



Dept. of Gynaecological Oncology and WICR,  
Westmead Millennium Institute, Westmead Hospital

**Standard Operating Procedures  
Version 2 - October 2010**

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## INTRODUCTION

The Gynaecological Oncology Biobank at Westmead was established to systematically collect high quality gynaecological biospecimens for research.

The main aim of the biobank has been to provide tissue to projects investigating the underlying molecular biology of gynaecological disease and to promote and facilitate collaboration between researchers to benefit gynaecological oncology research. In addition to frozen tissue specimens and a specimen of peripheral blood, comprehensive clinical data is collected so that molecular findings can be correlated with response to treatment and outcome. A key aim is to collect biospecimens in a way that maintains the flexibility required for the application of a range of current and emerging research technologies.

### Guiding principles

- Patients are provided with information about sample collection, research and ethical implications and if they agree to participate, sign a Patient Information and Consent Form.
- Only tissue in excess to that required for pathological assessment and diagnosis is collected.
- Protection of privacy of participating patients is paramount. Only the Biobank Managers have access to identifying information.
- The biospecimens are only to be used in projects that have been approved by the Sydney West Area Health Service Human Research Ethics Committee (SWAHS HREC) and the HREC at the Institution where the work will be performed, if relevant.
- Applications by researchers for material and information must be scientifically sound and make efficient use of the resources available.

The Gynaecological Oncology Biobank at Westmead is a member of Australasian Biospecimen Network-Oncology, an NHMRC funded network of Australian Tissue Banks that collect biospecimens for cancer research.

## PATIENT CONSENT PROTOCOL

The Gynaecological Oncology Biobank at Westmead complies with the NHMRC National Statement On Ethical Conduct In Human Research 2007 and the NSW Human Tissue Act 1983 and amendments 2006.

Tissue is collected under the project "Molecular Biology of Gynaecologic Disease", SWAHS ethics approval number HREC92/10/4.13.

Current versions of Patient Information Sheet and Consent Form are dated 28 July 2010. SWAHS HREC requires an annual report to be submitted every December for this project. See the Research Office for a proforma annual report document. All annual reports are filed in the "Molecular Biology of Gynaecologic Disease" ethics folder.

### Consenting Procedure

#### **All forms are in folder labelled "GYN ONC BIOBANK PROCEDURES"**

The Gynaecological Oncology Unit secretary starts all new patient files. The secretary will place the Gynaecological Oncology Biobank at Westmead Consent Form in the file for the initial consultation.

When discussing consent with the patient please ensure she understands each point on the Patient Information and Consent Form, highlighting the following:

- Biospecimens will be used in research projects investigating the underlying molecular biology of gynaecological disease.
- Only tissue excess to diagnostic requirements will be used and all types of gynaecological biospecimens (normal, benign, malignant) are useful in research.
- Your biospecimens will be stored for use in different research projects. Your sample will only be used for research that is approved by a Human Research Ethics Committee.
- In addition, if you are willing we also request that you provide a blood sample.
- Following the collection of a sample and blood, no further effort is required on your part.
- Participation is entirely voluntary and you are free to withdraw your consent at any time.

If the patient agrees, they sign the Consent Form and a copy of the Patient Information Sheet and Consent Form is given to them. The patient's file, with the signed Consent Form, is returned to the Gyn Onc secretary. The patient file will be given to the Gyn Onc Data Manager who will then give the Biobank Officer (BBO) the patient consent forms, and inform the BBO when the patients are booked for surgery.

BBO will then start a patient file on the Gyn Onc Biobank Database (for this procedure see data entry page 24).

## SPECIMEN COLLECTION & PRESERVATION PROTOCOL

On the day of surgery, the Gynaecological Oncology Biobank Officer (BBO) will attach a blood collection tube to the patient file for collection during surgery and call pathology to inform them of the name, DOB and MRN of consenting patients from which we are expecting to collect tissue. Blood (1 x 10 ml tube each of EDTA & Serum) will be collected before or during surgery.

### **Remember to treat all samples as potentially infectious.**

Specimen collection procedure is as follows:

- Surgeon will page BBO when biospecimen is ready for collection.
- BBO will take liquid nitrogen canister, tumour collection tubes (50 ml Falcon tubes with small hole in the top) and Tissue Collection Form to theatre to collect tumour and bloods.
- Theatre nurse will record the patient name, sample description and time sample was left in the red "urgent specimens" book at theatre reception. BBO needs to sign, date and record the number of samples taken in the red book.
- Specimens will be taken to Anatomical Pathology by the BBO. On arrival at pathology, the BBO will inform the staff that there is a sample "for frozen section".
- Pathologist will identify suitable tissue and if possible, excise an appropriate amount for immediate freezing in liquid nitrogen. The BBO will record when they were paged, when the specimen was collected and when it was frozen on the Tissue Collection Form (Appendix 2). The BBO will complete all other relevant information as requested on the form.

The tissue samples need to be snap frozen as soon as possible to maximise RNA quality. Request can be made for an adjacent portion to be formalin fixed and embedded in paraffin. This is to be processed in a green cassette and when ready, will be stored in the brown cardboard box labelled "Gyn Onc" in the Anatomical Pathology Processing Area.

Specimen preservation procedure is as follows:

- On returning to the lab, record patient Medical Record Number (MRN), date of birth (DoB) and name in the Gyn Onc Biobank Book.
- Label a 15 ml cryovial or 2 ml cryovial depending on the size of the sample with the next available lab number eg 533A. Patient initials and DoB should be recorded on the container.
- Remove sample from the 50 ml falcon tube and transfers to appropriately labelled cryovial. Cryovial is stored in liquid nitrogen.
- Process blood as per protocol (see page 6).
- Record relevant patient information on Gyn Onc Database and store paperwork in patient file in Gyn Onc office.

If there are any problems or you require further information, please contact:

CATHERINE KENNEDY/ YOKE-ENG CHIEW  
BIOBANK OFFICERS

(02) 9845 7306  
page \*2 08952

ANNIE STENLAKE  
RESEARCH NURSE

(02) 9845 6849

## BLOOD PROCESSING

Blood is to be collected from patients who have consented to participate in the study "Molecular Biology of Gynaecologic Disease".

**Pre-operative blood sample** collected from patient (record time of draw on tube) 1 x EDTA (10 ml) and 1 x plain for serum (10 ml)

Page \*2 08952 for blood to be collected.

Blood is collected and put on ice, before being transported to Gyn Onc lab.

Ensure that the Gyn Onc Blood Collection Form is completely filled in. The form is kept in the folder "Gyn Onc Tissue Bank Procedures"

The following aliquots are required:

1. Blood spots (protein saver cards)
2. EDTA whole blood
3. EDTA plasma
4. EDTA buffy coat
5. Serum

Perform the blood separation in the following order. Process the EDTA tube first while the serum clots on ice for at least 40 min. Plasma needs to be frozen ASAP to preserve protein integrity.

**Post-operative blood sample** collected from patient 1 x ACD tube (10 ml) – at time of medical oncology or gynaecological oncology clinic visits. BBO identifies consented patients from clinic list and supplies tubes to clinic nurse. If amenable, patient has tube filled by blood collectors in clinic and returns sample to nurse or reception. This is an ambient sample. Can be left to the following day to process. Clinic nurse will page BBO at end of clinic for bloods to be picked up.

The following aliquots are required:

1. Blood spots (protein saver cards)
2. ACD whole blood (4 x 1ml)
3. ACD plasma
4. ACD buffy coat

### 1. Blood Spot

Blood spot (Guthrie) cards (Whatman 903 protein saver cards) are stored at room temperature. These cards are kept on the bottom left hand side of Cath's shelf, behind the glass door.

Always handle cards wearing gloves and do not touch the circles where blood is deposited.

1. Mix EDTA blood thoroughly by inverting tube several times.
2. Wipe top of vacutainer with ethanol before opening.
3. Using a sterile pipette entirely fill the circles on the card taking care not to saturate the paper (each circle will take approximately 40 µl).
4. Label card with the next sequential tissue bank number (eg 533) and the blood spot suffix (BS). Also attach label from database with TB reference number, date and type of specimen details.

5. Air dry thoroughly in safety cabinet before folding over cover. Drying time will vary according to ambient conditions but will take at least 30 min. Cards may be left overnight to dry.
6. File in numerical order and store in locked drawer at room temperature (RT).
7. Record details on blood tracking form for entry into database.

## 2. EDTA whole blood, plasma and buffy coat

When labelling cryovials it is important to use the following suffixes to enable the type of sample to be readily identified. First use the tissue bank number allocated and then the suffixes below:

EDTA whole blood	WB
EDTA plasma	PB
Buffy coat	BC

1. Transfer two 1.0 ml aliquots of whole blood into pre labelled cryovials (eg 533WB).  
**For ACD tubes aliquot four 1.0 ml aliquots.**
2. Centrifuge remainder of EDTA blood tube at 3000 g for 20 min at 4°C.
3. Print labels for tubes from database with TB ref number, date and sample type.
4. Collect 0.5–1.0ml aliquots of plasma into pre labelled cryovials (eg 533PB). Take care not to disturb white cell layer. Record plasma volumes on blood tracking form.
5. Using a sterile pipette tip, remove the layer of white blood cells and platelets sitting on top of the red blood cells, together with a small amount of residual plasma. Take care not to lift red cells.
6. If it is difficult to cleanly separate this buffy coat layer, transfer to a small Eppendorf tube/micro centrifuge tube and centrifuge at 10,000 rpm for 2 min.
7. Place aliquots into pre labelled (eg 533BC) cryovials (approx 0.5 ml each).
8. Record details on blood tracking form.
9. Snap freeze vials by submersion in liquid nitrogen then transfer whole blood & plasma to storage box in -80°C freezer and buffy coat to liquid nitrogen or vapour phase tank. Record locations on tracking form.
10. The specimen vials should be split into 2 different storage boxes - eg if there are 4 vials containing plasma aliquots, 2 should be stored in one box and 2 in a different box. This is to aid the transfer of duplicate specimens to another collection centre for long term storage, ensuring back up in case of local freezer breakdown.

## 3. Serum

Note on tracking form if serum tube (red top) contains gel or not.

1. Allow blood to clot on ice for 40 min.
2. Centrifuge tube at 3000g for 10 min at 4°C.
3. Taking care not to disturb the clot, remove the serum and pipette into pre-labelled (eg 533SB) cryovials in aliquots of 0.5-1.0 ml.
4. Record bar code numbers and sample volumes on tracking form.
5. Attach cryo label from database, taking care not to obscure barcode number.
6. Snap freeze vials in liquid nitrogen before transferring to storage boxes in -80°C freezer, record locations on tracking form. Store the vials in 2 different boxes.
7. Retain clot in original tube and store at -80°C (as a back up source of DNA).

Discard all residual blood tubes in contaminated waste.

Enter all specimen details into database. The "Pathology" layout has a blood collection field to select a yes/no answer. However a more detailed layout is under "Blood" in the database. This allows for the recording of aliquot numbers and storage location. Fill in both layouts.

## ASCITES SEPARATION PROTOCOL

Two protocols are described. The first is a shorter method that is currently used in the laboratory. The second is based on Yamaue et al (1991), to obtain pure tumour cells from ascites and has not yet been tested in our lab.

### Current Method as of July 2008

- 2x cytopspins (or smears) of unpurified ascites – to assess tumour cell content
- Cell pellet fixed in 10% formalin for cytoblocks preparation – for IHC
- 1 ml aliquot of snap frozen concentrated unpurified ascites for DNA/RNA/protein extraction
- Aliquots of concentrated unpurified ascites with 10% DMSO – for cell culture

### Method

Ascites is collected from the ward in sterile catheter-bags containing 5000U unfractionated heparin and signed consent forms collected. Record volume and appearance of ascites on tracking log.

Aliquot 4x 250µl of the crude cell suspension into Nunc tubes and snap freeze  
Use 100ul of the crude cell suspension and make a smear on a microscope slide. Air dry and H&E stain the following day.

Transfer fluid to 4 x 250 ml centrifuge flasks and spin down at 400 g for 5 min at RT. The supernatant is discarded and the soft pellet is resuspended in an equal volume of complete medium (10N). This is divided into 2 aliquots and processed as follows:

#### A.

DMSO is added to one of the resuspended pellet to a final concentration of 10% (i.e to 9 mls of ascites pellet add 1 ml of sterile DMSO). 1 ml aliquots of ascites/DMSO are transferred to Nunc tubes and stored at -80°C overnight, and then transferred to liquid nitrogen the following day.

#### B.

10% Formalin is added to the other resuspended pellet (approx 4X volume of pellet, up to 50 ml) and then transferred to a 50 ml falcon tube. Store overnight at 4°C and then pellet at 1500rpm for 10 min. Discard supernatant and process as for paraffin embedded cytoblock preparation. (See Gyn Onc Methods folder for this protocol).

#### Note:

Cytopspins are air-dried and then stored at 4°C overnight and Giemsa stained the following day. For other staining protocols alternative fixatives may be required.

## Method 2

(Yamaue et al 1991, Eur J Cancer, 27 (10) 1258-1263)

1. Ascites is collected from the ward in sterile catheter-bags containing 5000U unfractionated heparin and signed Consent Forms collected.
2. Record volume and appearance of ascites on tracking log.
3. The fluid is immediately transferred to 4 x 250 ml centrifuge flasks and spun down at 400g for 5 min at RT.
4. The supernatant is discarded and the soft pellet is resuspended in an equal volume of complete medium (10N RPMI + 10% FCS)
5. Add 18 ml Ficoll-Paque Plus (FPP) to 2 x 50 ml centrifuge tubes
6. Layer 24 ml of the well mixed diluted ascites on to the FPP using a standard sterile plastic transfer pipette.
7. Centrifuge at 400g for 30 min at 20°C
8. Draw off most of the upper layer using a standard sterile plastic transfer pipette, leaving the mononuclear and tumour cells at the interface undisturbed. Leave a small amount of fluid above the interface.
9. Using a fine tipped sterile transfer pipette, remove the cells from the interface to a new 50 ml centrifuge tube. Take care to avoid transfer of too much FPP as it is toxic to the cells.
10. Wash cells with 30 ml complete media (or PBS?) and centrifuge at 100g (or more?) for 10 min.
11. While cells are spinning prepare the FPP discontinuous gradient. In 2 x 50 ml centrifuge tubes add 10 ml of 100% FPP and on top carefully layer 15 ml 75% FPP
12. When cells have finished spinning carefully remove and discard supernatant.
13. Count the cells and resuspend in complete medium at a concentration of  $1 \times 10^6$  /ml.
14. The cells are then layered on the discontinuous gradient and centrifuged at 400 g for 30 min
15. While the cells are spinning prepare the Percoll discontinuous gradient. In 2 x 15 ml centrifuge tubes carefully layer 4 ml each of 25%, 15% and 10% Percoll respectively.
16. After centrifugation a tumour rich fraction is collected from the 75% interface (according to the diagram this fraction will be in the middle of the 75% layer) using a fine tipped sterile pipette.
17. The tumour cell-enriched suspension is then directly layered onto the Percoll discontinuous gradient and centrifuged at 25 g for 7 min.
18. The tumour cells depleted of lymphoid cells are then collected from 25% interface (at the bottom of the tube) using a fine tipped sterile pipette.
19. Cells are then washed by transferring them to a 15 ml centrifuge tube, adding 10 ml of complete medium and centrifuging at 60-100g for 10 min.
20. Cells are then resuspended at  $1 \times 10^6$  /ml? (or depending what we want to do with them. We may resuspend and split into 4 different tubes and spin again to end up with 4 equal fractions or one for pellet, 1 for PCR)

According to the Yamaue paper the final fractions should have more than 90% tumour cell purity.

## **STORAGE OF BIOSPECIMENS**

Tissue samples are initially stored in the liquid nitrogen tanks in the Gyn Onc lab.

Samples are periodically moved to Westmead Millennium Institute Cryostorage facility on Level 1.

On the day of the move collect dry ice pellets from the Clinical Sciences wash up room and place into 2 eskies. The boxes for the isothermal are available from Ces Nast in the WMI Store room. Label the box with the next available isothermal shelf number eg 12L. Take the samples that are to be moved and place them in the new boxes on dry ice. Check the lab number eg 533 off against the location that the sample has come from eg 3E5 and write the new location in the lab book eg 12L. It is useful if there are 2 people performing this as it is quicker and allows someone to double check the storage location.

Move the samples as quickly as possible to the WMI facility. The key for the isothermal is kept in tissue culture in the co-ordinators top drawer.

Once the samples are in their new location, update the storage details in the Gyn Onc database.

Blood samples are stored in the box labelled "Gyn Onc Blood" on the 3<sup>rd</sup> shelf of the -80°C freezer in the Clinical Sciences Corridor. The key to the freezer is in the top drawer of Catherine E's bench.

Buffy Coat is stored in liquid nitrogen.

Guthrie cards are stored in a box labelled "Blood Spot Cards" on Catherine K's bench in the Gyn Onc lab.

## **MAINTENANCE OF TISSUE BANK**

Liquid nitrogen is filled every Tuesday by BOC, and every Friday afternoon each container is topped up by the BBO with approx 10 litres of liquid nitrogen from the dewars stored in the lab. After topping up, use the ruler on the bench behind the tanks to measure the level. It should be over 40 cm. If not, more liquid nitrogen is available from the Fertility Centre, room 2178. Ensure that the lids of the containers are pushed down firmly.

If the alarm goes off, check cause of alarm. If low level light is flashing, top up liquid nitrogen. The only other reason that the light will flash is if the power source to the alarm is interrupted.

## CRYOSECTIONING OF FROZEN TISSUE

### Materials:

- Super frost slides
- OMI #10 Disposable safety scalpel
- Forceps
- Paint brush
- Nunc vials
- Slide rack
- Kimwipes
- S35 Microtome blade
- OCT compound (Tissue-Tek)
- Hammer
- Safety glasses
- Canister of liquid nitrogen with tumours
- Microm HM505E Cryostat (Rm 3031, Level 3, WMI)

### Procedure:

\* Microtome training with WMI Histology is compulsory. \*

1. Reset temperature of Microtome to -30°C (normally its on at -20°C)
2. Spray paper towel/tissue with 70% ethanol & clean the stage of microtome & surrounding areas (do not spray ethanol directly)
3. Ethanol clean the forceps & paint brush. Place them in the -30C microtome.
4. Label 4 nunc vials & slides with:
  - tumour number
  - date
  - the corresponding number 1,2,3,4
5. Wrap labelled nunc vials in aluminium foil & place them in the Liquid nitrogen canister to freeze.
6. Place labelled slides on top of glass door of Microtome.
7. When ready to cut, place a kimwipe underneath the cutter to collect unwanted tissue shavings (makes cleaning after a lot easier).
8. Carefully insert the blade using forceps, secure the blade & flip back the metal piece to cover the blade.
9. Squeeze some OCT compound into the round mounting piece & place the tissue on it.
10. Leave the mounted tissue in the -30C microtome to set.
11. Insert the mould into the holder & when ready to cut, flip open the metal piece that covers the blade.
12. Cut 10 um section on to the slide for H&E – Slide 1.
13. Cut subsequent 4 X 100 um sections into the labelled Nunc vial 1. Place into liquid nitrogen canister.
14. Cut another 10 um section on to the slide for H&E – Slide 2.
15. Cut subsequent 4 X 100 um sections into the labelled Nunc vial 2. Place into liquid nitrogen canister.
16. Repeat H&E & cryosections for Slide 3, 4 & Vial 3,4 respectively.
17. Store cryosections in liquid nitrogen tank.

## H&E staining of frozen sections

1. 10-15s in Harris haemotoxylin
2. Rinse in water
3. 10s in Scots Bluing solution (3.5g sodium bicarbonate, 20 g magnesium sulphate in 1 L water)
4. Rinse in water
5. 10-15s in Eosin/Erythrosin B solution(ICPMR) # AEYWEST – specially prepared for ICPMR from Australian Biostain.
6. 2 x 10s Abs EtOH to wash & dehydrate
7. Dry in fumehood
8. 20s Histolene in fume hood
9. Mount.

## RNA extraction from cryosections

Materials:

- Absolute RNA microprep kit (Stratagene #40085)
- Canister of liquid nitrogen with cryosection vials
- Disposable scalpel (Feather Safety razor Co Ltd)
- Hand held mini homogeniser
- Plastic pestles (treated with RNase-out soln, cleaned & autoclaved)
- Heating block at 60C with an eppendorf of Elution buffer
- 100% & 70% AR grade Ethanol
- 1.5 ml eppendorf tubes

Preparing reagents from the kit:

- Add 290 ul DNase buffer to the lyophilised RNase-free DNase. Swirl to mix but try not to introduce bubbles. Store at -20°C
- Add 16 ml 100% ethanol to High Salt wash buffer bottle (HSB).
- Add 68 ml 100% ethanol to Low salt buffer bottle (LSB)
- Label date on bottles after addition.
- Store at RT

In the fume hood:

- Prepare fresh mixture of Lysis buffer & B-ME before use: 1.4 ul B-ME /200 ul
- Add 200 ul Lysis buffer-BME to the eppendorf tubes. Cap the lids before removing from fume hood.

On the bench:

1. Remove disposable scalpel from packaging & place them in the liquid nitrogen canister.
2. Insert a plastic pestle into the Hand held mini homogeniser.
3. Remove a cryosection from the vial using a frozen scalpel as quickly as possible & drop it into the eppendorf containing 200 ul of lysis-BME buffer.
4. Homogenized it for a couple of seconds.
5. Add equal vol of 70% ethanol. Vortex 5s.
6. Place RNA binding spin cup into 2 ml eppendorfs.
7. Spin 14,000 rpm, 1 min.
8. Remove & retain spin cup but discard filtrate.
9. Replace spin cup into eppendorf & add 600 ul LSN.
10. Spin 14,000 rpm, 1 min.
11. Discard filtrate & respin 14,000 rpm X 2 min.

12. Prepare DNase solution – gently mix 5 ul DNase + 25 ul Digest buffer.
13. Add DNase solution directly into the fiber matrix inside spin cup.  
Incubate at 37°C, 15 min.
14. Add 500 ul HSB into spin cup.
15. Spin 14,000 rpm, 1 min.
16. Discard filtrate & add 600 ul LSB into spin cup.
17. Spin 14,000 rpm, 1 min.
18. Discard filtrate & add 300 ul LSB into spin cup.
19. Spin 14,000 rpm, 2 min.
20. Transfer spin cup into 1.5 ml eppendorfs.
21. Add 30 ul Elution buffer (prewarmed to 60°C) directly into the fiber matrix inside spin cup.
22. Incubate RT, 2 mins.
23. Spin 14,000 rpm, 1 min.
24. Quantitate RNA using Quantagene Pro.
25. Run samples on Agilent 2100 Bioanalyser to determine RNA quality.

## RNA analysis on Agilent Bioanalyser

Book a time with by Gandhi Kaushal.  
Email him at [kaushal.gandhi@wmi.usyd.edu.au](mailto:kaushal.gandhi@wmi.usyd.edu.au)

Fill the Agilent BioAnalyser usage form & email this to Kaushal

Sample preparation:

\* avoid using powdered gloves, ART tips & arturus amplication kit elution buffer as they interfere with process. \*

- Samples must be 25-500 ng/ul & dissolved in 2 ul sterile water (water for irrigation) or low salt/EDTA buffer in 0.5 ml PCR tubes
- Take samples in esky of ice to Kaushal (Rm 3033, level 2 WMI)
- Results will be emailed following completion of run (usually within 2 hrs)

## DNA extraction from cryosections

Materials:

- DNeasy Tissue kit (Qiagen #69504)
- 100% ethanol
- Waterbath at 55°C
- Heating block at 70°C

Preparing reagents from the kit:

- Before using for the first time, add the appropriate amounts of ethanol (96-100%) to buffers AW1 and AW2 as indicated on the bottles.

On the bench:

1. Pipette 180 ul Buffer ATL into eppendorf tubes.
2. Remove a cryosection from storage.
3. Add 20 ul PK, vortex 5-10s, & incubate at 55°C until tissue is completely lysed. Vortex occasionally during incubation to disperse the sample. Lysis is usually 1-3 hr but over night is convenient. (will not affect them adversely)
4. Vortex for 15s. Add 200 ul Buffer AL & **immediately** vortex 5-10s. Incubate at 70°C, 10 min.
5. Add 200 ul 100% ethanol to the sample & mix thoroughly to yield homogenous solution.
6. Pipette the mixture to DNeasy spin column placed in a 2 ml collection tube provided.
7. Spin 8000 rpm, 1 min. Discard flow-through & collection tube.
8. Place the spin column in a new collection tube, add 500 ul Buffer AW1.
9. Spin 8000 rpm, 1 min. Discard flow-through & collection tube.
10. Place the spin column in another collection tube, add 500 ul Buffer AW2.
11. Spin 14000 rpm, 3 min. Discard flow-through & collection tube (ensure that no carry over ethanol by carefully removing column without contact with flow-through)
12. Place spin column in a clean 1.5 ml eppendorf & pipette 100 ul Buffer AE onto the DNeasy membrane.
13. Incubate RT, 1 min.
14. Spin 8000 rpm, 1 min to eluate.
15. Repeat elution with a new eppendorf tube to prevent dilution of first eluate.
16. Quantitate DNA using Qubit fluorometer.

## DNA extraction from EDTA/ heparin/ citrate blood samples

Materials:

- FlexiGene DNA kit (Qiagen #51204)
- 100% Ethanol
- Waterbath at 37°C, then 65°C.
- Heating block at 65°C

Preparing reagents from the kit:

- Resuspend the lyophilised Protease with 0.3 ml Buffer FG3
- Store at 4°C

Prepare Buffer FG2/Qiagen Protease mixture (Buffer FG2P) no more than 1 hr before use:

- For every 4 ml blood, need 2 ml Buffer FG2 & 20 ul Protease.
  - Hence for 8 X 4 ml blood, need 16 ml Buffer FG2 & 160 ul Protease.
1. Thaw frozen blood tubes quickly in 37°C waterbath & store on ice. (change temp of bath to 65°C for later use)
  2. Pipette 10 ml Buffer FG1 into a 50 ml centrifuge tube. Can do 8 at a time.
  3. In fumehood: Add 4 ml blood & mix by inverting the tubes 5X.
  4. Spin tubes at 2000g, 5 min on swing out rotor in 2 lots of 4
  5. Discard supernatant carefully as the pellet may be loose. Invert tube onto daylee towel to drain, taking care the pellet remains in the tube.
  6. Add 2 ml Buffer FG2P, close the tube & vortex immediately until pellet is completely homogenized. **Important** to vortex each tube immediately after addition of buffer.

7. Invert tube 3x, incubate in 65°C waterbath, 10 min (colour changes from red to olive green, indicating protein digestion)
8. Add 2 ml isopropanol & thoroughly mix by inversion until DNA precipitant becomes visible as threads or clump.
9. Centrifuge for 3 min at 2,000g.
10. Discard supernatant carefully as the pellet may be loose. Invert tube onto daylee towel to drain, taking care the pellet remains in the tube.
11. Add 2 ml 70% Ethanol & vortex for 5 seconds.
12. Centrifuge for 3 min at 2,000g.
13. Discard supernatant carefully as the pellet may be loose. Invert tube onto daylee towel to drain, taking care the pellet remains in the tube.
14. Air dry the DNA pellet for 5 min.
15. Add 100 ul Buffer FG3, vortex for 5 seconds at low speed & dissolve the DNA by incubating for 1 hr a 65°C in the heating block.
16. Quantitate DNA using Qubit flourometer.

## **DNA Quantitation**

### **Quant-iT™dsDNA HS (10 pg/ul – 1000 ng/ul) Assay**

#### **Critical Assay Parameters**

##### Assay Temperature

The Quant-iT™ dsDNA HS assay for the Qubit™ fluorometer delivers optimal performance when all solutions are at RT (22–28°C). The Quant-iT™ assays were designed to be performed at RT, as temperature fluctuations can influence the accuracy of the assay. To minimize temperature fluctuations, store the Quant-iT™dsDNA HS reagent and the Quant-iT™ dsDNA HS buffer at room temperature and insert all assay tubes into the Qubit™ fluorometer only for as much time as it takes for the instrument to measure the fluorescence, as the Qubit™ fluorometer can raise the temperature of the assay solution significantly, even over a period of a few minutes. Do not hold the assay tubes in your hand before reading, as this will warm the solution and result in a low reading.

##### Incubation Time

In order to allow the Quant-iT™ assay to reach maximum fluorescence, incubate the tubes for the DNA and RNA assays for 2 minutes after mixing the sample or standard with the working solution. After this incubation period, the fluorescence signal is stable for 3 hours at RT.

##### Photobleaching of the Quant-iT™ Reagent

The Quant-iT™ reagents exhibit high photostability in the Qubit™ fluorometer, showing <0.3% drop in fluorescence after 9 readings and <2.5% drop in fluorescence after 40 readings. It is important to remember, however, that if the assay tube remains in the Qubit™ fluorometer for multiple readings, a temporary reduction in fluorescence will be observed as the solution increases in temperature. (The temperature inside the Qubit™ fluorometer may be as much as 3°C above room temperature after 1 hour.) For this reason, if you want to perform multiple readings of a single tube, you should remove the tube from the instrument and let it equilibrate to room temperature for 30 seconds before taking another reading.

## Experimental Protocol

1. Set up the number of 0.5 mL tubes you will need for standards and samples. The Quant-iT™ dsDNA HS assay requires 2 standards (Std 1 & 2)  
**Note:** Use only thin-wall, clear 0.5 mL PCR tubes. Acceptable tubes include Qubit™ assay tubes (500 tubes, Invitrogen Cat. no. Q32856) or Axygen PCR-05-C tubes (VWR, part number 10011-830).
2. Label the tube lids.
3. Make the Quant-iT™ working solution by diluting the Quant-iT™ dsDNA HS reagent 1:200 in Quant-iT™ dsDNA BR buffer. Do not mix the working solution in a glass container.  
**Note:** The final volume in each assay tube must be 200 µL. Each standard tube will require 190 µL of Quant-iT™ working solution, and each sample tube will require anywhere from 180 µL to 199 µL. Prepare sufficient Quant-iT™ working solution to accommodate all standards and samples. For example, for 8 samples, prepare enough working solution for the samples and 2 standards: ~200 µL per tube in 10 tubes yields 2 mL of working solution (10 µL of Quant-iT™ reagent plus 1,990 µL of Quant-iT™ buffer).
4. Load 190 µL of Quant-iT™ working solution into Std 1 & Std 2 tubes.
5. Pipette 10 µL of Quant-iT™ standard 1 & 2 into the appropriate tube and mix by vortexing 2–3 seconds, being careful not to create bubbles.
6. Load Quant-iT™ working solution into individual assay tubes so that the final volume in each tube after adding sample is 200 µL.  
**Note:** Your sample can be anywhere between 1 µL and 20 µL, therefore, load each assay tube with a volume of Quant-iT™ working solution anywhere between 180 µL and 199 µL.
7. Add each of your samples to assay tubes containing the correct volume of Quant-iT™ working solution (prepared in step 6) and mix by vortexing 2–3 seconds. The final volume in each tube should be 200 µL.
8. Allow all tubes to incubate at room temperature for 2 minutes.
9. Calibrate the standards & measure concentrations of assay tubes on the Qubit™ fluorometer.
10. Record the reading given by the Qubit™ fluorometer.

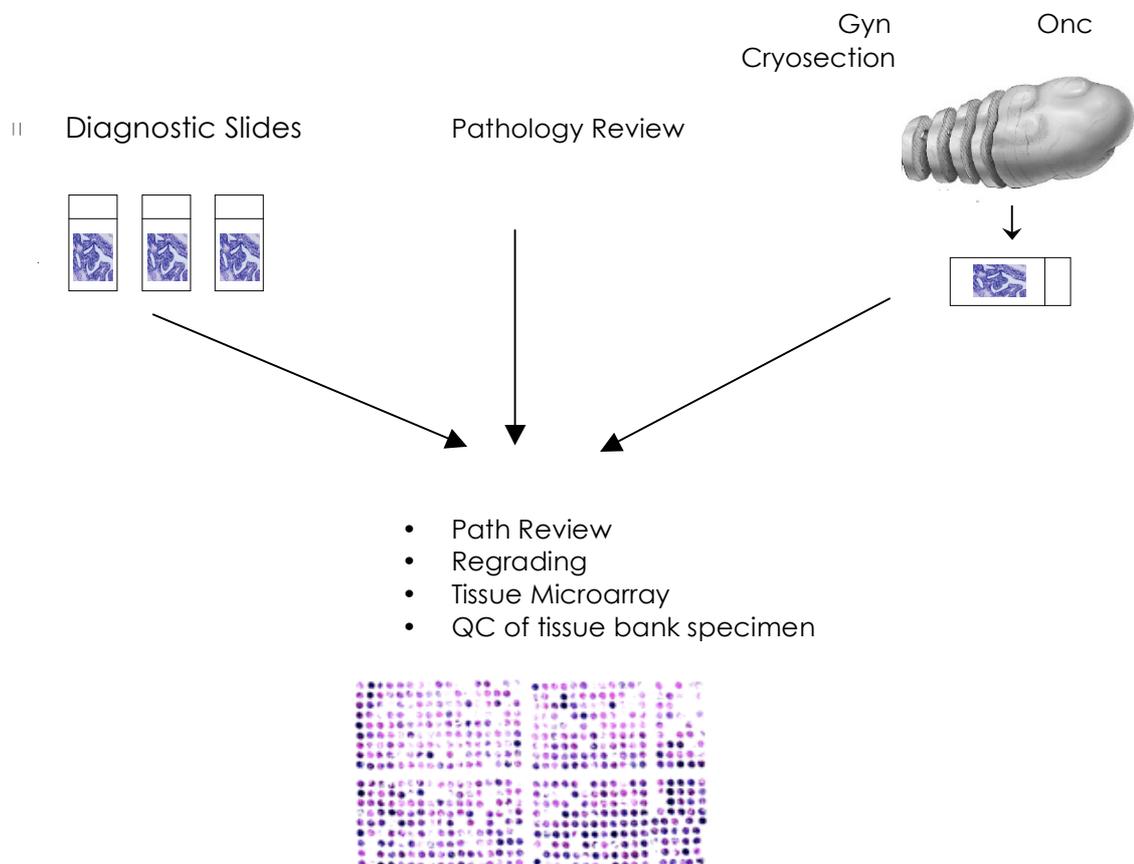
## **PATHOLOGY REVIEW PROCEDURE**

### **AIMS**

1. To review histopathology and confirm that the frozen specimen held in the Gynaecological Oncology Biobank at Westmead contains tissue of the same type and histology as the primary tumour described in the diagnostic pathology report.
2. To determine the quantity and quality of tumour tissue within the specimen held in the Gynaecological Oncology Biobank at Westmead.
3. To re-grade the case using a consistent, pre-defined grading system on diagnostic H&E slides using the Silverberg grading system.
4. To check for and record any interesting co-existing pathology.
5. To select a representative block that may be cored (1.0 mm diameter) for tissue microarray (TMA) construction and to mark 3 selected areas on the corresponding H&E.

## FLOW CHART FOR PATH REGRADING, CRYOSECTION COMPARISON AND TISSUE MICROARRAY CORE SELECTION

1. Relevant H&E cryosection from biobank specimen will be selected by the Gynaecological Oncology Biobank at Westmead team.
2. Tissue from the Gynaecological Oncology Biobank at Westmead will be matched to specimen code on pathology report. 3x H&E's will be selected from blocks/slides held in Anatomical Pathology (eg A1-A3).
3. The Gynaecological Oncology Biobank at Westmead Path Review Form, along with diagnostic H&Es selected above, a copy of the pathology report and a H&E of the cryosection, will be given to the pathologist.
  - **Diagnostic slides will be used to:**
    1. Review and re-grade the case using the Silverberg criteria.
    2. Pathologist will then select the most suitable section/block that concurs with the pathology report for tissue microarray (TMA). Pathologist then circles two appropriate areas on the slide corresponding to the block to be cored (0.6 mm diameter) for TMA construction.
  - **Cryosection** from frozen tissue specimen will be reviewed for tissue content and concordance with diagnostic Pathology Report



## **TISSUE ARRAY PROTOCOL**

On completion of the pathology review process a mud map is made from each slide.

The selected blocks that correspond to the chosen slides are collected from Anatomical Pathology. When blocks or slides are collected from pathology ensure that you record this in the black research book in the administrative area. Record the Westmead Hospital Accession number eg SW-08012345, the patients name, amount taken, your name, department and extension number.

The block and selected slide are compared and the area on the block that corresponds to the area chosen by the pathologist during the path review is circled.

The blocks that have been chosen are then ready for use in a tissue microarray.

## PATHOLOGY REVIEW

### GRADING OF OVARIAN CARCINOMA (Rosemary Balleine)

Ref: Silverberg S G, Histopathologic grading of ovarian carcinoma: a review and proposal  
International Journal of Gynaecological Pathology (2000) 19: 7-15

#### ARCHITECTURE

- Decide on the histological pattern displayed by the majority of the fields of a given tumour
  1. Predominantly glandular (note: must be true round glands of uniform or variable size)
  2. Papillary (includes slit like spaces)
  3. Solid

#### NUCLEAR GRADE

- Chose area with greatest pleomorphism in half a low power field
  1. Regular uniform vesicular nuclei  
Variation in diameter no more than 2:1  
Low n:c  
No chromatin clumping or prominent nucleoli
  2. Variation in size from 2:1 to 4:1  
Intermediate variation in shape  
Nucleoli recognisable but small  
Some chromatin clumping  
No bizarre cells
  3. Marked variation in nuclear size (>4:1) and shape  
High n:c  
Prominent chromatin clumping  
Thick nuclear membrane  
Large eosinophilic nucleoli  
Bizarre cells often present

#### MITOTIC ACTIVITY

Assess most active area (usually the periphery)

Assess 30 high power fields and nominate highest score per 10 hpf.

Nikon Optiphot microscope with field diameter of 0.663 and field area of 0.345 mm<sup>2</sup>

1. <9
2. 10-24
3. >25

Rosemary Balleine microscope details: Olympus BX51 field diameter of 0.545, area 0.233 mm<sup>2</sup>

Raghwa Sharma microscope details: Olympus BX40, field diameter of 0.545, area 0.233 mm<sup>2</sup>

1. <6
2. 7-16
3. >17

#### FINAL GRADE

Total score    3 to 5 = grade 1  
                     6 to 7 = grade 2  
                     8 or 9 = grade 3

## TISSUE BANK OVARIAN PATHOLOGY SLIDE REVIEW

**PATHOLOGIST** \_\_\_\_\_ **DATE** \_\_\_\_\_

### 1. ANATOMICAL PATHOLOGY DIAGNOSTIC H&E SLIDES

**BLOCK IDs** \_\_\_\_\_

**SLIDE IDs** \_\_\_\_\_

**PATIENT INITIALS** \_\_\_\_\_ **MICROSCOPE USED** \_\_\_\_\_

**TUMOUR TYPE**     Benign     LMP     Malignant

**HISTOLOGY**     Serous     Mucinous     Endometrioid     Clear Cell

Mixed (specify) \_\_\_\_\_

Other (specify) \_\_\_\_\_

**GRADE** (please refer to grading notes)

ARCHITECTURAL PATTERN

NUCLEAR ANAPLASIA

MITOTIC COUNTS

--	--	--

	<b>1</b>	<b>2</b>	<b>3</b>
<input type="checkbox"/> Glandular	<input type="checkbox"/> Papillary	<input type="checkbox"/> Solid	
<input type="checkbox"/> Mild	<input type="checkbox"/> Moderate	<input type="checkbox"/> Severe	
<input type="checkbox"/> ≤6	<input type="checkbox"/> 7-16	<input type="checkbox"/> ≥17	
<input type="checkbox"/> 1 (3-5)	<input type="checkbox"/> 2 (6-7)	<input type="checkbox"/> 3 (8-9)	

OVERALL GRADE

COMMENT (necrosis, vessels, other?) \_\_\_\_\_

**CO-EXISTING OVARIAN PATHOLOGY IN SECTION** (eg adjacent LMP, cystadenoma, endometriosis)

Please specify (including subtype if applicable) \_\_\_\_\_

Does the section appear representative of the lesion as described in the pathology report ?  
 \_\_\_ YES \_\_\_ NO

**COMMENT**

\_\_\_\_\_

### 2. GYN ONC TISSUE BANK H&E CRYOSECTION

**SLIDE ID** \_\_\_\_\_ Tissue Bank Specimen Received \_\_\_\_\_

Does cryosection concur with the path report and above slide? \_\_\_ YES \_\_\_ NO

**TUMOUR COMPONENT**

0 – 25%     25 – 50%     50 – 75%     75 – 100%

**NORMAL TISSUE COMPONENT**

TYPE(S) eg Fallopian tube \_\_\_\_\_

0 – 25%     25 – 50%     50 – 75%     75 – 100%

**PROPORTION NECROTIC**

0 – 25%     25 – 50%     50 – 75%     75 – 100%

**COMMENT**

\_\_\_\_\_

## SELECTION OF BLOCK FOR TISSUE ARRAY CORING

---

**SLIDE ID** \_\_\_\_\_ **PATIENT INITIALS** \_\_\_\_\_

**BLOCK ID** \_\_\_\_\_

Please select two areas for tissue array coring that are representative of the main pathology.  
Please circle selected areas with pen.

Areas selected **YES** \_\_\_\_\_ **NO** \_\_\_\_\_

Biobank Officer will photocopy slide and keep a hard copy in the tissue bank file.

**COMMENTS** \_\_\_\_\_

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## PATHOLOGY REVIEW TRACKING LOG

PATIENT INITIALS \_\_\_\_\_ GYN ONC TUMOUR ID \_\_\_\_\_

GYN ONC TISSUE BANK H&E CRYOSECTION SLIDE ID \_\_\_\_\_

PATHOLOGY REPORT COPIED DATE \_\_\_\_\_ PATH ID \_\_\_\_\_

POTENTIAL BLOCKS FOR SLIDE SELECTION FROM PATH REPORT

\_\_\_\_\_

ID OF 3 BLOCKS/SLIDES SELECTED FOR PATH REVIEW

\_\_\_\_\_

CORRESPONDING H&E SECTIONS OBTAINED FROM ANATOMICAL PATHOLOGY

SLIDE NUMBER	DATE OBTAINED	DATE RETURNED

- SLIDES, PATH REPORT & REVIEW FORM DELIVERED TO PATHOLOGIST  
 PATHOLOGIST NAME \_\_\_\_\_ DATE \_\_\_\_\_
- SLIDES, PATH REPORT & REVIEW FORM RETURNED DATE \_\_\_\_\_
- INFORMATION FROM REGRADING ENTERED INTO DATABASE DATE \_\_\_\_\_
- MUD MAP MADE OF CHOSEN SECTION/SLIDE DATE \_\_\_\_\_
- BLOCK FOR TMA OBTAINED FROM ANATOMICAL PATHOLOGY  
 BLOCK ID \_\_\_\_\_ DATE TAKEN \_\_\_\_\_ DATE RETURNED \_\_\_\_\_
- BLOCK SENT FOR ARRAYING DATE SENT \_\_\_\_\_ DATE RETURNED \_\_\_\_\_

## **DATABASE, DATA ENTRY AND BACK-UP PROCEDURES**

It is critical to the operation of the tissue bank that the database is up to date. As soon as any patient information is received it should be entered into the database. The greater the collection of information for a particular sample, the more valuable it is.

The database is password protected and only accessible by the BBO.

### **DATA ENTRY**

#### **Consent**

When a patient consent form is collected, a patient file is started by opening the database, selecting the "Pathology" Layout, then selecting "New Record" from the "Record" window.

The minimum information that needs to be recorded is:

- Name
- Date of Birth
- Medical Record Number (MRN)
- Select the "Yes" tick box for the Consent field

When this is done, place the consent form in a plastic sleeve and print out a label using the "Label" layout. The form is then placed in the "signed consent forms" folder ready for the additional information that will follow when the patient has surgery.

#### **Sample Collection**

When a sample has been collected (eg tissue, blood, ascites) ensure that the appropriate form has been filled in (see Appendix 2). On return to the lab record the following in the Red Gyn Onc Biobank Book in room 2204:

- Next available Tissue Bank Number (eg 533)
- Name
- Date of Birth
- MRN
- Anatomical site of sample (eg right ovary)
- Sample code (eg 533A)
- Storage location
- Date of surgery and surgeon

If a sample from more than one anatomical site is collected from a single patient then additional letters need to be used, eg 533A ovary, 533B omentum. Blood is recorded using the codes in the Blood Processing protocol on pages 6 & 7.

Using the information in the Red Biobank book and the white "Tissue Collection Form" enter the following information into the "Pathology" Layout in the database:

- Tumour Number
- Tumour Availability eg "Fresh frozen tissue"
- Primary site
- Collection Date
- Age at surgery
- Surgeon

Then go to the "tumour location" layout and fill in the storage location and the time the specimen was collected and frozen.

If Blood was collected, tick "Yes" in the Blood field in the Pathology Layout, then enter specific information such as volume and amount of tubes frozen in the "Blood" layout.

Take the patient's plastic sleeve from the "signed consent forms" folder, add the white "Tissue Collection Form and move it to the "files awaiting path reports" folder.

### **Tumour Board**

Every Wednesday the Gynaecological Oncology Multidisciplinary Team Tumour Board meeting is held in the Anatomical Pathology Department from 8.30 till 10 am.

At the meeting take notes relevant to patients from whom samples have been obtained, in particular, primary site, FIGO stage, largest diameter of residual disease, grade and co-existing pathology.

On return from the meeting enter the information into the "Pathology" Layout in the database:

- Tick "yes" in the "tumour board report" field

- Primary Site

- Stage (eg 3C)

- Grade

- Any other relevant information (such as previous cancer or synchronous tumour) in the "Comments" field.

Then go to the "Clinical Review" layout and fill in the "Residual Disease" field.

Patients that have benign disease will not usually be discussed at tumour board.

### **Pathology Report**

The pathology report is available on the Cerner database about a week after the patient's surgery. Yoke-Eng has access to this database. Alternatively, the pathology report can be photocopied from the patients Gyn Onc file located in the secretary's office. Also copy and file the Operation Report. There is a field in the "Pathology" layout to record this.

Information needed from the Pathology report:

- Tumour Type (eg serous adenocarcinoma)

- Grade

- Silverberg grade (if applicable)

- Coded Nomenclature (ICD-O code)

The pathology report may also contain immunohistochemical staining results. These are to be recorded in the "Tumour markers/Immuno" layout. Occasionally, the report will mention that an additional report will be issued (eg if mismatch repair staining is requested) ensure that this is copied.

## **BACK-UP PROCEDURE**

Every day the server automatically creates a back up file of all the Gyn Onc Filemaker Pro databases. The last 7 days of back up files may be accessed through the server if there is a problem with the current file.