Development of NUT Carcinoma Organoids

Information:
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Approved by: ISoOR-ISOB-Technical Committee

Statement of Alignment
This Standard Operating Procedure (SOP) aligns with the principles and quality framework outlined in the *ISoOR-ISOB: International Standard for Organoid Biobanking* established by the International Society of Organoid Research (ISoOR). It is designed to uphold the ISoOR-ISOB's core values of global reproducibility, transparency, and standardization in organoid research, ensuring the reliable development and characterization of NUT carcinoma organoids.

1 Purpose

This SOP aims to provide a unified, auditable, and reproducible operational framework and quality requirements for the establishment and cultivation of NUT carcinoma organoids. It supports model construction, quality control, sample preservation, and translational research. Through controlled culture formulations, key parameters, and standardized processes, this SOP promotes consistency, data comparability, and resource sharing across teams and institutions, ultimately establishing a standardized technical system to enhance the reliability and efficiency of NUT carcinoma research.

NUT carcinoma is a rare and highly aggressive malignancy, with its core pathological mechanism driven by chromosomal translocations involving the NUTM1 gene and other genes (such as BRD4, BRD3, or NSD3), forming fusion oncoproteins that lead to epigenetic reprogramming (e.g., super-enhancer formation and histone acetylation abnormalities), thereby promoting tumorigenesis and progression. This tumor primarily originates from squamous epithelium and is considered a specific

subtype of squamous cell carcinoma (SCC) in the lung and head and neck regions, commonly occurring in the thorax (lungs, mediastinum) and head and neck (sinuses, pharynx), with rare involvement of bone/soft tissue or abdominopelvic areas. Clinically, it manifests as rapidly growing masses, often accompanied by local compression symptoms, with non-specific imaging features. Pathologically, it is characterized by poorly/undifferentiated squamous phenotypes, with immunohistochemistry showing diffuse nuclear positivity for NUT. Epidemiologically, it mainly affects young and middle-aged adults, with no gender predominance and no clear risk factors; over 90% of cases are diagnosed at stage III/IV, with frequent distant metastases (most commonly to bone). The prognosis is extremely poor, with a median survival of approximately 6–7 months, and limited response to conventional treatments (e.g., chemotherapy, radiotherapy), often resulting in brief remissions followed by rapid progression. Currently, BET inhibitors represent the only targeted therapy, but they are prone to resistance and significant adverse effects, with a lack of effective personalized strategies. To address these challenges, it is essential to develop standardized, scalable in vitro models that preserve the molecular (NUTM1 fusion status), phenotypic, and heterogeneous characteristics of primary tumors.

Tumor organoids, as a novel three-dimensional culture technology, can maintain tumor heterogeneity and genomic features of primary tumors in vitro and have been widely applied in various cancer studies. However, there is currently no specialized organoid culture system for NUT carcinoma. Existing culture methods fail to meet the needs of this rare and highly aggressive tumor. Additionally, inter-patient heterogeneity in NUT carcinoma is significant, with varying responses to drugs based on different NUTM1 fusion types, and there is a lack of effective in vitro drug sensitivity testing methods to identify potential responder subgroups. Furthermore, no reports exist on establishing NUT carcinoma organoids from thoracic or abdominal effusions, which offer advantages of easier access and lower invasiveness compared to tissue biopsies, making them particularly suitable for rare tumor research. Finally,

current culture systems lack regulatory factors targeting NUT carcinoma-specific signaling pathways, hindering the effective maintenance of organoid growth and characteristics. Therefore, there is an urgent need to develop standardized NUT carcinoma organoid culture methods to fill this technical gap and support drug screening and mechanistic exploration.

2 Scope

Four types of patient-derived biosamples—tumor tissues, pleural effusions, fine-needle aspirates (FNAs), and transbronchial lung biopsies (TBLBs)—are applicable for NUT cancer organoid culture. The standardized construction of NUT carcinoma organoid models holds multifaceted strategic importance, particularly in rare tumor research, where its normalization can significantly enhance research reliability and translational potential:

- **Providing High-Fidelity Models and a Foundation for Mechanistic Studies**: Organoids can preserve the heterogeneity, NUTM1 fusion status, and key pathways of primary tumors in a three-dimensional system, more closely mimicking the in vivo environment than two-dimensional cell lines, ensuring accuracy and reproducibility in mechanistic studies and enabling in-depth exploration of fusion protein oncogenic mechanisms and downstream signaling.
- **Supporting Drug Screening, Stratification Assessment, and Precision Medicine**: Standardized models can evaluate the in vitro efficacy of BET inhibitors and combination regimens, identifying sensitive subgroups, resistance risks, and potential synergies (e.g., BETi with immune checkpoint inhibitors) through cell viability grading, optimizing clinical trial enrollment, and providing data support for personalized treatments.
- **Enhancing Accessibility of Rare Tumor Samples and Longitudinal Monitoring**: Using minimally invasive samples like thoracic/abdominal effusions as starting points, the construction pathway offers convenience and repeatability, ideal for rapidly progressing rare tumors like NUT carcinoma, enabling personalized assessments

across different NUTM1 fusion types and dynamic monitoring of tumor evolution while reducing invasive procedures for patients.

- **Establishing Quality Systems and Ethical Compliance Frameworks**:

  By defining key quality control thresholds (e.g., Morphological evaluation, viability ≥80%, sterility testing, NUTM1 fusion protein positivity confirmation, and genomic stability verification), the SOP ensures model reliability and batch consistency while incorporating ethical standards (e.g., informed consent and data privacy) to minimize research variability and enhance credibility.

3 Responsibilities

1. **Principal Investigator (PI): Professor Mingzhu Yin**
2. **Laboratory Staf: Chongyang Shen, Minghui zhang, Xin Li, Zhuomiao Ye, Liang Dong**
3. **Quality/Compliance Officer: Jun Fu**

4 Materials & Equipment

1. Culture Media & Reagents

• Basal media (DMEM/F12, advanced RPMI-1640)

• Antibiotics (Penicillin/Streptomycin, Primocin)

• Defined supplements (B27, N2)

• Dissociation enzymes (collagenase/Dispase, TrypLE)

• ECM hydrogels (e.g., Matrigel)

• Cryopreservation medium (DMSO, FBS, basal medium)

• Nutrients (N-Acetylcysteine, Nicotinamide, GlutaMax)

• Inhibitors (A83-01, Y-27632, Losmapimod)

2. Growth Factors & Cytokines

• Essential recombinant proteins: EGF, bFGF, FGF10, Noggin, R-spondin, Wnt3a)

3. Labware & Consumables

• Sterile tissue-culture plastics: 24-well plates, pipettes, filter tips, microcentrifuge tubes

• Cryovials (1.5–2 mL, leak-proof)

• Hemocytometers or automated cell counters

• Sterile Phosphate Buffer Saline (PBS), wash buffers, collection containers

4. Major Equipment

• 37 °C, 5 % CO_2 incubators (humidity controlled)

• BSL-2 certified biosafety cabinets

• Centrifuges (swing-bucket, 300 ×g capability)

• Inverted bright-field & fluorescence microscopes

• Programmable –80 °C freezers & liquid nitrogen (LN_2) cryo-storage (vapor phase)

• Controlled-rate freezing containers

• 4 °C refrigerators & –20 °C freezers for reagents

• Water baths (37 °C)

• pH meters, osmometers, analytical balances

5 Safety & Ethics

5.1 Biosafety & Regulatory Framework

All steps are performed under BSL-2 containment as mandated for human, potentially infectious, primary tumor material. Institutional biosafety officers reviewed and approved the protocol before release; no work may begin until the local Institutional Review Board (IRB) and, when applicable, an institutional stem-cell or genetic-modification committee have granted written authorization. Transport of patient-derived samples between sites follows national dangerous-goods regulations and the Material Transfer Agreement (MTA) signed by provider and recipient institutions.

5.2 Personal Protective Equipment

Minimum PPE within the BSL-2 cabinet consists of disposable gloves (double-gloving during tissue mincing), fully buttoned lab coat with knit cuffs, sleeve guards if desired, and ANSI-approved eye protection. Aerosol-producing steps (pipetting ECM domes, vortexing digests) require a face shield; when LN_2 is handled

for banking, cryo-apron and insulated gloves are added. All PPE is removed on exit and discarded into regulated infectious-waste containers.

5.3 Documentation & Training

Staff must document completion of institutional biosafety manual training modules and maintain current annual certificates. Ethical approval letters, donor consent forms, and IRB amendment numbers are stored in the secure electronic database referenced by each batch ID; paper copies remain in the QA archive for the organoid life-cycle plus five years.

5.4 Genetic Modification & Zoonotic Considerations

The protocol as written uses unmodified patient cells; any future introduction of lentiviral or CRISPR constructs requires re-submission to the institutional Genetic Modification Safety Committee for elevated BSL-2 enhanced containment and additional PPE (N95 respirators, sealed centrifuge buckets). Because samples are human-only, zoonotic risk is negligible; nevertheless, all primary cells are screened every two passages for mycoplasma and adventitious agents.

6 Procedure Overview

6.1 Cell Source

(1) Patient-derived biosamples for NUT cancer organoid culture

Four types of patient-derived biosamples—tumor tissues, pleural effusions, fine-needle aspirates (FNAs), and transbronchial lung biopsies (TBLBs)—are applicable for NUT cancer organoid culture, each differing in collection method, suitability, and purpose: tumor tissues, obtained via surgical resection or core biopsy, serve as the "gold standard" with high organoid success rates (preserving full tumor heterogeneity), making them ideal for drug testing and tumor biology research; pleural effusions, minimally invasive fluid samples collected via thoracentesis, work for metastatic cancers but require tumor cell enrichment and are valuable for modeling metastasis and longitudinal monitoring; FNAs, ultrasound or CT-guided cell samples aspirated with a thin needle, have low cell yield and lack stromal components (needing specialized matrices) but are well-suited for small, hard-to-reach tumors or

urgent testing; TBLBs, lung tissue samples collected via bronchoscopy, provide moderate yield with partial tumor microenvironment components.

(2) Approved sources of biosamples

Patient-derived biosamples are sourced primarily from two approved channels: institutional biobanks and collaborative partners.

a. Institutional biobanks: These are facilities managed by hospitals, universities, or research institutions. They handle the collection, storage, and management of biosamples, and any request for samples must be approved by an ethical committee or Institutional Review Board (IRB).

b. Collaborative partners: These refer to external organizations such as other research institutions, biotech companies, or hospitals. Biosamples are obtained through formal agreements; for example, acquiring cell lines in accordance with the terms outlined in a Material Transfer Agreement (MTA).

(3) Crucial Referenced Consent Forms

Several key consent forms are required depending on the sample donor:

a. Adult consent forms: Mandatory for collecting samples from adult donors and are a requirement of biobanks.

b. Parental permission plus minor assent forms: Used for donors under 18 years old. Specifically, donors aged 7 to 17 must provide joint assent along with parental permission.

c. Surgical consent forms: These forms not only cover consent for the surgery itself but also address the potential use of intra-operative samples for research purposes.

6.2 Tissue Processing and Pre-culture Preparation for Organoid Culture

The preparation of starting materials for organoid culture involves sourcing, processing, and pre-treating primary cells or tissues to create a suitable environment for 3D organoid formation. We use resected tumor samples as an example to describe the sample processing procedure before the initiation of organoid culture. Given the unique characteristics of organoid culture (e.g., use of 3D matrices, long-term culture), operational procedures must be refined within the BSL-2 framework.

- Collection: Surgically removed tissue should be gathered as fresh as possible — ideally within 30 minutes post-surgery. To transport the tissue, place it in a chilled tissue preservation solution (such as DMEM/F12 supplemented with antibiotics). The tumor's classification as NUT cancer must be confirmed by pathological diagnosis (FISH/NGS confirmation of NUTM1 rearrangement or positive NUT protein by immunohistochemistry).
- Washing and Mincing: In a sterile environment, rinse the tissue three times using PBS to eliminate blood and impurities. Next, use scalpels to mince the tissue thoroughly into tiny fragments, each around 0.5 mm^3 in size.
- Tissue dissociation: Submerge the minced tissue in an enzyme cocktail solution (5-10 mL) and incubate it at 37°C for a set duration (ranging from 30 minutes to 1 hour) with agitation. This step breaks down the tissue into cell clusters.

Enzyme Cocktail Components	**Supplier**	**Catalogue Number**	**Final Concentration**
Collagenase/Dispase	Roche	10269638001	1 mg/mL
Hyaluronidase	Sigma	H3506	0.1 mg/mL
Deoxyribonuclease I	Sigma	DN25	40 μg/mL
DMEM/F12	Invitrogen	11320033	1x

6.3 Organoid Development

(1) Initiation of 3D Culture: Embedding in Extracellular Matrix (ECM)

This step is critical: it involves encapsulating cells in ECM to enable 3D growth.

- ECM Preparation: Thaw Matrigel (Corning 356255) overnight at 4°C on ice. Ensure all pipette tips and tubes are pre-cooled to avoid the ECM polymerizing prematurely.
- Mixing and Seeding: Gently resuspend the cell pellet in the cold, liquid ECM, taking care not to create bubbles. Use a pre-chilled pipette tip to dispense the mixture onto the surface of a pre-warmed culture plate in the form of small droplets (approximately 10-25 μL per droplet, typically 1,000–10,000 cells per well for 24-well plates).

- Polymerization: Incubate the culture plate at 37°C for 15-30 minutes. This allows the ECM droplets to solidify into stable "domes."
- Medium Addition: Once polymerization is complete, carefully add pre-warmed, NUT cancer organoid culture medium over each dome.

Organoid Media Component	**Supplier**	**Catalogue Number**	**Final Concentration**
EGF	Sino Biological	10605-H01H	50 ng/ml
bFGF	Sino Biological	10014-HNAE	20 ng/ml
WNT3A	Sino Biological	WNT007-02H	50 ng/ml
R-Spondin 1	Sino Biological	11083-H08H1	100 ng/mL
FGF10	Sino Biological	10573-HNAE	50 ng/mL
Noggin	Sino Biological	10267-H08H	200 ng/mL
A83-01	Tocris	2939	500 nM/L
Y-27632	Tocris	7000	10 μM/L
Losmapimod	Sigma	SML3596	1 μM/L
B27 supplement 50×	Gibco	12587010	1×
N2 supplement 100×	Gibco	17502048	1×
N-Acetylcysteine	Tocris	7874	1.25 mM/L
Nicotinamide	Tocris	4106	2 mM/L
GlutaMax 100×	Invitrogen	35050061	1×
Penicillin / Streptomycin	Invitrogen	15140122	100 U/ml
Primocin	Invivogen	ant-pm-05	100 μg/ml
Advanced RPMI-1640	Invitrogen	12633012	1×

(2) Culture Maintenance and Passaging

Organoids require regular monitoring and care to maintain healthy growth.

- Incubation and Monitoring: Culture the plate in the **37°C, 5% CO_2 incubator**. Regularly observe the organoids under a microscope every 2-3 days. Healthy organoids will appear as bright, spherical structures and will increase in size (~200 μm) and number over 7-14 days.
- Medium Changes: Change the culture medium every **2-4 days** by carefully aspirating the old medium and adding fresh, pre-warmed medium without disturbing the Matrigel domes. Change the medium more frequently if it appears yellow (indicating acidification).

- Passaging (Sub-culturing): When organoids become large (~200 μm) and dense (typically after 1-2 weeks), they need to be passaged.

a. Dissolution: Gently break up the Matrigel dome using a cold PBS buffer to dissolve the Matrigel.

b. Collection and Digestion: Collect the organoids, let them sediment, and then digest them with TrypLE digestion solution (Gibco, 12605010) for 5-10 minutes until they break into smaller cell clusters — not single cells, as clusters have higher viability.

c. Re-seeding: After washing and centrifugation, resuspend the cell clusters in fresh, cold Matrigel and seed them into new droplets at an appropriate split ratio (e.g., **1:2 to 1:4).**

(3) Molecular/genetic characterization: Characterize NUT organoids using methods like immunostaining/western blotting/Fluorescence *In Situ* Hybridization (FISH) for NUT fusion, genomic sequencing (WES/WGS) and RNA sequencing to confirm they retain the key characteristics of the original tissue.

(4) Drug sensitivity testing

The following procedures focus on standardized operation steps for evaluating organoid sensitivity to drugs, covering pre-experiment preparation, drug treatment, detection, and data processing, ensuring reproducibility and accuracy of results. In organoid drug sensitivity testing, the setting of drug concentrations requires careful consideration of both empirical experience and physiological relevance, with key principles including:

• Empirical setting (for investigational drugs): Design 3-5 gradients (In organoid drug sensitivity testing, targeted drug concentrations are usually kept below 10 μM).

• Reference to Cmax (for approved drugs): Use 0.1×–10× Cmax to match *in vivo* plasma levels (Cmax from clinical data).

Final concentration of solvents (e.g., DMSO) should be ≤0.1% to avoid affecting organoid viability. Both morphological imaging-based assays and cell viability-based assays (including CCK-8 and CellTiter-Glo) can be employed to assess the sensitivity of organoids to drugs. Here we use CCK-8 assay as an example to evaluate the cell

viability of organoid after drug treatment.

a. Organoid preparation: Harvest log-phase growth organoids, count them using a hemocytometer, and seed 2000–3000 organoids per well (24-well plate). Incubate the plate at 37°C with 5% CO_2 for 24 hours to allow organoids to attach to the Matrigel and adapt to the new environment.

b. Aspirate the old medium from each well gently (avoid disturbing organoids). Add 500 μL (24-well plate) of drug working solution (experimental groups) or control solution (control groups) to the corresponding wells. Each group should have 3–4 technical replicates. Incubate the plate at 37°C with 5% CO_2 for 48-72 hours (adjust incubation time based on drug action kinetics—e.g., 48 hours for fast-acting chemotherapeutics, 72 hours for targeted drugs).

c. Add 50 μL CCK-8 reagent to each well. Incubate at 37°C for 2–4 hours (CCK-8). Measure absorbance at 450 nm using a microplate reader. Calculate cell viability:

Cell Viability Rate = $(OD_{experimental}-OD_{blank}) / (OD_{control}-OD_{blank}) \times 100\%$.

Use GraphPad Prism software to plot the dose-response curve (drug concentration vs. cell viability). If necessary, calculate IC_{50} (half-maximal inhibitory concentration) via nonlinear regression.

6.4 Quality Control

Proper characterization ensures organoids are valid models. This document details standardized quality control (QC) measures for NUT cancer organoids—covering morphology assessment, viability assays, molecular characterization, and sterility checks—to ensure consistency, functionality, and safety throughout their culture, cryopreservation, and banking lifecycle.

(1). Morphology Assessment

Morphological integrity reflects organoid health and functional potential; it should be checked at key stages (pre-cryopreservation, post-thaw recovery, and during routine culture).

a. Observation Methods

- Use bright-field microscopy (4× or 10× objective field) to examine organoids in culture dishes or Matrigel domes. For high-resolution details (e.g., lumen

formation, cell layer structure), use phase-contrast or confocal microscopy.

- Document morphology via standardized imaging: Capture 3–5 representative fields per sample (ensuring ≥10 organoids are visible per 4× objective field) and label with sample ID, passage number, and observation date.
- b. Pass/Fail Criteria (Measure size via ImageJ software)

Healthy Morphology (Pass)	**Abnormal Morphology (Fail)**
Compact 3D aggregates (80–250 μm), retains tumor-specific features (e.g., irregular borders), consistent size across batch.	Diffuse necrosis (>40% of organoid), cytoplasmic vacuolation (>30% of cells), disrupted budding, or shrunken, dark-appearing cores.

- Rejection Rule: Samples with >30% abnormal organoids are discarded; batches with inconsistent morphology (e.g., >20% size variation) require re-evaluation of culture conditions.

(2). Viability Assays

Viability measures the proportion of living cells within organoids, with a minimum threshold of 80% viability for cryopreservation, banking, or experimental use. The Trypan Blue Exclusion Assay serves as a recommended method for assessing the cell viability of cultured NUT cancer organoids.

- Sample Preparation: Harvest 10–20 organoids per sample; dissociate them into single cells or small clusters using TrypLE at 37°C for 5–10 mins) to ensure dye penetration.
- Staining and Counting: Mix 10 μL of cell suspension with 10 μL of 0.4% trypan blue solution (21-23°C); incubate for 1–2 mins. Load the mixture into a hemocytometer and count cells under a light microscope.
- Living Cells: Unstained (trypan blue is excluded by intact cell membranes).
- Dead Cells: Blue-stained (membrane damage allows dye entry).
- Calculation: Viability (%) = (Number of living cells / Total number of cells) × 100.

(3) Molecular characterization

Immunohistochemistry (IHC) is used for specific detection the expression of NUT fusion protein in cell smears, with clear steps and key parameters to ensure experimental reproducibility.

- Cell smear preparation: Collect TrypLE-dissociated organoid cells (1000-2000 cells in 50 μl PBS, evenly spread them on clean glass slides, and air-dry at 21-23°C for 1 hour.
- Immerse the air-dried smears in 95% alchohol for 10–15 minutes to preserve cell structure and protein integrity. Rinse the fixed smears with PBS for 2 times, 3 minutes each time, to remove residual fixative. Incubate the smears with 3% H_2O_2 for 15 minutes to block the activity of endogenous peroxidase. Rinse the fixed smears with PBS for 2 times, 3 minutes each time.
- Dilute rabbit anti-NUT antibody (Cell Signaling, 3625) with 3% BSA in PBS (dilution1:100–1:200). Set positive/negative controls. Incubate smears with diluted antibody (30-50 μl per slide) for 1 hours at 37°C in a humidified box. Rinse with PBS for 3 times, 5 minutes each time. Incubate with SignalStain® Boost IHC Detection Reagent (30-50 μl per slide, Cell Signaling, 8114) for 30 minutes at 21-23°C. Rinse with PBS for 3 times, 5 minutes each time.
- Add fresh DAB solution (SignalStain® DAB Substrate Kit, 8059), incubate for 3-5 minutes at 21-23°C; monitor under light microscope. Stop with distilled water and rinse for 2 times, 3 minutes each time.
- Hematoxylin stain (1-2 minutes); differentiate with 1% HCl-ethanol (5–10s). Blue in tap water (5–10 minutes); dehydrate in serial ethanol (70% → 100%, 2 minutes each). Clear in xylene for 2 times, 5 minutes each time.
- Mount with neutral balsam and dry in 37°C oven. Observe (100×/400×): Nuclear brown = positive. A nuclear NUT positivity rate of ⩾ 80% is regarded as qualified.

(4) Sterility Checks

Sterility ensures organoids are free of microbial contamination (bacteria, fungi,

mycoplasma), which can compromise culture integrity and experimental results. Checks are mandatory before cryopreservation, after thawing, and every 2–3 passages during long-term culture.

a. Routine Microbial Screening (Bacteria/Fungi)

- Visual Inspection: Check culture medium daily for turbidity (bacterial contamination), white/colorless colonies (fungal contamination), or unusual color changes (e.g., yellowing from bacterial metabolism).
- Culture-Based Testing: Transfer 100 μL of organoid culture supernatant to two sterile media: Tryptic Soy Broth (TSB): Incubate at 37°C for 48–72 hours (detects bacteria). Sabouraud Dextrose Broth (SDB): Incubate at 25°C for 5–7 days (detects fungi).
- Result: Turbidity or colony growth—contaminated (discard sample; decontaminate culture equipment with 70% ethanol).

b. Mycoplasma Testing (Obligate Intracellular Pathogens)

Mycoplasma is often undetectable via visual inspection; use sensitive methods:

- PCR-Based Assay: Use a commercial mycoplasma detection kit (MycoAlert, Lonza, LT07-418) to amplify mycoplasma-specific DNA from culture supernatant. Positive results show a color change or fluorescence signal.
- Fluorescent Staining: Stain organoid cultures with DAPI solution (Beyotime, C1006) for 2-5 min, and visualize via fluorescence microscopy.
- Frequency: Test every 2 passages for long-term cultures; mandatory test before banking human-derived organoids.

c. Contamination Response

- If contamination is detected: Immediately isolate the affected culture; discard all contaminated materials (per institutional biosafety waste protocols); decontaminate the biosafety cabinet (BSC) with 10% bleach followed by 70% ethanol; test adjacent cultures to prevent spread.

(4). QC Documentation

- Record all QC results in a digital database (e.g., LabWare, Excel) linked to the organoid’s batch ID, including:

- Morphology: Images, % of healthy organoids.
- Viability: Assay type, viability percentage, operator.
- Sterility: Test method, result (positive/negative), date.
- Retain records for the duration of the organoid's banking lifecycle (plus 5 years post-disposal) to ensure traceability and compliance with ethical guidelines.

6.5 Cryopreservation / Banking

For long-term storage, organoids can be cryopreserved and subsequently thawed for downstream applications.

(1). Pre-Cryopreservation Preparation

a. Sample Selection and Quality Check

- Select log-phase, healthy organoids (typically 3–7 days post-passage) with intact morphology (no signs of necrosis, dissociation, or microbial contamination).
- Perform a viability test using trypan blue staining: only organoids with >80% viability are eligible for cryopreservation.

b. Reagent and Equipment Preparation

- Cryopreservation Medium: DMEM/F12 + 10% DMSO + 40% fetal bovine serum (FBS).
- Equipment: label cryovials (1.5–2 mL) with organoid type, passage number, date, and biosafety label (if applicable); For the cryopreservation of organoids, a programmed cooling container is utilized to facilitate freezing, with subsequent storage in a -80°C ultra-low temperature refrigerator**.**

(2). Cryopreservation Procedure

a. Organoid Harvesting and Processing

- Aspirate culture medium from organoid cultures; gently wash organoids twice with cold PBS to remove residual medium.
- For Matrigel-embedded organoids: Add sufficient cell recovery solution to the Matrigel to cover all of the Matrigel. Incubate at room temperature (21-23°C) for 5 to 20 minutes, allowing the cell recovery solution to dissolve the

Matrigel and release the organoids. Centrifuge at 300×g for 5 min to pellet organoids**.**

- Resuspend the organoid pellet in pre-chilled cryopreservation medium (100–200 μL per cryovial, ensuring ~1×10^4–1×10^5 organoids/mL for optimal recovery).

b. Gradual Cooling (Critical for Viability)

- Place cryovials in a pre-cooled (4°C) programmed cooling container filled with isopropanol (-1 ° C/min); store at -80°C overnight; transfer to LN_2 the next day.
- Biosafety Note: For BSL-2/3 organoids, use sealed, leak-proof cryovials and decontaminate the exterior with 70% ethanol before placing in LN_2 storage**.**

(3). Organoid Banking Management

a. Storage Organization

- Store cryovials in LN_2 tanks (vapor phase, not direct immersion) or -196°C cryogenic freezers.
- Maintain a digital and physical inventory system recording:
- Organoid details (type, donor source, passage number, genetic modifications).
- Storage location (tank ID, rack number, position).
- Freezing date, operator, and thawing history (if applicable).
- Implement a regular LN_2 level monitoring schedule (daily checks) to prevent sample thawing.

b. Quality Control (QC) for Banked Organoids

- Perform QC every 6–12 months: Thaw a representative cryovial from each batch; assess viability (trypan blue) and morphology (via bright-field microscopy).
- Critical Thresholds : **Pre-Cryopreservation**: ≥80% viability required to proceed (lower viability increases post-thaw cell death); **Post-Thaw Recovery**: ≥50% viability acceptable after organoids recover over 3–5 days; <30% viability indicates cryopreservation failure (batch discarded).

- Discard batches with <50% viability or loss of organoid-specific functionality.

(4). Thawing and Recovery (for Post-Banking Use)

- Remove the cryovial from LN_2; thaw rapidly in a 37°C water bath (2–3 mins).
- Decontaminate the vial exterior with 70% ethanol; transfer the organoid suspension to a centrifuge tube containing 5× volume of pre-warmed culture medium (to dilute DMSO, which is toxic at high concentrations).
- Centrifuge at 300×g for 5 mins; aspirate the supernatant; resuspend the organoid pellet in fresh, pre-warmed complete culture medium.
- Plate organoids in Matrigel (per standard organoid culture protocols) and incubate at 37°C, 5% CO_2. Replace medium after 24 hours to remove residual DMSO; monitor recovery over 3–5 days.

(5). Biosafety Considerations

- All steps must be performed in a biosafety cabinet (BSC) BSL2 to prevent aerosol exposure to viable organoids.
- Dispose of used cryovials, pipette tips, and residual medium as infectious waste (per institutional biosafety protocols for organoid waste handling).
- For human-derived organoids, ensure compliance with ethical guidelines (e.g., informed consent for donor materials) and data privacy regulations.

6.6 Disposal

Institutional biosafety protocols for organoid waste handling (aligned with WHO/national guidelines) target risks from organoid-specific waste (e.g., viable cells, culture reagents) to protect humans, the environment, and labs.

(1). Core Principles

- Risk Classification: Group by biological risk (e.g., Risk 2 for common NUT cancer organoids; Risk 3 for genetically modified or pathogen-infected organoids) to guide handling.
- Containment: Prioritize preventing viable organoid cell release—use sealed containers for culture waste and avoid aerosol generation during disposal.

- Traceability: Document organoid type, waste generation date, and treatment method for accountability.

(2). Biohazard Labeling

- Design: Universal biohazard symbol (red circle + 3 prongs) + additional "Organoid Waste" label in high contrast.
- Placement: Affix to culture waste containers, sharps bins holding organoid manipulation tools, and transport carts.
- Extra Info: Include organoid risk group, generating lab, and whether waste contains genetically modified cells.

(3). Organoid Waste Segregation

Category	**Description (Organoid-Specific)**	**Rules**
Infectious Organoid Waste	Viable organoids, contaminated culture media, used well plates/flasks	Dedicated red biohazard containers; pre-treat with disinfectant (e.g., 70% ethanol) before sealing.
Organoid-Related Sharps	Contaminated pipette tips, scalpel blades (for organoid sectioning), broken culture dishes	Rigid puncture-resistant bins; seal at ¾ full; avoid mixing with non-organoid sharps.
Pathological Organoid Waste	discarded tumor organoids, cell pellets .	Sealed containers with absorbent lining; autoclave before final disposal.
Non-Biological Organoid Waste	Empty media bottles (uncontaminated), unused pipette wrappers	Regular recycling/trash; confirm no organoid residues before disposal.

(4). Post-Segregation Handling

- Treatment: Inactivate viable organoid waste via autoclaving (121°C, 15 psi for 20 mins) or chemical disinfection (e.g., 10% bleach soak for 30 mins);

genetically modified organoid waste requires additional regulatory-compliant treatment.

- Transport: Use leak-proof, labeled carts; transport only to dedicated organoid waste treatment areas (avoiding general lab zones).
- Disposal: Autoclaved organoid waste → regular hazardous landfill; incinerate high-risk organoid waste (e.g., pathogen-infected) per local rules.

6.7 Troubleshooting

Success relies on attention to detail and problem-solving.

Challenge/Situation	**Recommended Action**
Contamination (Bacterial/Fungal)	Discard the culture. Maintain strict aseptic technique. If mycoplasma is suspected, use a removal agent, but discard if contamination persists.
ECM Not Solidifying	Ensure the culture plate is pre-warmed and that the ECM is handled on ice until seeding. Work quickly to prevent the ECM from warming up prematurely.
Handling Variability	Be aware that components like Matrigel and some medium supplements are undefined and can have batch-to-batch variation. Test new batches before full-scale use.
Molecular Drift	Be aware that long-term passaging (over 20 passages) can lead to genetic and phenotypic drift from the original tissue. Use early-passage organoids (under 10 passages) for experiments that require high fidelity to the patient sample.

7 Documentation

Maintain complete, auditable records throughout the organoid lifecycle.

- Batch Record: link every batch to donor ID, biosample type, passage number, operator, date, ECM lot, medium lot, and freezing/thawing history.
- Culture Log: daily entries of medium changes, microscopic observations, split ratios, and incubation conditions.
- QC Report: Store morphology images, viability percentage, sterility test results (TSB, SDB, mycoplasma), IHC/IF NUT protein staining results, operator initials, and pass/fail verdict; retain electronic files for 5 years post-disposal.
- Deviation Log: record any non-conformance (e.g., delayed processing, equipment failure), root-cause analysis, and corrective actions; close out with QA sign-off before next passage or release.
- Training Sign-off: verify completion of BSL-2, aseptic technique, and organoid-specific modules; keep certificates in personnel files and link to each batch record.

8 References

1. **ISoOR-ISOB -** International Standards for Organoid Biobanking.

2. **ISO 20387:2018** - Biotechnology — Biobanking — General requirements for biobanking.

3. **ISO 15189:2022** - Medical laboratories — Requirements for quality and competence.

4. **ISBER BEST PRACTICES: RECOMMENDATIONS FOR REPOSITORIES FIFTH EDITION** - International Society for Biological and Environmental Repositories.

5. **IARC TECHNICAL PUBLICATION NO. 44** - Common minimum technical standards and protocols for biobanks dedicated to cancer research.

6. **ISO/IEC 17025:2017** - General requirements for the competence of testing and calibration laboratories.

7. **ISO 9001:2015** - Quality management systems — Requirements.