your Matrigel aliquot to thaw on ice before use. An 800µl aliquot of frozen Matrigel takes ~1 hour and 15 min to thaw on ice, and a full vial takes ~8 hours. To aliquot a full vial, thaw on ice overnight, and aliquot into pre-chilled tubes. For testing, both normal and tumor organoids are cultured in a new batch (or batches) of Matrigel, in respective culture media and their growth is monitored.

2.7. Quality Control

1. Monitor culture by microscopic examination regularly and examine for signs of microbial contamination or cell distress. Make notes of observations in the cell culture lab notebook.
2. Regular mycoplasma testing of the organoid culture is recommended, since mycoplasma contamination cannot be detected easily by visual examination.
3. Record culturing, passaging and freezing of cells in notebook. Be sure to also include the date on which media was changed, cells were split or frozen. You should also update the liquid

nitrogen logbook if removing a vial to start new batch of cells or when transferring cryogenic vials with frozen cells to the liquid nitrogen.
4. Each time you split the organoids, you increase the passage number by one. When you start cells from a freeze down, it is recommended that you start with the passage number at which the organoids were frozen and every time the organoids are passaged, you increase the passage number by one.
5. The personnel should also document any anomalies and/or deviations from the specified SOP.
6. All the tubes, bottles, cell culture well-plates and cryovials should be labelled properly and legibly. Labels should contain information such as organoid identifier number, passage number since original isolation from tissue, passage date and initials
7. All growth factors, stock media and supplemented media should be stored at the appropriate storage temperature.
8. When preparing media, make sure you date and put your initials on the media bottle and enter this information diligently in your lab notebook. It is also recommended to note the lot numbers of media and other reagents in your lab notebook.
9. During processing, it is recommended that tissue and organoids are kept on ice, except unless specified.

2.8. Procedures

Note:

- The process and reagent volumes described below are applicable for culturing organoids in a 24-well plate. Scale-up accordingly if using different culture conditions, as indicated below.

Culture Dish	No. of Matrigel Domes/Well	Matrigel Volume/Dome	Culture Media/Well
24-well plate	1	50µl	0.5ml
6-well plate	8	50µl	3.0ml

2.8.1. Procedure to establish a human pancreatic cancer tumor organoid culture

Day 0:

1. Thaw Matrigel overnight at 4ºC and maintain on ice when not refrigerated.
2. Warm up 24-well cell culture plate(s) overnight in the 37ºC incubator.
3. Store cryovials and Mr. Frosty Freezing Container at 4ºC.

Day 1:

4. Tissue sample is collected at the tissue source site in a 50ml Falcon tube containing HSM+++. After the sample arrives in the culture lab, keep sample on ice until start of isolation.
5. When the pancreatic tumor sample is received at the laboratory, it is associated with a code referred to as ID1, which is assigned by the tissue source site. Make sure that designated personnel from the laboratory have recorded ID1 and have linked the sample to a new code designated as ID2.
   Note: Only authorized personnel should have access to the ID1 code and the ID1-ID2 link.
6. If bar-coding system is available, create an ID2 barcode label for the sample.

7. Start-up biosafety cabinet, disinfect the work area surface and P1000 and P200 pipets by spraying liberally with 70% ethanol and allowing to air dry.
8. Prepare fresh DM and hCPLT. Protocol included in Section 1.9 and 1.8 respectively.
9. Label 2-4 cryovials and 1-2 24-well culture plates with ID2-coded bar-code or non-bar-code labels. The 24-well culture plates will be used for plating cells for organoid culture. The cryovials will be used for snap freezing primary tumor tissue and for freezing digested material. Label the cryovials clearly to indicate that their contents contain primary tissue or digested material.
10. Transfer the tumor tissue to a 10cm Petri dish.
11. Examine the tissue macroscopically, and record the size, shape and gross morphological characteristics such as amount of fat content, vasculature, necrotic content if applicable, etc. Take a picture of the tumor if possible.
12. Excise out approximately 50mg tumor tissue, preferably from the center of the tissue, and place into an ID2 labeled cryovial. Snap-freeze cryovial in liquid nitrogen.
13. Mince the remaining tissue specimen into small fragments (1 mm^3 or less) using sterile scalpels. **Note:** If there is fat present, try to dissect it away from the specimen. Pancreatic tumors are highly fibrotic, and therefore, the tissue is usually hard.
14. Transfer the remaining tissue pieces into a 15ml conical tube and add 8ml of pre-warmed DM.
15. Place the tube on rotator (37ºC), with rotation (35 rpm) for an initial digestion of 1h.
16. Check the digestion by sampling a small volume (10µl) of the digestion media and spotting it in a petri dish. Observe the material under an inverted microscope and if several clusters of cells are visible, stop the reaction, by placing the tube on ice and adding ice-cold +++ media.
17. If clusters of cells are not visible, continue with digestion and check every 30 min.
18. Stop reaction after 2hrs, quench enzymatic reaction placing 15ml falcon tube in ice.
19. Centrifuge for 5 min at 200g at 4ºC.
20. Remove media to leave 1.5ml behind and add 8.5ml of ice-cold +++.
21. Centrifuge the tube at 200g for 5 min at 4ºC.
22. In the unlikely event that the tumor is well vascularized and a noticeable amount of blood is visible, perform red blood cell lysis using ACK lysing buffer.
    a) Add 4ml of ACK lysing buffer. Invert the tube gently a few times to dislodge the pellet.
    b) Centrifuge the tube at 200g for 5 min at 4ºC.
23. Remove supernatant, add 1.5ml TrypLE Express supplemented with 15µl DNAseI (10mg/ml) for 5-10 min (no more than 10 min), and incubate in a 37ºC water bath.
    Note: Every 5 min pipette up and down with 1000 pipette tip to break large clumps.
24. At the end of 10 min, pipette the suspension with P1000 for 10 times to ensure large clumps are broken down.
25. Stop the reaction by placing the tube on ice.
    Note: For cryopreserve digested material, follow the procedure to cryopreserve digested material starting from tumor tissue.

Cryopreserve digested material starting from tumor tissue

i. After step 25, pipette the suspension with P200 for 5 times and transfer 200µl of cell suspension in a 1.5ml Eppendorf tube and add 800µl of ice-cold +++.
ii. Centrifuge the Eppendorf tube at 400g for 5 min at 4ºC. Without disturbing the pellet, carefully remove the supernatant.
iii. Resuspend the cell pellet in 500µl of Recovery Cell Freezing Medium.
iv. Using a P1000 pipette tip, transfer 500µl of suspension resuspended in the freezing medium to sterile labelled cryovial.
v. Move vial to Mr. Frosty Freezing container. Incubate for 24 hours at -80ºC. After 24 hours transfer the vial to liquid nitrogen on dry for long term storage.

26. Add ice-cold+++ up to 10ml.
27. Spin down at 200g for 5 min at 4ºC. Without disturbing the pellet, carefully remove the supernatant.
28. Resuspend the pellet in Matrigel. Volume of Matrigel depends on pellet size. Usually the pellet

from 30mg primary tumor tissue is resuspended in about 400µl of Matrigel, and 50µl of this suspension is plated per well in a 24-well plate.
29. Place the plate into a 37°C tissue culture incubator until Matrigel solidified (10-15 min).
30. Add 10.5mM Rho Kinase Inhibitor stock to hCPLT. You will need 500µl of media per well plated.
31. Add 500µl of pre-warmed hCPLT supplemented with Rhoki to the wells of the 24-well plate containing Matrigel domes. Return the 24-well plate to the 37ºC cell culture incubator.

Day 2:
32. 24 hrs after plating, observe the Matrigel domes under the microscope and take microscopic images using 4X and/or 10X objectives.

2.8.2. Procedure to maintain a human pancreatic tumor organoid culture

Note:
- Media is refreshed every 2-3 days after passaging.
- Pre-warm hCPLT in a 37ºC waterbath.

1. Start-up biosafety cabinet, disinfect the work area surface and P1000 and P200 pipets by spraying liberally with 70% ethanol and allowing to air dry.
2. Prepare fresh hCPLT.
3. Carefully remove the medium in each well using a Pasteur pipet connected to a suction system.
4. Carefully add 500µl fresh pre-warmed hCPLT per well to the pancreatic organoids.
5. Put the culture plate back into the cell culture incubator.

2.8.3. Procedure to passage a human pancreatic tumor organoid culture

Note:
- Although most tumor organoids do not have a visible lumen, presence of dead cells around the organoids indicate that the organoids are due to be passaged. Splitting ratio is usually 1:2-1:8.

Day 0:

1. Thaw Matrigel overnight at 4ºC and maintain on ice when not refrigerated.
2. Warm up 24-well cell culture plate(s) overnight in the 37ºC incubator.

Day 1:

3. Start-up biosafety cabinet, disinfect the work area surface and P1000 and P200 pipets by spraying liberally with 70% ethanol and allowing to air dry.
4. Prepare fresh hCPLT. Add Y-27632 – Rho kinase inhibitor to a final concentration of 10nM.
   Note: RhoKi is added only to single cell suspensions or during passaging. A 10000X (100mM) RhoKi stock solution is made by resuspending 50mg RhoKi in 1.5ml of Ultrapure RNAse and DNAse-free water. 1000X (10mM) diluted stocks (in ultrapure RNAse and DNAse-free water) are stored as 100µl aliquots at -20ºC.
5. Wipe all tubes/containers with 70% ethanol and transfer organoids and hCPLT, TrypLE Express, and CRS into the biosafety cabinet.
6. Prepare ID2-coded bar-coded or non-bar-coded labels for culture plates. Include passage no. details (such as P1, P2, etc.) on the labels.
7. Aspirate media from each of the wells.
8. Add 1ml of ice cold CRS to alternate wells of the plate.
9. Starting with a well that had CRS added to it, pipette the liquid up and down until the Matrigel

falls apart and the solution is homogeneous.

10. Take the mix of organoids, Matrigel, and CRS and pipette it into a well that does not have CRS added to it.
11. Pipette up and down again, until the solution is homogeneous.
12. Move the suspension to a 15ml conical tube on ice.
13. Repeat this procedure for the remaining wells. Harvest up to 24 Matrigel domes per 15ml conical tube.
14. Incubate on ice for 30 min, inverting the tube every 10 min.
    Note: For cystic organoids, follow procedure for Mechanical Dissociation. For solid organoids, follow procedure for Enzymatic Dissociation.

ENZYMATIC DISSOCIATION:

i. After step 14, centrifuge the tube at 200g for 5 min at 4ºC.
ii. Aspirate the supernatant, being careful not to disturb the pellet.
iii. Resuspend the pellet in 4ml of TrypLE Express supplemented with 4µl 10mg/ml DNAse I stock.
iv. Incubate conical tube at 37ºC with 35 rpm rotation for 5 min.
v. Examine the contents of the conical tube under an inverted microscope, checking to see if the organoids have dissociated into cell clumps or single cells. If most of the organoids are still intact, continue the dissociation, checking every 3 to 5min. If sufficient cell clumps are observed, continue to vi.
vi. Centrifuge the tube at 200g for 5 min at 4ºC.
vii. Aspirate the supernatant, being careful not to disturb the pellet.
viii. Add 1ml of ice-cold HSM+++ media to the pellet, and pipette up and down at least 20 times, making sure to hit the bottom of the tube with the pipette tip to resuspend the pellet.
ix. Add additional HSM+++ media to a total volume of 10ml and invert the tube a couple of times.
x. Centrifuge the tube at 200g for 5 min at 4ºC.
xi. Aspirate the supernatant, being careful not to disturb the pellet.

MECHANICAL SHEARING:

i. After Step 14, centrifuge the tube at 200g for 5 min at 4ºC.
ii. Aspirate the supernatant, being careful not to disturb the pellet.
iii. Add 1ml of ice cold HSM+++ to the pellet.
iv. Pipette up and down making sure to hit the bottom of the conical tube with the pipette tip to properly resuspend the pellet and shear the organoids.
v. Add additional HSM+++ media to bring the volume to 10ml total and invert the tube a couple of times to resuspend pellet.
vi. Centrifuge the tubes at 200g for 5 min at 4ºC.
vii. Aspirate the supernatant, being careful not to disturb the pellet.
viii. Repeat steps iii-vii, until the pellet is clean and free of Matrigel (typically done 1 to 3 times).

15. Resuspend the pellet in Matrigel. The volume of Matrigel depends on the density of the organoids and the desired plating ratio. For eg: For 1:2 plating of an entire 24 well plate of organoids, add 2.4 ml of Matrigel.
16. Plate 50µl Matrigel domes into pre-warmed 24-well plates on top of a pre-warmed hot water bottle.
17. Place the plate into a 37ºC tissue culture incubator until Matrigel solidifies (typically 5-15 min).
18. Add 500µl of pre-warmed hCPLT.
19. Return organoids to 37ºC tissue culture incubator.

Note:

- Media is replenished every 3-4 days.

2.8.4. Procedure to freeze down a human pancreatic tumor organoid culture

Note: To increase survival post-cryofreezing, organoids should be frozen down 1-2 days after trypsinization, when they have a smaller size.

Day 1:

1. Start-up biosafety cabinet, disinfect the work area surface and P1000 and P200 pipets by spraying liberally with 70% ethanol and allowing to air dry.
2. Wipe all tubes/containers with 70% ethanol and transfer organoids and Recovery Cell Freezing Media into the biosafety cabinet.
3. Prepare ID2-coded bar-coded or non-bar-coded labels for cryovials. Include passage no. details (such as P1, P2, etc.) on the labels. Attach labels on the cryovials.
4. A picture of the organoids, prior to resuspension, may be taken for records.
5. Aspirate media from each of the wells.
6. Add 1ml of ice cold CRS to alternate wells of the plate.
7. Starting with a well that had CRS added to it, pipette the liquid up and down until the Matrigel falls apart and the solution is homogeneous.
8. Take the mix of organoids, Matrigel, and CRS and pipette it into a well that does not have CRS added to it.
9. Pipette up and down again, until the solution is homogeneous.
10. Move the suspension to a 15ml conical tube on ice.
11. Repeat this procedure for the remaining wells. Harvest up to 16 Matrigel domes per 15ml conical tube.
12. Incubate on ice for 1h, inverting the tube every 10 min.
    Note: For cystic organoids, follow procedure for Mechanical Dissociation. For solid organoids, follow procedure for Enzymatic Dissociation.

ENZYMATIC DISSOCIATION:

i. After step 12, centrifuge the tube at 200g for 5 min at 4ºC.
ii. Aspirate the supernatant, being careful not to disturb the pellet.
iii. Resuspend the pellet in 4ml of TrypLE Express supplemented with 4µl 10mg/ml DNAse I stock.
iv. Incubate conical tube at 37ºC with 35 rpm rotation for 5 min.
v. Examine the contents of the conical tube under an inverted microscope, checking to see if the organoids have dissociated into cell clumps or single cells. If most of the organoids are still intact, continue the dissociation, checking every 3 to 5 min. If sufficient cell clumps are observed, continue to vi.
vi. Centrifuge the tube at 200g for 5 min at 4ºC.
vii. Aspirate the supernatant, being careful not to disturb the pellet.
viii. Add 1ml of ice-cold HSM+++ to the pellet, and pipette up and down at least 20 times, making sure to hit the bottom of the tube with the pipette tip to resuspend the pellet.
ix. Add additional HSM+++ to a total volume of 10ml and invert the tube a couple of times.
x. Centrifuge the tube at 200g for 5 min at 4ºC.
xi. Aspirate the supernatant, being careful not to disturb the pellet.

MECHANICAL SHEARING:

i. After Step 12, centrifuge the tube at 200g for 5 min at 4ºC.
ii. Aspirate the supernatant, being careful not to disturb the pellet.
iii. Add 1ml of ice cold HSM+++ to the pellet.
iv. Pipette up and down making sure to hit the bottom of the conical tube with the pipette tip to properly resuspend the pellet and shear the organoids.
v. Add additional HSM+++ media to bring the volume to 10ml total and invert the tube a couple of times to resuspend pellet.
vi. Centrifuge the tubes at 200g for 5 min at 4ºC.
vii. Aspirate the supernatant, being careful not to disturb the pellet.
viii. Repeat steps iii-vii, until the pellet is clean and free of Matrigel (typically done 1 to 3 times).

13. Resuspend the cell pellet in Recovery Cell Freezing Medium. 500µl of freezing medium will be used for each 400µl of Matrigel.
14. Using a P1000 pipette tip, transfer 500µl of organoids resuspended in the freezing medium to sterile labelled cryovials.
15. Move vials to Mr. Frosty Freezing container. Incubate for 24 hours at -80ºC. After 24 hours transfer the vials to liquid nitrogen on dry for long term storage.

2.8.5. Procedure to collect pellet from human pancreatic tumor organoid culture

Note:

- Organoid should be harvested at 70-80% of confluency. Four domes of Matrigel (each of 50µl) are sufficient to obtain at least 1µg of nucleic acids (either DNA or RNA) for downstream analysis. If DNA and RNA from organoid cultures at the same passage are needed, it is advisable to harvest 8-10 domes of Matrigel.

Day 1

1. Start-up biosafety cabinet; disinfect the work area surface and P1000 and P200 pipets by spraying liberally with 70% ethanol and allowing to air dry.
2. Wipe all tubes/containers with 70% ethanol and transfer organoids, TrypLE Express, and CRS into the biosafety cabinet.
3. Prepare ID2-coded bar-coded or non-bar-coded labels for 1.5 ml Eppendorf. Include passage no. details (such as P1, P2, etc.) on the labels.
4. Aspirate media from each of the wells.
5. Add 1ml of ice-cold CRS to alternate wells of the plate.
6. Starting with a well that had CRS added to it, pipette the liquid up and down until the Matrigel falls apart and the solution is homogeneous.
7. Take the mix of organoids, Matrigel, and CRS and pipette it into a well that does not have CRS added to it.
8. Pipette up and down again, until the solution is homogeneous.
9. Move the suspension to a 15ml conical tube on ice.
10. Repeat this procedure for the remaining wells. Harvest a maximum of 10 Matrigel domes per 15ml conical tube.
11. Incubate on ice for 30min, inverting the tube every 10min.
12. Centrifuge the tubes at 200g for 5min at 4ºC
13. Carefully aspirate the supernatant and add 10 mL of ice-cold DPBS. Invert the tube a couple of times to dissolve Matrigel.
14. Centrifuge the tube at 200g for 5min at 4ºC.
15. Carefully aspirate the DPBS trying not to disturb the pellet and resuspend it 1ml of ice-cold DPBS.
16. Centrifuge 6200g 5 min at 4ºC.
17. Carefully aspirate the DPBS without disrupting pellet.
18. Put pellet in dry ice and storage in -80ºC.

3. Establishing Pancreatic Tumor Organoids From Cryofrozen Cultures

3.1. Purpose: To describe the procedures for establishing human pancreatic cancer organoid cultures, from cryofrozen cultures, maintenance of these cultures, passaging them and freezing them down. This is a general procedure and certain details, and reagents may vary depending on the particular tumor type and characteristics and site of model generation.

3.2. Scope: This SOP applies to all laboratory personnel at University of Verona, and other end-users who are involved in establishing and maintaining pancreatic cancer organoids from cryofrozen organoids.

3.3. Responsibilities: Authorized personnel processing the pancreatic tumor organoids must ensure that:

- All samples used for establishing the tumor organoids are obtained lawfully and with appropriate consent;
- All procedures are followed correctly and are compliant with the SOP;
- All samples are adequately labeled during processing;
- All the applicable documentation and records are collected, updated and maintained for all samples.

Authorized personnel responsibilities also include:

- Preparation of stock and supplemented media, recognizing when additional stock medium needs to be prepared, and when media and other reagents need to be ordered;
- Culturing, splitting, freezing and thawing the organoids as required;
- Recognizing when new cells need to be thawed and when cells have to be frozen down;
- Recognizing when organoid culture stocks are getting low and when more cells need to be frozen down.

3.4. Health and Safety: Authorized personnel carrying out this procedure should maintain safe working practices and observe all relevant environmental health and safety guidelines. This includes the appropriate use of personal protective equipment, and procedures for waste disposal, disinfection, and biosafety. Gloves and lab coat are the minimal amount of protection that should be worn. Snug fitting non-permeable (waterproof) gloves are best. Eye protection is also recommended when exposure to splashing liquid nitrogen, solvents and biological active material is possible.

3.5. Definition and Acronyms

- SOP: Standard Operation Procedure
- HSM+++: Human Splitting Medium
- hCPLT: Human Complete Medium
- RhoKi: Y-27632 – Rho kinase inhibitor

3.6. Equipment, Materials & Reagents

Equipment

- Biosafety Cabinet
- Suction system to aspirate liquids using a Pasteur pipet, set up inside the biosafety cabinet
- Light Microscope
- Centrifuge
- Gas Burner
- Pipet aid
- P200
- P1000

- Cell Culture Incubator (37ºC & 5% CO_2)

Supplies
- 15 ml falcon tubes (Falcon, # F2097)
- 50ml falcon tubes (Falcon, # F2070)
- 5ml plastic pipettes (Falcon, # F7543)
- 10ml plastic pipettes (Falcon, # F7551)
- 25ml plastic pipettes (Falcon, # F7525)
- Sterile glass Pasteur pipettes (VWR, # 14673-010)
- 24-well Suspension Plates (Greiner # 662 102)
- 6-well Suspension Plates (Sarstedt, # 833-920-500)
- Ice bucket with ice
- Waste container (for pipet tips, etc)
- Dewar flask or ice bucket with sufficient liquid Nitrogen for snap-freezing cryovial.

Reagents
- 70% ethanol
- HSM+++ (section 1.7)
- hCPLT: Used for culture of organoids from PDAC
- Matrigel, growth factor reduced phenol red free (Corning, # 356231)
- Y-27632 – Rho kinase inhibitor (Sigma, #Y0503)

Note:
- Matrigel should be stored in a -20ºC freezer. Matrigel begins to harden and polymerize at temperatures above 4ºC. If polymerization has occurred, do not reuse the Matrigel. Allow enough time for your Matrigel aliquot to thaw on ice before use. An 800µl aliquot of frozen Matrigel takes ~1 hour and 15min to thaw on ice, and a full vial takes ~8 hours. To aliquot a full vial, thaw on ice overnight, and aliquot into pre-chilled tubes.

3.7. Quality Control

1. Monitor culture by microscopic examination regularly and look out for signs of microbial contamination or cell distress. Make notes of observations in the cell culture lab notebook.
2. Regular mycoplasma testing of the organoid culture is recommended, since mycoplasma contamination cannot be detected easily by visual examination.
3. Record culturing, passaging and freezing of cells in notebook. Be sure to also include the date on which media was changed, cells were split or frozen. You should also update the liquid nitrogen logbook if removing a vial to start new batch of cells or when transferring cryogenic vials with frozen cells to the liquid nitrogen.
4. Each time you split the organoids, you increase the passage number and total number of passages by one. When you start cells from a freeze down, it is recommended that you start with the passage number at which the organoids were frozen and every time the organoids are passaged, you increase the passage number by one.
5. The personnel should also document any anomalies and/or deviations from the specified SOP.
6. All the tubes, bottles, cell culture well-plates and cryovials should be labelled properly and legibly. Labels should contain information such as organoid identifier number, passage number since original isolation from tissue, passage date and initials.
7. All growth factors, stock media and supplemented media should be stored at the appropriate storage temperature.
8. When preparing media, make sure you date and put your initials on the media bottle and enter this information diligently in your lab notebook. It is also recommended to note down the lot numbers of media and other reagents in your lab notebook.
9. Tissue and organoids should be kept on ice when they are being processed.

10. It is recommended to check viability of cryofrozen organoids by thawing a vial of early passage and late passage organoids, established from a tumor tissue.

3.8. Procedures

Note: The process and reagent volumes described below are applicable for culturing organoids in a 24-well plate. Scale-up accordingly if using different culture conditions, as indicated below.

Culture Dish	No. of Matrigel Domes/Well	Matrigel Volume/Dome	Culture Media/Well
24-well plate	1	50µl	0.5ml
6-well plate	4	50µl	2.0ml

3.8.1. Procedure to establish an organoid culture from cryofrozen pancreatic tumor organoids

Day 0:

1. Thaw Matrigel overnight at 4ºC and keep cold.
2. Warm up 24-well cell culture plate(s) overnight in the 37ºC incubator.

Day 1:

Note: Cell membranes can be damaged due to osmotic shock caused by the DMSO present in the Recovery Cell Freezing medium. Organoid cultures should be started up individually, so as to avoid cross contamination. It is very important that one sample is handled at a time.

3. Start-up biosafety cabinet, clean the work area surface and clean the P1000 and P200 pipets with 70% ethanol.
4. Wipe the exteriors of all tubes/containers with 70% ethanol and transfer them into the biosafety cabinet.
5. Prepare ID2-coded bar-coded or non-bar-coded labels for culture plates. Include passage no. details (such as P1, P2, etc.) on the labels.
6. HSM+++ medium should be out of the fridge for at least 30min to reach room temperature and then warm up at 37ºC before starting the procedure.
   Note: While thawing organoids, the frozen organoids should be diluted in warm HSM+++ medium as quickly as possible. If the cells remain in the freezing medium for too long the viability will be poor.
7. Wipe all tubes/containers with 70% ethanol and transfer organoids and Recovery Cell Freezing Media into the biosafety cabinet.
8. Take a tweezer, cryo-protection gloves and a box filled with sufficient amount of dry ice. Remove a vial of organoids from the liquid nitrogen tank and place it on the dry ice. Remember to be very careful as the vials are extremely cold and could cause damage to skin if held for too long.
9. Thaw the vial rapidly by agitation in a 37°C water bath until there is still little frozen material (the additional frozen material will thaw by the time you take the vial out of the water bath and proceed to culture). Thawing should be rapid (within 60-120 seconds). Remove the vial from water bath and wipe it down with 70% ethanol at room temperature and transfer to a biosafety cabinet. All following steps must be done under aseptic conditions.
10. When a small bit of ice remains in the vial, pre-wet a P1000 with HSM+++ and pipette the cell suspension into a 15ml conical tube containing 8.5ml of cold (not ice-cold) HSM+++.
11. Wash the cryovial with 1ml of room temperature HSM+++, and add to the conical tube containing the rest of the organoids.

12. Centrifuge tube at 200g for 5min at room temperature.
13. Aspirate the supernatant carefully, using a Pasteur pipette connected to a suction system, without disturbing the pellet. Re-suspend the pellet in the 350µl of Matrigel, so the organoids are seeded in approximately same volume of Matrigel that was frozen in the vial (about 400µl).
    Note: Matrigel and pelleted organoids should stay on ice during this procedure.
14. Plate 50µl cell suspension as a single droplet, in a pre-warmed 24-well tissue culture plate. Transfer the plate to a 37ºC incubator. Leave for 20-30min for Matrigel to solidify.
15. Add 500µl of pre-warmed hCPLT supplemented with 10.5 µM Rho Kinase Inhibitor to each well.
16. Transfer the plate back to the 37ºC incubator.

Day 2:

17. Look at the organoid culture under the microscope and make notes on culture characteristics, such as recovery rate, density, etc. Take microscopic images of the organoids to document post-freeze recovery.

Note: Change media about 3 days after thawing and passage as required.

4. Establishing Pancreatic Tumor Organoids From Cryopreserved Digested Materials

4.1. Purpose: To describe the procedures for establishing human pancreatic cancer organoid cultures, from cryopreserved materials. This is a general procedure and certain details and reagents may vary depending on the particular tumor type and characteristics and site of model generation.

4.2. Scope: This SOP applies to all laboratory personnel at University of Verona, and other end-users who are involved in establishing and maintaining pancreatic cancer organoids from cryopreserved digested materials starting from tumor tissue.

4.3. Responsibilities: Authorized personnel processing the pancreatic tumor organoids must ensure that:

- All samples used for establishing the tumor organoids are obtained lawfully and with appropriate consent;
- All procedures are followed correctly and are compliant with the SOP;
- All samples are adequately labeled during processing;
- All the applicable documentation and records are collected, updated and maintained for all samples.

Authorized personnel responsibilities also include:

- Preparation of stock and supplemented media, recognizing when additional stock medium needs to be prepared, and when media and other reagents need to be ordered;
- Culturing, splitting, freezing and thawing the organoids as required;
- Recognizing when new cells need to be thawed and when cells have to be frozen down;
- Recognizing when organoid culture stocks are getting low and when more cells need to be frozen down.

4.4. Health and Safety: Authorized personnel carrying out this procedure should maintain safe working practices and observe all relevant environmental health and safety guidelines. This includes the appropriate use of personal protective equipment, and procedures for waste disposal, disinfection, and biosafety. Gloves and lab coat are the minimal amount of protection that should be worn. Snug fitting non-permeable (waterproof) gloves are best. Eye protection is also recommended when exposure to splashing liquid nitrogen, solvents and biological active material is possible.

4.5. Definition and Acronyms

- SOP: Standard Operation Procedure
- HSM+++: Human Splitting Medium
- hCPLT: Human Complete Medium
- RhoKi: Y-27632 – Rho kinase inhibitor

4.6. Equipment, Materials & Reagents

Equipment

- Biosafety Cabinet
- Suction system to aspirate liquids using a Pasteur pipet, set up inside the biosafety cabinet
- Light Microscope
- Centrifuge
- Gas Burner
- Pipet aid
- P200

- P1000
- Cell Culture Incubator (37°C & 5% CO_2)

Supplies
- 15 ml falcon tubes (Falcon, # F2097)
- 50ml falcon tubes (Falcon, # F2070)
- 5ml plastic pipettes (Falcon, # F7543)
- 10ml plastic pipettes (Falcon, # F7551)
- 25ml plastic pipettes (Falcon, # F7525)
- Sterile glass Pasteur pipettes (VWR, # 14673-010)
- 24-well Suspension Plates (Greiner # 662 102)
- 6-well Suspension Plates (Sarstedt, # 833-920-500)
- Ice bucket with ice
- Waste container (for pipet tips, etc)
- Dewar flask or ice bucket with sufficient liquid Nitrogen for snap-freezing cryovial.

Reagents
- 70% ethanol
- HSM+++ (section 1.7)
- hCPLT: Used for culture of organoids from PDAC
- Matrigel, growth factor reduced phenol red free (Corning, # 356231)
- Y-27632 – Rho kinase inhibitor (Sigma, #Y0503)

Note:

- Matrigel should be stored in a -20ºC freezer. Matrigel begins to harden and polymerize at temperatures above 4ºC. If polymerization has occurred, do not reuse the Matrigel. Allow enough time for your Matrigel aliquot to thaw on ice before use. An 800µl aliquot of frozen Matrigel takes ~1 hour and 15min to thaw on ice, and a full vial takes ~8 hours. To aliquot a full vial, thaw on ice overnight, and aliquot into pre-chilled tubes.

4.7. Quality Control

1. Monitor culture by microscopic examination regularly and look out for signs of microbial contamination or cell distress. Make notes of observations in the cell culture lab notebook.
1. Regular mycoplasma testing of the organoid culture is recommended, since mycoplasma contamination cannot be detected easily by visual examination.
2. Record culturing, passaging and freezing of cells in notebook. Be sure to also include the date on which media was changed, cells were split or frozen. You should also update the liquid nitrogen logbook if removing a vial to start new batch of cells or when transferring cryogenic vials with frozen cells to the liquid nitrogen.
3. Each time you split the organoids, you increase the passage number and total number of passages by one. When you start cells from a freeze down, it is recommended that you start with the passage number at which the organoids were frozen and every time the organoids are passaged, you increase the passage number by one.
4. The personnel should also document any anomalies and/or deviations from the specified SOP.
5. All the tubes, bottles, cell culture well-plates and cryovials should be labelled properly and legibly. Labels should contain information such as organoid identifier number, passage number since original isolation from tissue, passage date and initials.
6. All growth factors, stock media and supplemented media should be stored at the appropriate storage temperature.
7. When preparing media, make sure you date and put your initials on the media bottle and enter this information diligently in your lab notebook. It is also recommended to note down the lot numbers of media and other reagents in your lab notebook.
8. Tissue and organoids should be kept on ice when they are being processed.

9. It is recommended to check viability of cryofrozen organoids by thawing a vial of early passage and late passage organoids, established from a tumor tissue.

4.8. Procedures

Note: The process and reagent volumes described below are applicable for culturing organoids in a 24-well plate. Scale-up accordingly if using different culture conditions, as indicated below.

Culture Dish	No. of Matrigel Domes/Well	Matrigel Volume/Dome	Culture Media/Well
24-well plate	1	50µl	0.5ml
6-well plate	4	50µl	2.0ml

4.8.1. Procedure to establish an organoid culture from cryopreserved digested materials

Day 0:

1. Thaw Matrigel overnight at 4ºC and keep cold.
2. Warm up 24-well cell culture plate(s) overnight in the 37ºC incubator.

Day 1:

Note: Cell membranes can be damaged due to osmotic shock caused by the DMSO present in the Recovery Cell Freezing medium. Cells should be started up individually, so as to avoid cross contamination. It is very important that one sample is handled at a time.

3. Start-up biosafety cabinet, clean the work area surface and clean the P1000 and P200 pipets with 70% ethanol.
4. Wipe the exteriors of all tubes/containers with 70% ethanol and transfer them into the biosafety cabinet.
5. Prepare ID2-coded bar-coded or non-bar-coded labels for culture plates. Include passage no. details (such as P1, P2, etc.) on the labels.
6. HSM+++ medium should be out of the fridge for at least 30min to reach room temperature and then warm up at 37°C before starting the procedure.
   Note: While thawing digested materials, the frozen cryopreserved cell suspensions should be diluted in warm HSM+++ medium as quickly as possible. If the cells remain in the freezing medium for too long the viability will be poor.
7. Wipe all tubes/containers with 70% ethanol and transfer organoids and Recovery Cell Freezing Media into the biosafety cabinet.
8. Take a tweezer, cryo protection gloves and a box filled with sufficient amount of dry ice. Remove a vial of organoids from the liquid nitrogen tank and place it on the dry ice. Remember to be very careful as the vials are extremely cold and could cause damage to skin if held for too long.
9. Thaw the vial rapidly by agitation in a 37°C water bath until there is still little frozen material (the additional frozen material will thaw by the time you take the vial out of the water bath and proceed to culture). Thawing should be rapid (within 60-120 seconds). Remove the vial from water bath and wipe it down with 70% ethanol at room temperature and transfer to a biosafety cabinet. All following steps must be done under aseptic conditions.
10. When a small bit of ice remains in the vial, pre-wet a P1000 with HSM+++ and pipette the cell suspension into a 15ml conical tube containing 8.5ml of cold (not ice-cold) HSM+++.
11. Wash the cryovial with 1ml of room temperature HSM+++, and add to the conical tube containing

the rest of the organoids.

12. Centrifuge tube at 200g for 5min at room temperature.
13. Aspirate the supernatant carefully, using a Pasteur pipette connected to a suction system, without disturbing the pellet. Re-suspend the pellet in 50µl of Matrigel.
    Note: Matrigel and pelleted cells should stay on ice during this procedure.
14. Plate 50µl cell suspension as a single droplet, in a pre-warmed 24-well tissue culture plate. Transfer the plate to a 37ºC incubator. Leave for 20-30 min for Matrigel to solidify.
15. Add 500µl of pre-warmed hCPLT supplemented with 10.5 µM Rho Kinase Inhibitor to each well.
16. Transfer the plate back to the 37ºC incubator.

Day 2:

17. Look at the organoid culture under the microscope and make notes on culture characteristics, such as recovery rate, density, etc. Take microscopic images of the organoids to document post-freeze recovery.
    Note: Change media about 3 days after thawing and passage as required.

5. Isolation of Nucleic Acids From Tissue and Organoid Models

5.1. Purpose: To describe the procedures for isolation of nucleic acids (DNA and RNA) from human cancer organoid cultures and pancreatic cancer tissues. Different procedures for isolation of nucleic acids are described. The choice of the procedure will depend on the input material (e.g., organoid pellet or organoids embedded in Matrigel) and the scope of the isolation (simultaneous vs individual isolation of DNA and RNA). All the procedures described for the isolation of RNA will lead to the loss of small RNA species.

5.2. Scope: This SOP applies to all laboratory personnel at University of Verona, and other end-users who are involved in the extraction of nucleic acids from human cancer organoid cultures and cancer tissues.

5.3. Responsibilities: Authorized personnel processing sample must ensure that:
- All samples used were obtained lawfully and with appropriate consent;
- All procedures are followed correctly and are compliant with the SOP;
- All samples are adequately labeled during processing;
- All the applicable documentation and records are collected, updated and maintained for all samples.

5.4. Health and Safety: Authorized personnel carrying out this procedure should maintain safe working practices and observe all relevant environmental health and safety guidelines. This includes the appropriate use of personal protective equipment, and procedures for waste disposal, disinfection, and biosafety. Gloves and lab coat are the minimal amount of protection that should be worn. Snug fitting non-permeable (waterproof) gloves are best.

5.5. Definition and Acronyms

- SOP: Standard Operation Procedure
- DPBS: Dulbecco's Phosphate Buffered Saline

5.6. Equipment, Materials & Reagents

Equipment
- Centrifuge
- Microcentrifuge
- Cryostat
- P10
- P20
- P200
- P1000
- NanoDrop™ 2000/2000c Spectrophotometers
- QuBit 2.0 Fluorometer
- Agilent 2100 Bioanalyzer Instrument
- Thermomixer
- Vortex

Supplies
- Waste container (for pipet tips, etc)
- Microcentrifuge tubes 1.5ml (#L024664)
- AllPrep DNA/RNA Mini Kit (Qiagen #80204)
- DNeasy® Blood&Tissue Kit (Qiagen, #69504)

- PureLink® RNA Mini Kit (Life Technologies, #12183018A)
- 1ml Syringe 25G x 5/8", 0,5 x 16 mm (Pic, #02.071250.000.350)
- 1000/200/20/10 µl tips

Reagents

- 96-100% ethanol in water
- 70% ethanol in water
- 14.3 M β-mercaptoethanol (β-ME)
- DPBS (Gibco, # 14190-144)
- Ultrapure DNase/RNAase Free Distilled water (Thermo Fisher Scientific, #10-977-015)
- Chloroform
- Trizol^{TM} (Thermo Fisher Scientific, #12183555)
- **Alternative NucleoSpin RNA Plus (M&N 740984.50)**
- RNAse Zap® (Thermo Fisher Scientific, #AM9780)
- Qubit RNA BR Assay Kit (Thermo Fisher Scientific, #Q10210)
- Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, #Q32850)
- RNA 6000 Nano Kit (Agilent 5067-1511)

Note:

- For simultaneous isolation of DNA and RNA from the same organoid culture or tissue sample refer to the section 5.10 (Simultaneous isolation of genomic DNA and RNA using AllPrep DNA/RNA mini Kit).
- Perform all centrifugation steps at room temperature (15-25ºC) for nucleic acid extraction unless otherwise noted.
- Tumor tissues are kept frozen in -80ºC for long term storage.
- Solutions containing ethanol are considered flammable. Use appropriate precautions when using this chemical.
- RNA and DNA samples must be kept in -80ºC for long term storage.
- Preheat thermomixer to 56°C.
- Wear disposable gloves while handling reagents and RNA samples to prevent RNAase contamination from the surface of the skin; change gloves frequently, particularly as the protocol progresses into purified materials. Use aseptic techniques when working with RNA.
- RNA samples must be kept on ice.

5.7. Quality Control

1. The personnel should always document any anomalies and/or deviations from the specified SOP.
2. All the tubes, bottles, cell culture well-plates and cryovials should be labelled properly and legibly. Labels should contain information such as organoid/tissue identifier number, passage number for organoids, date and initials.
3. Follow proper aseptic RNA handling techniques to prevent RNase contamination of reagents and RNA samples.
4. Keep freshly harvested sample on ice and quickly proceed to lysis and homogenization or freeze samples immediately after collection in liquid nitrogen or on dry ice and keep at -80ºC for later use.
5. Always maintain a ratio of 10:1 between the volume of TRIzol Plus RNA Purification Kit and the mass of the samples (example 50-100mg of tissue for 1 ml of TRIzol).
6. Do not exceed the RNA binding capacity of the spin cartridge by adding samples containing more than 1mg of total RNA. If working with samples containing more than 1 mg of RNA, divide the sample into aliquots in different spin cartridge.

5.8. Procedures to Isolate genomic DNA using the DNeasy® Blood&Tissue Kit

Note:

- Redissolve any precipitates in Buffer AL and Buffer ATL
- Add ethanol to buffer AW1 and AW2 concentrates.

5.8.1 Procedure to prepare organoid pellet samples for genomic DNA isolation

1. Equilibrate the tube containing organoid pellet (Section 2.8.5) to room temperature.
2. Prepare ID2-coded bar-coded or non-bar-coded labels for 1.5 ml Eppendorf. Include details such as passage number of organoids, date and initials.
3. Resuspend the pellet in 200µl of DPBS and then add 20µl of proteinase K.
4. Add 200µl Buffer AL. Mix thoroughly by vortexing.
5. Incubate in thermomixer with 300-400 rpm rotation at 56ºC for 10 min.
6. Continue to the point n 12 of the Section 5.8.3.

5.8.2 Procedure to prepare tumor tissue samples for genomic DNA isolation

1. Prepare 1.5 ml microcentrifuge tube(s) with 180µl Buffer ATL for each sample.
2. Retrieve frozen tissues from the biobank and cut tissue slices using a cryostat. For nucleic acid isolation, cut around 20-30 slices of tutor tissue (7 µm thick). The number of slices depends on the dimension of tumor tissue. If possible, perform H&E staining on 4 µm thick tissue slices prepared before (top slide) and after (bottom slide) cutting the 7µm thick slices for isolation. The top and bottom slides can be reviewed by a pathologist to assess neoplastic cell content and also pathological characteristics of the tissue.
3. Using a needle 25G, transfer individual tissue slices into a 1.5ml microcentrifuge tube containing ATL buffer.
4. Pass the solution through the 1 ml syringe 25G x 5/8” to homogenize tissue.
5. Place the tube in -80ºC for at least 2 hours to perform a heat shock. Alternatively, tubes can be kept in the -80ºC over-night.
6. Continue to point n 7 below of the Section 5.8.3.

5.8.3 Genomic DNA Isolation

7. Equilibrate the tube containing the homogenized tissue at room temperature.
8. Prepare ID2-coded bar-coded or non-bar-coded labels for Eppendorf tube. Include details such as tissue type, date and initials.
9. Add 20µl proteinase K to the tube, mix by vortexing, and incubate at 56ºC for up to 3 hours until tissue lysis is complete. Use thermomixer with 300-400 rpm rotation and vortex occasionally during incubation.
10. At the end of the incubation, vortex the tube for 15 seconds.
11. Add 200µl Buffer AL. Mix thoroughly by vortexing.
12. Add 200µl Ethanol (96-100%). Mix thoroughly by vortexing.
13. Pipet the mixture into a DNeasy Mini Spin column placed in a 2 ml collection tube. Centrifuge at >6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.
14. Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW1. Centrifuge at >6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.
15. Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW2. Centrifuge at >20.000 x g (14,000 rpm) for 3 min. Discard the flow-through and collection tube.
16. Transfer the spin column to a new 1.5ml microcentrifuge tube.
17. Elute the DNA by adding 60 µl of Buffer EB to the center of the spin column membrane. Incubate for 1 min at room temperature (15-25ºC). Centrifuge for 1 min at >6000 x g.

Notes: While the manual is suggesting Buffer AE for elution, use Buffer EB or Nuclease Free-water.

18. Repeat elution to increase the DNA yield.
19. Quantify samples using the Nanodrop and QuBit Fluorometer.
20. Store the purified DNA at room temperature if used within few hours. For long-term storage, store the purified DNA at -80ºC.

5.9. Procedures to Isolate RNA using PureLink® RNA Mini Kit

Note:

- Both Lysis Buffer and Wash Buffer I (PureLink® RNA Mini Kit) contain guanidine isothiocyanate. Do not add bleach or acidic guanidinium isothiocyanate, as reactive compounds and toxic gases are generated.

5.9.1. Procedure to prepare organoid pellet samples for RNA extraction

1. Prepare ID2-coded bar-coded or non-bar-coded labels for 1.5 ml Eppendorf. Include details such as passage number for organoid, date and initials.
2. Retrieve organoid pellet from the biobank.
3. Add directly 1 ml of TRIzol Reagent directly to the pellet.
4. Pipet up and down the lysate several times to obtain a homogenous solution.
5. Incubate for 5 min at room temperature to permit complete dissociation of the nucleoprotein complex.
6. Put in -80ºC for at least 2 hours to perform a heat shock. Alternatively, tubes can be kept in the -80ºC over-night.
7. Continue to the point n 9 of the Section 5.9.4

5.9.2. Procedure to prepare samples directly from organoid culture for RNA extraction

Note: RNA can be harvested directly from Matrigel domes containing organoids (at around 70-80% confluency). For obtaining at least 2 µg of RNA, it is advisable to start from 4 domes of Matrigel (each 50µL). In any case, do not collect more than 8 domes with 1mL of TRIzol reagent.

1. Prepare ID2-coded bar-coded or non-bar-coded labels for 1.5 ml Eppendorf. Include details such as passage number, date and initials.
2. Aspirate the media from each well of the 24-well plate.
3. Working under the chemical hood, add 1mL of TRIzol Reagent to one well of the 24-well plate and dissolve Matrigel by pipetting up and down several times.
4. Transfer the solution to the next well of the 24-well plate and repeat the procedure for the wells that need to be harvested.
5. Transfer the solution to a pre-labeled 1.5 ml Eppendorf and pipet up and down several times to obtain a homogenous solution.
6. Incubate for 5 min at room temperature to permit complete dissociation of the nucleoprotein complex.
7. Put in -80ºC for at least 2 hours to perform a heat shock. Alternatively, tubes can be kept in the -80ºC over-night.
8. Continue to the point n 9 of the Section 5.9.4.

5.9.3. Procedure to prepare frozen tissue samples for RNA extraction

1. Prepare additional ID2-coded bar-coded or non-bar-coded labels for 1.5 ml Eppendorf. Add 1ml of TRIzol Reagent to each tube.

2. Retrieve frozen tissue from the biobank and cut tissue slices using the cryostat. For RNA isolation, cut around 20-30 slices of tumor tissue (7 µm thick). The number of slices depends on the dimension of tumor tissue. If possible, perform H&E staining on 4 µm thick tissue slices prepared before (top slide) and after (bottom slide) cutting the 7µm thick slices for isolation. The top and bottom slides can be reviewed by a pathologist to assess neoplastic cell content and also pathological characteristics of the tissue.
3. Using a needle, transfer tissue slices with a needle into the 1.5 ml microcentrifuge tube containing TRIzol Reagent.
4. Pass the solution through the 1 ml syringe 25G x 5/8" to homogenize tissue until the attainment of a homogenous solution.
5. Incubate for 5 min at room temperature to permit complete dissociation of the nucleoprotein complex.
6. Put in -80ºC for at least 2 hours to perform a heat shock. Alternatively, tubes can be kept in the -80ºC over-night.
7. Continue to the point n 9 of the Section 5.9.4

5.9.4. RNA extraction

9. Equilibrate frozen tubes at room temperature.
10. Prepare additional ID2-coded bar-coded or non-bar-coded labels for 1.5 ml Eppendorf. Include details such as tissue type, date and initials.
11. Add 200 µl of chloroform per 1ml of TRIzol Reagent used for lysis and securely cap the tube.
12. Incubate for 2-3 min.
13. Centrifuge the samples for 15 min at 12,000 x g at 4ºC.
14. The mixture separates into a lower red phenol-chloroform, interphase, and a collarless upper aqueous phase.
15. Transfer ≈ 600 µl of the colourless, upper aqueous phase containing the RNA into a new tube.
16. Add an equal volume of 70% ethanol, then mix well by vortexing.
17. Invert the tube to disperse any visible precipitate that may be formed after adding ethanol.
18. Transfer up to 700 µl of the sample to a spin cartridge (with collection tube).
19. Centrifuge at 12,000 x g for 15 seconds.
20. Discard the flow-through and reinsert the spin cartridge into the same collection tube.
21. Repeat steps 18-20 until the entire sample has been processed.
22. Add 700 µl of Wash Buffer I to the spin cartridge.
23. Centrifuge at 12,000 x g for 15 seconds.
24. Discard the flow-through, then reinsert the spin cartridge into the same collection tube.
25. Add 500 µl of Wash Buffer II to the spin cartridge.
26. Centrifuge at 12,000 x g for 15 seconds.
27. Discard the flow-through, then reinsert the spin cartridge into the same collection tube.
28. Repeat steps 26-27 once.
29. Centrifuge at 12,000 x g for 1 min to dry the membrane.
30. Discard the collection tube, then insert the spin cartridge into a 1.5 ml microcentrifuge tube.
31. Add 50 µl of DNase-RNase-free water to the centre of the membrane in the spin cartridge.
32. Incubate 1 min.
33. Centrifuge at >12,000 x g for 2 min.
34. Repeat steps elution to increase the RNA yield.
35. Discard the spin cartridge.
36. Quantify samples with the Nanodrop and QuBit Fluorometer.
37. Store the purified DNA at room temperature if used within few hours. For long-term storage, store the purified DNA at -80ºC.

5.10 Simultaneous isolation of genomic DNA and RNA using AllPrep DNA/RNA mini Kit

Note:

- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT plus containing β-ME can be stored at room temperature (15–25°C) for up to 1 month.
- Dithiothreitol (DTT) must be added to Buffer ALO before use. Add 8 mg DTT per 1 ml Buffer ALO.
- Buffer RPE, Buffer AW1, and Buffer AW2 are each supplied as a concentrate. Before using for the first time, add the appropriate volume of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Buffer RLT plus may form a precipitate during storage. If necessary, redissolve by warming, and then place at room temperature.
- Preheat Buffer EB to 70°C to ensure optimal DNA elution.

5.10.1. Procedure to prepare organoid pellet samples for simultaneous DNA/RNA extraction

1. Collect organoid pellet as described in section Section 2.8.5. If simultaneous isolation of DNA and RNA is desired, make sure to collect organoid pellet from at least 8 domes of Matrigel containing organoids at 70-80% confluency (each dome of 50µl).

We estimate that on average there are 1-3 x 10^6 cells per 8 domes of MG (about 600 µl of RLT plus buffer)

2. Prepare ID2-coded bar-coded or non-bar-coded labels for 1.5 ml Eppendorf. Include details such as passage number for organoid, date and initials.
3. Retrieve organoid pellet from the biobank.
4. Loosen the cell pellet thoroughly by flicking the tube. Add 600 µl of Buffer RLT plus and pipet up and down several times.
5. Pass the lysate at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free 1 ml syringe. Transfer the homogenized lysate to an AllPrep DNA spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 30 s at for 30 seconds at ≥ 8000 x g (≥ 10'000 x rpm).
   Note: Make sure that no liquid remains on the column membrane after centrifugation. If necessary, repeat the centrifugation until all liquid has passed through the membrane.
6. Place the AllPrep DNA spin column in a new 2 ml collection tube (supplied), and store at room temperature (15–25°C) or at 4°C for later DNA purification in sections 5.10.3. Use the flow-through for RNA purification in section 5.10.4.
   Note: Do not store the AllPrep DNA spin column at room temperature or at 4°C for long periods. Do not freeze the column.

5.10.2. Procedure for prepare frozen tissues for DNA/RNA purification

1. Prepare 1.5 ml microcentrifuge tube(s) with 600µl Buffer RTL plus containing β-ME to each 1.5 ml safe lock Eppendorf tube for each sample.
2. Retrieve frozen tissues from the biobank and cut tissue slices using a cryostat. Cut around 20-30 slices of tumor tissue (7 µm thick). The number of slices depends on the dimension of tumor tissue. If possible, perform H&E staining on 4 µm thick tissue slices prepared before (top slide) and after (bottom slide) cutting the 7µm thick slices for isolation. The top and bottom slides can be reviewed by a pathologist to assess neoplastic cell content and also pathological characteristics of the tissue.

3. Using a needle, transfer individual tissue slices into a 1.5ml microcentrifuge tube containing RLT buffer with β-ME.
4. Pass the solution through the 1 ml syringe 25G x 5/8" to disrupt and homogenize tissue.
5. Place the AllPrep DNA spin column in a new 2 ml collection tube (supplied), and store at room temperature (15–25°C) or at 4°C for later DNA purification in sections 5.10.3. Use the flow-through for RNA purification in section 5.10.4.
   Note: Do not store the AllPrep DNA spin column at room temperature or at 4°C for long periods. Do not freeze the column.

5.10.3. Genomic DNA purification

1. Add 500 µ Buffer AW1 to the AllPrep DNA spin column.
2. Close the lid gently and centrifuge for 15 seconds at 8,000 x g (10,000 x rpm) at RT to wash the spin column membrane. Discard the flow-through.
3. Add 500 µl Buffer AW2 to the AllPrep DNA spin column.
4. Close the lid gently and centrifuge for 2 minutes at 14,000 x g at room temperature to wash the spin column membrane. The long centrifugation dries the membrane ensuring that no ethanol is carried over during DNA elution that could interfere with downstream reactions.
5. Place AllPrep DNA spin column in the previously prepared 1.5 mL collection tube. Add 50 µl of Buffer EB directly to the spin column membrane and close the lid.
6. Incubate at RT for 1 min.
7. Centrifuge at room temperature for 1 min at 8,000 x g to elute the DNA.
8. Repeat steps 5-7 to increase DNA yield by adding 50 µl of EB.
9. If multiple columns were required for a sample, combine all eluent into a single tube with a single channel pipette. Discard the empty tubes.
10. Quantify samples with the Nanodrop and QuBit Fluorometer.
11. Store the purified DNA at room temperature if used within few hours. For long-term storage, store the purified DNA at -80°C.

5.10.4. Total RNA purification

12. Add 1 volume (around 600µl) of 70% ethanol to the flow-through from steps 6 and 5 of Sections 5.10.2 and 5.10.3, respectively. Mix well by pipetting (do not centrifuge) and proceed directly with step 13.
    Notes: If some lysate was lost during homogenization and DNA binding to the AllPrep DNA spin column, adjust the volume of ethanol accordingly.
    When purifying RNA from certain samples, precipitates may be visible after addition of ethanol. This does not affect the procedure.
13. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to a RNeasy spin column placed into a 2 ml collection tube (supplied).
14. Close the lid gently and centrifuge for 15 seconds at ≥ 8000 x g (≥ 10'000 x rpm). Discard the flow-through and reuse the collection tube.
    Notes: If samples volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.
15. Add 700 µl Buffer RW1 to the RNeasy spin column.
16. Close the lid gently and centrifuge for 15 seconds at ≥ 8000 x g (≥ 10'000 x rpm) to wash the spin column membrane. Discard the flow-through and reuse the collection tube. Attention: after centrifugation, carefully remove the RNeasy spin column from the collection tube, so that the column does not contact the flow-through. Be sure to empty completely the tube.
17. Add 500 µl Buffer RPE to the RNeasy spin column.
18. Close the lid gently and centrifuge for 15 seconds at ≥ 8000 x g (≥ 10'000 x rpm) to wash the spin column membrane. Discard the flow-through and reuse the collection tube.
19. Add 500 µl Buffer RPE to the RNeasy spin column.

20. Close the lid gently and centrifuge for 2 min at ≥ 8000 x g (≥ 10'000 x rpm) to wash the spin column membrane. Discard the flow-through and reuse the collection tube.
21. Place the RNeasy spin column in a new 2 ml collection tube (supplied) and discard the old collection tube with the flow-through. Centrifuge full speed for 1 min.

Perform this step to eliminate any possible carryover of Buffer RPE or residual flow-through remains on the outside of the RNeasy spin column after the previous step.

22. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied).
23. Add 30-50 µl Nuclease-free water directly to the spin column membrane.
24. Close the lid gently and centrifuge for 1 min at ≥ 8000 x g (≥ 10'000 x rpm) to elute the RNA.
25. Repeat step 24 using the eluate to increase RNA yield, in the same collection tube.
26. Quantify samples with the Nanodrop and QuBit Fluorometer.
27. Determine RNA quality.
28. Store the purified RNA in ice if used within few hours. For long-term storage, store the purified RNA at -80°C.

5.11. Alternative Total RNA preparation from organoids with the M&N kit

5.11.1. Procedure to prepare samples directly from organoid culture for RNA extraction

Note: RNA can be harvested directly from Matrigel domes containing organoids (at around 70-80% confluency). For obtaining at least 2 µg of RNA, it is advisable to start from 4 domes of Matrigel (each 50µL). In any case, do not collect more than 6 domes with 350 µl LBP reagent. You can routinely cultivate 6 domes with 50 µl Matrigel each in one well of a six well plate.

5.11.2. Total RNA purification

1. Prepare all the needed components from the NucleoSpin RNA Plus (M&N 740984.50) as described in the manufacturer's manual.
2. Remove the media from the well(s) containing the matrigel domes with organoids.
3. Add a total of 350 µl of LBP to the domes.
4. Lyse the domes and the containing organoids by pipetting until everything is in solution.
5. Proceed with RNA isolation as recommended in the protocol.

5.12. Measurement of nucleic acids

Note: Please read the manufacturer's instruction for the Qubit assays and the RNA 6000 Kit. It can be useful to measure the RNA concentration via a photometer, but you need an additional assay to determine the amount of DNA contamination. Everything above 1% of DNA contamination is unacceptable.

6. Measure the amount of RNA using a Qubit assay.
7. Measure the amount of DNA using Qubit. (Note you expect the percentage of DNS in this preparation to be less than 1% of the measured RNA).
8. Measure the RIN using the RNA 6000 Nano Kit using an Agilent Lab on a Chip system.