International Study of Embryonal Tumors:
Preparation of biological samples

I. BLOOD SAMPLES

A. Material used:

- Vacutainer tubes using EDTA as an anticoagulant
- Vacutainer tubes using acid-citrate-dextrose (ACD) as an anticoagulant
- 1.8 ml Cryotubes (color coded with red, white and yellow tops)
- pipettes

Note: Each centre should contact IARC if they experience any problems in obtaining either Vacutainer tubes or cryotubes. Heparin may be used only when EDTA is difficult to obtain.

B. Blood extraction:

10ml of blood with EDTA should be obtained. This will be divided into plasma, leucocytes and red blood cells. If the subject is willing to give 2 tubes of blood, the second 10 ml of blood should be collected with ACD tube and be used for viable lymphocyte.

**If 1 tube (10ml) of blood is obtained→ for plasma, buffy coat, RBC

**If >=1 tube of blood is obtained→ 1st tube (10ml with EDTA) for plasma, buffy coat, RBC; 2nd tube (10ml with ACD) for viable lymphocyte; the remaining tubes for plasma, buffy, and RBC.

C. (1) Blood processing for buffy coat, RBC and plasma (EDTA tube)

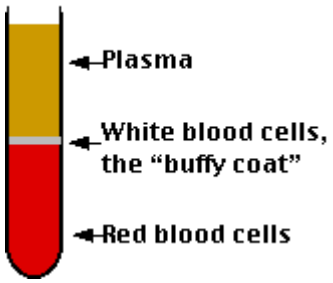
Blood samples can stay at room temperature for a maximum of 12 hours before being processed in a laboratory.

From 8-10 ml of blood with EDTA the following should be obtained :

- 3-6 1.8ml cryotubes of plasma
- 2-3 cryotubes of leukocytes
- 2-3 cryotubes with red blood cells

Blood will be processed and frozen under the following protocol:

The blood with EDTA is centrifuged for 10 minutes at 2000 rpm. We get 3 layers (plasma, leucocytes, and red blood cells):



- All of the plasma (about 50%-60% of sample) is extracted and distributed into 3-6 different cyrotubes (**Yellow top**).
- After the plasma has been removed collect the top 2ml of the remaining specimen making sure that the buffy coat (white layer on top of the red blood cells. *Note that this layer is sometimes hard to visualize*) is included.
  To make sure that the contents in each cryotube is homogeneous, take the buffy coat into 1 tube, invert the tube 2-3 times to make it homogenous, then divide into 2 cryotubes. Identify these tubes with a **white top**.
- Finally, the remaining red blood cells are extracted and put into 2 - 3 cryotubes with a **red top.**

*Each tube will be labeled and coded with the subjects full identification number. This number will be protect using transparent tape..

C (2) Blood processing for viable lymphocyte (ACD tube)
Note: Simple processing should be batched to limit the time between addition of DMSO to initiation of freeze to 35 minutes.

I. Whole blood should be mixed by inversion and then transferred to a 50ml tube on ice.

II. The volume of blood should be noted and sterile DMSO should be added to a final concentration of 10%.

III. The sample and DMSO should be mixed by inversion and then aliquoted into 1.8ml cryotubes **(brown top).**

IV. These cryotubes should then be placed in a controlled-rate freezer (Mr. Frosty) until frozen to -80ºC and then transferred to a regular -80ºC freezer.

V. Ship the frozen sample to IARC.

D. Sample storage:
Samples will be stored at -70ºC. If possible they will be equally distributed between different freezers in order to avoid the loss of material in case of any problem with one of the freezers.

E. Record keeping
Make a record of the number of samples available on the case/control logsheet.

II. SALIVA COLLECTION PROCEDURE

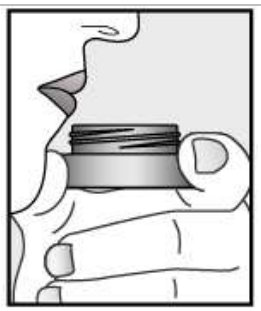
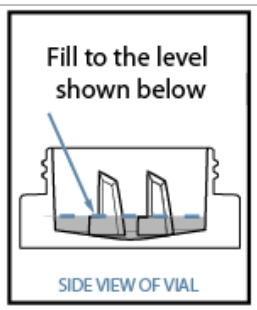
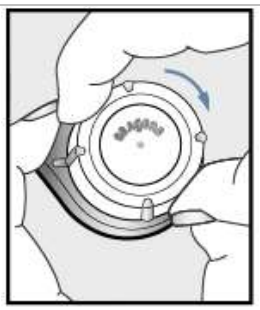
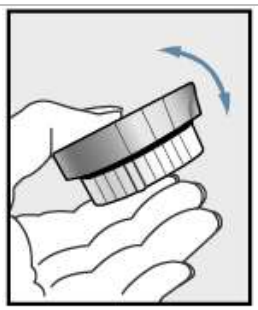
Saliva will be collected when subjects do not wish to provide blood samples.

A. <u>For Adult Subjects</u>

a. Material used:

- 1 Oragene saliva collection vial in plastic package
- Barcode labels

***b.* Collection procedure:**

			
Step 1	**Step 2**	**Step 3**	**Step 4**
Rinse mouth with water and wait at least 30 seconds. Put ¼ tsp sugar on your tongue. Spit your saliva into blue container.	Spit until the amount of liquid saliva (not foam) reaches the level shown in **picture #2.**	Cap the blue container with the white lid.	Tighten firmly and mix gently.

Step 5. Label the container with preprinted barcode labels

Contents: Each collection vial contains 2ml of Oragene liquid. Before use, the solution in the cap should be clear and colorless.

Warnings: Wash with water if the Oragene liquid comes in contact with eyes or skin. Do not ingest.

Oragene Storage: Store at room temperature 15-30˚C (59-86˚F) before DNA extraction.

<u>B. For Infants and Children</u>

a, Materials used:

- 1 Oragene saliva collection vial in plastic package
- 5 saliva collection sponges with scissors
- Barcode labels

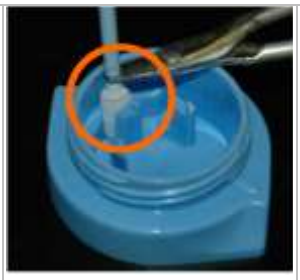
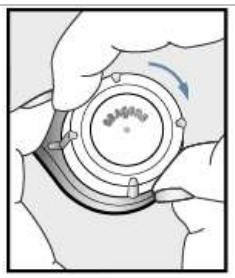
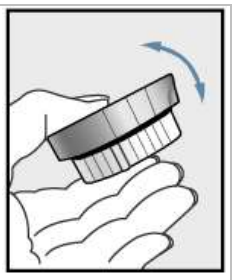
b. Collection procedures:

Before collection:

- Caution should always be used when inserting anything into a child's mouth.

- Do not rinse or brush for at least 10 minutes before collection. If your child is nursing, wait 15-20 min after feeding before collection.
- Some children find that the saliva sponge tickles their gums.
- Try not to rub the sponge directly on the child's teeth to minimize collection of bacteria.
- A maximum of 5 sponges should be used per person.

Collection:

				
Step 1	**Step 2**	**Step 3**	**Step 4**	**Step 5**
Dip the sponge in sugar and place in the child's mouth between the gums and the inner cheek. Gently move the sponge around the upper and lower cheek for at least 30 seconds to soak up as much saliva as possible. There is no need to scrape the cheek.	*Next, place the sponge firmly into the blue container, between the 'teeth' and inner wall, as shown in the **picture above.** Using the scissors provided, carefully cut the narrow part of the handle just above the sponge. Discard the handle.*	*Repeat Steps 1 and 2 for as many of the remaining sponges as possible, up to a total of 5. It helps to wait 3 minutes after each sponge.*	*When finished collecting sponges, cap the blue container with the white lid.*	*Tighten firmly and mix gently.*

Step 6. Label the container with preprinted barcode labels

Contents: Each collection vial contains 2ml of Oragene liquid. Before use, the solution in the cap should be clear and colorless.

Warnings: Wash with water if the Oragene liquid comes in contact with eyes or skin. Do not ingest.

Oragene Storage: Store at room temperature 15-30˚C (59-86˚F) before DNA extraction.

III: FRESH TISSUE SAMPLES

Because collection of fresh tissue requires a high level of coordination with surgeons it will only be feasible in some centers.

A. Material used
Store in a cryotube or plastic bags

B. Sample collection
Obtain at least 0.5g of fresh tumor tissue from biopsy or surgery and place it in a cryotube or small sterile plastic bag. It would be preferable to snap freeze the sample in liquid nitrogen. Close the tube or bag and write the full identification number on it. Cover the identification number with transparent tape to protect it.

C. Sample storage
If sample collection takes place in an area far from the freezer use small "cold bags" to keep the specimens cool.
If the samples have not been snap frozen, place them in an ice-bath prior to freezing. Store the specimens at -70^{o}C if possible until enough samples have been collected for shipment.
Move the samples to the freezer the same day they are obtained.

D. Record keeping
Record the availability of fresh tissue on the case log sheet. Indicate whether the samples are snap frozen on the case log sheet. If the samples are not snap frozen, indicate approximately how long it has been chilled on ice before it was frozen.

IV. PARAFFIN EMBEDDED TUMOR TISSUE SAMPLES
Note: this work will not be conducted immediately but when appropriate funds have been obtained. The main priority is to ensure that pathologists obtain and preserve tumour tissue on as many cases as possible.

Types of samples
The best fixation solution for the preservation of DNA is ethanol. If not possible then buffered formalin is accepted but the fixation time should not exceed 24 hours, and be preferably no more than six hours.

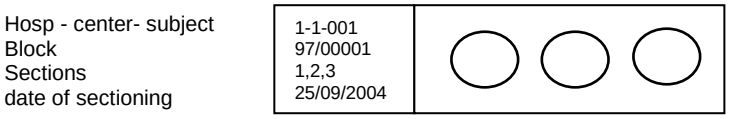
The samples can be cut either at IARC or within each centre. To ensure a comparable standard they should be cut using the following protocol.

A. Amount of tissue
40 sections of 5μ thickness for the molecular analysis,
15 sections of 5μ thickness for the immunohistochemical analysis.

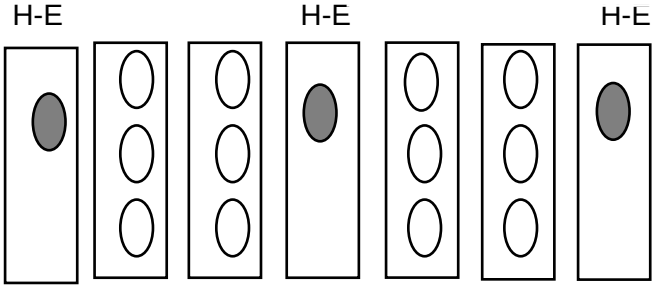
B. Material used
- glass slides and coverslips,
- poly-L-Lysine treated slides,
- microtome and disposable blades,
- haematoxylin-eosin dye,
- slide boxes,
- copy of the pathological diagnosis report.

C. Sample collection and preparation

1. Identify <u>all</u> paraffin-embedded blocks -histology and cytology- related to the disease of interest. Priorities are:
    1. primary tumoral tissue
    2. metastatic tumoral tissue
    3. normal tissue
    4. tissue with preneoplastic lesions
2. In order to obtain homogeneous sections, shave the block until there is a straight phase then start sectioning.
3. Cut 30 consecutive sections, place them on glass slides, and label them with the hospital, center, and subject code numbers, the block code, and the section number, date of sectioning:



4. With the exception of the 1st, 8th, and 15th sections that will be used for pathological verification, multiple sections can be placed on one glass slide, depending on the surface of the sample, as shown in the figure:



5. Stain control sections (1, 8, and 15) with hematoxylin-eosin.
6. Cut 15 additional sections (3 μm thick) and place each one of them in an individual treated glass slide for the immunohistochemical analyses.
7. one H-E control staining (section 8) in the last 15 sections to confirm that tumor is still being cut.
8. Change the blade before cutting a new block to avoid inter-block contamination.

D. Sample storage

1. Store slides from each block in a separate box.
2. Label every box with the hospital, center, subject, and block code numbers.
3. Keep the boxes at 4°C, otherwise, store in a safe cupboard placed in a cool area.