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Table of Contents

1. Purpose

"National Biobank Consortium of Taiwan" (hereinafter referred to as the "Integration Platform") primarily integrates domestic human biobanks — their data, biospecimens, and related information (hereinafter referred to as "Integration Platform biospecimen data") — through a cloud-based system, making them available for application and use by medical, clinical, industry, and related biomedical research investigators nationwide. To achieve cross-institutional collaboration, it is necessary to establish consistent quality standards for dispensed biospecimens and consistent clinical data content. To maintain good specimen quality and to ensure consistency in the steps for extracting tissue RNA, so as to facilitate future related research, this Standard Operating Procedure is hereby established.

2. Scope of Application

This SOP applies to the entire process from fresh frozen tissue specimen collection through tissue RNA extraction and completion of quality testing.

3.Responsibilities

All human biobanks that have joined the Integration Platform, when extracting RNA from human tissue they have collected,are recommended to follow this Standard Operating Procedure, or must otherwise achieve the same quality and yield.

4. Description

This SOP can be divided into four stages, all of which are performed manually

1. Fresh frozen tissue specimen collection from surgical resection or biopsy
2. Frozen tissue embedding and section staining interpretation
3. Frozen tissue RNA extraction
4. Frozen tissue RNA quality testing (horizontal electrophoresis)

5. Operating Procedures

5.1 Fresh Frozen Tissue Specimen Collection from Surgical Resection or Biopsy

Materials and Equipment:

cryotubes, sterilized fine-tipped forceps (11cm), scalpel blade, aluminum foil, liquid nitrogen, thermos flask, freeze-resistant labels, -80°C freezer or liquid nitrogen tank

Procedure: After obtaining case consent, first prepare cryotubes for the tissue to be preserved, affix pre-numbered freeze-resistant labels, and draw an appropriate amount of liquid nitrogen into a thermos flask beforehand.Under the guidance of a pathologist, cut the remaining tissue specimen from the surgical resection or biopsy after pathological examination, dividing it into tumor tissue and surrounding non-tumor tissue; place it on aluminum foil, and immediately cut it into small pieces using a scalpel blade (approximately 5x5x5 mm^3), then rapidly freeze it in liquid nitrogen. Using fine-tipped forceps, place the pieces into a cryotube (a single tube can hold many pieces), and store at -80°C in a freezer or liquid nitrogen tank.

Precautions:

1. Because collecting fresh tissue specimens involves many variables, handling must be adapted to the actual situation.
2. Priority must be given to having sufficient tissue for pathological diagnosis; if the remaining tissue is insufficient, do not retain frozen tissue.
3. If the tumor tissue specimen cut is already very small, there is no need to cut it into smaller pieces.
4. Sometimes surgery finishes very late and same-day collection is not possible; if operating room staff can assist by first refrigerating the specimen at 4°C (it must not be placed in formalin), it may be collected the next day (this should be noted as "collected the next day") — do not discard this specimen. If a fresh specimen is quickly placed in refrigeration, the DNA quality of most tissues can still be suitable for molecular biology experiments even if collected the next day. However, for RNA extraction, the result may not necessarily be ideal.

5.2 Frozen Tissue Embedding and Section Staining Interpretation

Equipment:

−-80℃ freezer, cryostat, stainer, coverslipper

Consumables:

1. Aluminum foil molds (Note: cut aluminum foil into squares of approximately 4 cm, use the back end of a marker pen as a form to make the aluminum foil molds)
2. OCT
3. Sterilized fine-tipped forceps (11cm)
4. 100ml beaker
5. Specimen storage box (10x10 grid) (the inner grid dividers need to be removed, so that the grid space becomes 5 x 5, allowing 25 aluminum foil molds to be placed)
6. 1.5ml microcentrifuge tube (eppendorf)
7. Foam box (for holding frozen tissue and dry ice)
8. Dry ice

Procedure:

1. Before tissue RNA extraction, prepare an expanded polystyrene container filled with dry ice and the specimen retrieval list. Remove the 9 × 9-compartment specimen storage box containing the cryovials from the −80 °C freezer and place it in the container filled with dry ice. Remove the frozen tissue specimens individually and transfer each specimen into the corresponding pre-labeled microcentrifuge tube. Return each cryovial to its original position after the tissue specimen has been removed. This procedure shall be performed by two staff members: one person shall perform the specimen transfer, while the other shall verify that each specimen is placed into the correctly labeled microcentrifuge tube.

2. Place the microcentrifuge tubes containing the tissue specimens listed on the retrieval list into a 9 × 9-compartment specimen storage box and store the box in a −80 °C freezer until use.

3. Label the side of each aluminum foil mold with the corresponding

specimen identification number and set it aside for use.

4. Remove the specimen storage box containing the microcentrifuge tubes with the frozen tissue specimens and place it in an expanded polystyrene container filled with dry ice to keep the specimens frozen until use.

5. Perform the following procedure inside the expanded polystyrene container filled with dry ice. Place the aluminum foil mold labeled with the specimen identification number on the dry ice. Using sterilized forceps, remove the tissue specimen from the microcentrifuge tube and rapidly place it at the bottom of the aluminum foil mold with its largest surface facing downward. Add optimal cutting temperature (OCT) compound until the specimen is completely covered. Minimize the risk of tissue thawing throughout the entire procedure.

6. Place the used forceps in a beaker containing clean water. The forceps shall subsequently be cleaned and sterilized together.

7. Once the OCT compound has frozen and turned white, place the OCT-embedded specimen into a specimen storage box modified to contain 5 × 5 compartments. Store the box in a −80 °C freezer. Frozen sectioning may be performed after the specimen has been frozen for 30 minutes. The specimen identification number corresponding to each compartment shall be clearly documented.

8. Remove the frozen OCT-embedded tissue specimen from the −80 °C freezer, peel away the aluminum foil, and place the specimen in the cryostat. Cut the specimen into 4 μm-thick sections and mount the sections onto pre-labeled glass slides. Allow the slides to air-dry at room temperature for at least 2 hours before hematoxylin and eosin (H&E) staining. Air-drying for 4 hours or overnight is preferable to reduce the risk of tissue sections detaching from the slides during staining.

9. After sectioning has been completed, rewrap the remaining OCT-embedded tissue specimen in its original aluminum foil bearing the specimen identification number and return it to the specimen storage box modified to contain 5 × 5 compartments.
10. A pathologist shall evaluate the frozen sections to determine the tumor diagnosis and tumor cell percentage. The evaluation results shall be documented in the relevant records.

Notes:

1. The consistency of all specimen identification numbers shall be verified throughout the entire procedure.
2. When embedding frozen tissue in OCT compound for subsequent tissue RNA extraction, the tissue must remain frozen at all times and must not be allowed to thaw. Consequently, the OCT compound may not adhere closely to the frozen tissue, making cryosectioning more difficult. In addition, RNA in frozen tissue that has undergone cryosectioning is more susceptible to degradation. Therefore, for tissue RNA extraction, cases in which frozen sections have already been prepared and evaluated for tissue DNA extraction should preferably be selected, thereby avoiding the need for additional cryosectioning.

5.3 Frozen Tissue RNA Extraction

Equipment:

1. Benchtop high-speed refrigerated microcentrifuge (eppendorf refrigerated Microcentrifuge 5415 R or equivalent product)
2. Bead-based tissue homogenizer (MagNA Lyser instrument)
3. Ultra-micro spectrophotometer (Thermo Nanodrop 2000 or equivalent product)
4. –80℃ freezer
5. Water purification system (Milli-Q® Integral)
6. Pipetman (pipetman) (1,000ul, 200ul, 10ul, 2ul)
7. Chemical fume hood (chemical hood)

Reagents:

1. TRIzol™ Reagent (PRO TECH Cat no.15596018)
2. Chloroform
3. 100% isopropanol
4. 75% ethanol (75% EtOH)
5. Sterilized secondary distilled water (ddH_2O), abbreviated as secondary water (ddH_2O)

Consumables:

1. 1.5ml microcentrifuge tube (Eppendorf)
2. Bead tube (MagNA Lyser Green Beads)
3. Dry ice
4. Ice bucket
5. Foam box
6. Filter tip (1,000ul, 200ul, 10ul, 2ul)

Procedure: (the entire process must be performed in a chemical fume hood)

1. Prepare one bead tube (MagNA Lyser Green Beads) and two 1.5ml microcentrifuge tubes (Eppendorf), and write the specimen number on the cap and side of each.
2. Using a 1,000ul pipetman, draw up 1 ml of Trizol and add it to the pre-numbered bead tube, then place it in an ice bucket containing crushed ice.
3. Take –the 80°C
4. freezer's microcentrifuge tube containing frozen tissue (with tumor diagnosis and percentage already confirmed), and place it in a foam box containing dry ice.
5. Without letting the frozen tissue thaw, quickly pour it into the bead tube containing Trizol — the Trizol must completely cover the tissue. Shake up and down, then place it in the ice bucket containing crushed ice.Place the bead tube into the bead-based tissue homogenizer, and homogenize the tissue at 6,

   500 rpm for 40 seconds, up to a maximum of five times. Note:
6. If more than one round of homogenization is needed, after each round the tube must be placed in the ice bucket containing crushed ice to cool for 2-3 minutes before proceeding to the next round.Using a 1,
7. 000ul pipetman, draw up all of the tissue homogenate from the bead tube into a first new microcentrifuge tube, and let it stand at room temperature for 5 minutes.Add 200 ul of chloroform
8. , shake up and down for 15 seconds, and let stand at room temperature for 2 minutes.Place it in the pre-chilled benchtop high-speed refrigerated microcentrifuge (^{4}oC) and centrifuge at 12,000 xg
9. for 15 minutes.

   Draw off the supernatant and transfer it to a second new microcentrifuge tube. Note:
10. Draw off only about 80% of the supernatant — do not draw up any of the pellet underneath.Add 500 ul of 100% isopropanol (100% isopropanol)
11. , invert to mix thoroughly, and let stand at room temperature for 15 minutes.Place it once more in the pre-chilled benchtop high-speed refrigerated microcentrifuge (^{4}oC) and centrifuge at 12,000 xg,

12. for 10 minutes.
13. Discard the 100% isopropanol, retaining the pellet (pellet).Place it once more in the pre-chilled benchtop high-speed refrigerated microcentrifuge ($4^{o}C$) and centrifuge at 12,000 xg for 30 seconds, then use a 200ul pipetman to remove the remaining 100% isopropanol (100% isopropanol)
14. .
15. Add 1 ml of 75% ethanol to the 1.5ml microcentrifuge tube.Place it in the pre-chilled benchtop high-speed refrigerated microcentrifuge ($4^{o}C$), and centrifuge at 7,500 xg
16. for 5 minutes.
17. Discard the 75% ethanol, retaining the pellet (pellet).Place it in the pre-chilled benchtop high-speed refrigerated microcentrifuge ($4^{o}C$) and centrifuge at 7,500 xg for 30 seconds, then use a 200ul pipetman to remove the remaining 75% ethanol, and air dry (air dry)
18. for 3-5 minutes. Depending on the size of the pellet (pellet), add 2~3 times its volume of sterilized secondary water (ddH_2O) to resuspend the pellet, wait 3 minutes, then place it in the ice bucket containing crushed ice.
    Note: Because the size of the pellet extracted differs from case to case, if the pellet is too small to see, resuspend it using 10ul of sterilized secondary water (ddH_2O); if the pellet is approximately 1~2mm thick, resuspend it by adding 50-100ul of sterilized secondary water (ddH_2O).
19. Once the pellet has fully dissolved, gently tap the bottom of the 1.5 ml microcentrifuge tube to mix the RNA within thoroughly.Place it in the benchtop high-speed refrigerated microcentrifuge ($4^{o}C$) and centrifuge at 7,500 xg for 30 seconds, then quickly place the 1.5 ml microcentrifuge tube in the ice bucket containing crushed ice; using a 2ul pipetman, draw 1.2ul, and use the ultra-micro spectrophotometer (Nanodrop) to measure the RNA concentration and O.D. value: 260/280, 260/230. The ideal 260/280 value should fall between 1.8-2.0. (See Attachment 1, Figure B.)

20. Take the tissue RNA specimen, once its concentration has been measured, and aliquot approximately 1μg~2μg for gel electrophoresis into another 1.5 ml microcentrifuge tube, and place it in the ice bucket containing crushed ice, then store it in the 4℃ freezer until gel preparation is complete and electrophoresis can be run (if electrophoresis can only be run the next day, it must instead be stored in the ℃ freezer at -20 until then).The remainder of the tissue RNA specimen shall be aliquoted into several tubes of 10ug each, placed in numerical order by specimen number into a specimen storage box (9x9 grid) (with the specimen number and specimen type noted on the lid and side of the box), and promptly stored in the -80℃ freezer.

5.4 Frozen Tissue RNA Quality Testing (Horizontal Electrophoresis)

Equipment:

1. High-sensitivity gel imaging system (SYNGENE In Genius 3 or equivalent product)
2. Microwave oven
3. Mini electrophoresis tank (Mini Gel Migration Through)
4. Micro analytical balance(JADEVER SKY300 or equivalent product)
5. Benchtop horizontal shaker

Reagents:

1. UltraPure™ TAE Buffer, 10X (Thermo Cat no.15558042)
2. Agarose gel powder, Agarose-Molecular Biology Grade (Invitrogen Cat no.75510-019)
3. Ethidium bromide (EtBr)

Consumables:

1. Weighing paper (12x12 cm)
2. Spatula
3. Plastic wrap
4. 500ml sterilized Erlenmeyer flask
5. 1,000ml sterilized serum bottle
6. Graduated cylinders (50ml and 1L)

Procedure:

1. Preparing the Agarose Gel (1.5% gel)

1. Prepare 1X TAE Buffer: using a 1L graduated cylinder, pour 100ml of UltraPure™ TAE Buffer (10X) into a 1L serum bottle, then add 900ml of secondary water (ddH_2O), shake up and down to mix thoroughly, and store at room temperature.
2. Using a 50ml graduated cylinder, pour 40ml of the 1X TAE Buffer into the sterilized Erlenmeyer flask.
3. Zero the microbalance with weighing paper on it, then use a spatula to weigh out agarose gel powder (Agarose) 0.6 g, and add it into the Erlenmeyer flask containing the 1X TAE Buffer, mixing them together.
4. Cover the Erlenmeyer flask with plastic wrap, poke several small holes in the plastic wrap using a filter tip, place it in the microwave, and microwave for 30 seconds, then shake to mix thoroughly. This step must be repeated until the agarose gel powder (Agarose) is completely dissolved in the 1X TAE Buffer and appears completely transparent. (Repeat at least three times.)
5. Take the thoroughly mixed agarose gel solution out of the microwave and place it on the benchtop horizontal shaker for 3-5 minutes.
6. Using a 2ul pipetman, draw up 2 ul of EtBr and add it to the Erlenmeyer flask, mixing thoroughly.
7. Pour into the casting tray, to about half the height of the comb teeth, and let cool for 1 hour to solidify into the agarose gel (gel).

2. Performing Horizontal Electrophoresis

1. Place the solidified agarose gel into the mini electrophoresis tank, and pour in 450ml of TAE Buffer (1X).
2. Mix 1.2μg of RNA with 2ul of 6X Loading dye, then add sterilized secondary water (ddH_2O) to bring the total volume to 10ul.
3. Using a 10ul pipetman, inject the entire volume of the above thoroughly mixed liquid into a well of the agarose gel in the mini electrophoresis tank.
4. Perform horizontal electrophoresis for 35 minutes at a voltage of 85 volts (current of 500 mA)
5. Remove the agarose gel (gel) from the mini electrophoresis tank, and take it to the high-sensitivity gel imaging system to confirm the 18S and 28S bands. Save the file, and print and photograph it for the record (see Attachment 1, Figure A).

6. Quality Standards for Dispensing

Tissue RNA dispensed by the Integration Platform shall be accompanied by the following information:

1. Tumor component (must be interpreted and confirmed by a pathologist)
   -- Tumor tissue: the tumor component (tumor %) of the original tissue specimen should, in principle, be >50%; the higher the better.
   -- Non-tumor tissue surrounding the tumor: absence of tumor cell contamination must be confirmed.

   Note: Because the nature of each specimen differs, the tumor component requirement may be lowered for specimens that are difficult to obtain. In addition, if the research concerns the tumor microenvironment, applicants may also have different requirements for tumor component; the tumor component level may be adjusted in accordance with the applicant's requirements.
2. Whether the horizontal electrophoresis gel image shows both the 18S and 28S bands
   -- Presence of both the 18S and 28S bands in the tissue RNA indicates good RNA quality, and is required for the specimen to be eligible for dispensing.
   Note: Because the nature of each specimen differs, some specimens are difficult to obtain and are extremely precious, or some applicants' research methods are not affected by whether the RNA shows the 18S and 28S bands; such specimens may still be dispensed. Therefore, specimens should not be discarded simply because RNA quality is not fully ideal.
3. The tissue RNA's optical density (O.D) value should also be provided to the applicant for reference
   -- 260/280 ratio: for an ideal specimen, this should fall between 1.6-2.0
   -- 260/230 ratio: not important, and need not be provided
   Note: It is recommended that the 260/280 ratio preferably fall between 1.8~2.0, as nucleic acid purity is high in this range; if the ratio is <1.6 or the ratio is >2.0, it is recommended that the tissue RNA be re-extracted.

7. References:

1. Chomczynski, P. and Sacchi, N. Single-Step Method of RNA Isolation by Acid Guanidine-Thiocyanate-Phenol-Chloroform Extraction. Anal Biochem. 1987;162:156-159.
2. Sambrook, J., Fritsch, R. F., and Maniatis, R. Molecular Cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989).

3. RNA Extraction Procedure: http://www.bio-protech.com.tw/upload/20181115042308.pdf

8. Attachments

Attachment 1: Frozen Tissue RNA Horizontal Electrophoresis Gel Image and O.D. Value Testing Example

Figure A

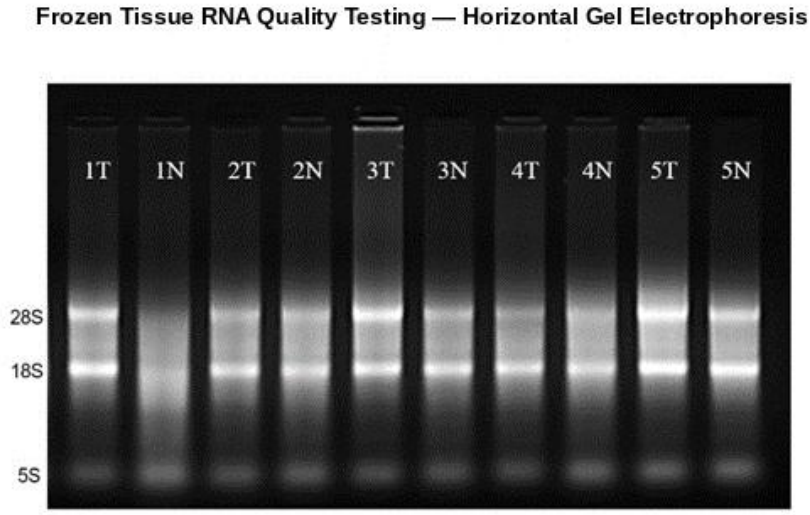

Figure B

Frozen Tissue RNA Quality Testing — O.D. Values

	ng/uL	260/280	260/230	Volume (ul)	Yield(ug)	Remarks	QC
1T	1812.3	1.98	1.87	100	181.2		X
1N	1997.4	2.01	2.06	130	259.7	No 18S	X
2T	1917.8	2	1.98	120	230.1		5.4
2N	1805.1	1.99	1.46	150	270.8		3.6
3T	2136.1	1.97	1.97	120	256.3		7.7
3N	1804	1.99	1.87	120	216.5		5.4
4T	1368.8	1.93	2.24	70	95.8	Weak 28S	X
4N	2419.4	2.02	1.93	150	362.9		X
5T	2198.7	1.99	1.97	170	373.8		7.5
5N	1782.1	2	1.94	120	213.9		4.3

Notes:

-- 1N shows no 18S or 28S bands on the gel, but has good O.D. values, indicating that O.D. values alone are not fully reliable for assessing RNA quality.

-- All other samples show both the 18S and 28S bands, which already meets the requirements for general RNA research use.

-- Although 2N's 260/230 value is relatively poor, its gel result is ideal, showing that the 260/230 ratio is not critical.

-- RIN (RNA Integrity Index) testing is now also available for QC (Quality Check), intended for more advanced genetic testing needs. For human tissue RNA, a RIN of 5 is already good, and 4 should also be usable.