1. Scope and Application

This manual is applicable for collection, handling, and logistics of samples for tests provided at CellCarta. The procedures described here are considered as advisable and most optimal.

2. Responsibilities

It is the responsibility of the section head of the Biosample Management unit to keep this procedure up to date and to inform stakeholders on changes made to this procedure.

3. Abbreviations, Definitions

BE : Belgium	NBF : Neutral Buffered Formalin
CSU : Cold Storage Unit	RCF : Relative Centrifugal Force
FFPE : Formalin Fixed Paraffin Embedded	rpm : Revolutions per minute
g : Gram	RT : Room Temperature
IHC : Immunohistochemistry	TAT : Turnaround Time
ml : Milliliter	VIP-6 : Vacuum Infiltration Processor

4. Sample Collection and submission

4.1. Fresh Tissue Biopsy

Before initiation of sample collection, fixative and fixation containers should be at hand. NBF (Neutral Buffered Formaldehyde 4% / Neutral buffered formalin 10%, pH 6,8-7,2) can be purchased (e.g. VWR cat. no. FOR0150AF59001) or prepared in house if not provided by a collection kit supplier. For in house preparation of 4% neutral buffered formaldehyde for histology, the following is suggested:

Product	**Amount**
Formaldehyde (40% w/v)	100 mL
Sodiumdihydrogenphosphate*H2O	4.0 g
Disodiumhydrogenphosphate	6.5g
Distilled water	Fill up to 1L solution

It is advised to collect and ship fresh samples at cold in 10% NBF. The shipment of samples in cold formalin can span up to one week without an adverse effect on sample quality (Bauer, D. et al., 2018). Ship fresh samples so that they are on Fridays received at CellCarta before noon. If needed, place collected biopsies in cold formalin for shipment on Monday.

Follow your institution’s guidelines and procedures to procure tumor tissue biopsy samples. The general rule accepted for optimal fixation is to prepare a minimum fixative volume of 10 times the biopsy/specimen volume and to use appropriate container sizes. After biopsy, immediately place the sample into a labeled (preferably cold) 10% NBF vial for tissue fixation. Any delays in fixation can compromise the specimen quality. The time to fixation is preferably shorter than 5 minutes. Record the start of fixation.

For small, e.g. core needle, biopsies, the use of a Cell Safe Biopsy Capsule (e.g. Fisher Scientific, cat. no. 22-500-463)is advised. The use of bio wrap or sponges is not advised since tissue tends to stick to it and may break upon removal. For core needle biopsy, use of an 18 gauge needle is advised but 14-21 gauges needles can be used as well. Fine needle aspirates, smaller than 14 gauge, are also

processed and classified as “needle” biopsy at CellCarta. For resections, the specimens should be cut into slices with a maximal thickness of 5mm, and a maximal size of 1.8cm x 3cm because each slice needs to be well-fixed and mountable onto individual histocassettes.

Figure 1: Histocassette with Cell Safe Biopsy Capsule for small biopsies

Bone marrow biopsies can also be shipped in 10% NBF to CellCarta for further tissue processing. For bone samples requiring decalcification, CellCarta performs optimized decalcification upon receipt of the fresh tissue biopsy, using a soft decalcifier.

Re-secure the vial lid to assure that the specimen has been immersed into the 10% NBF and that it has not adhered to the lid or sides of the vial (if not placed into a cassette). A slight swirling motion with the lid will confirm immersion of the specimen and absence of leaks in the container. When shipping fresh samples in a vial (with 10% NBF) use Parafilm Wrap to further secure the lid of the vial, by placing the Parafilm over the lid and vial (i.e. do not place the Parafilm under the lid).

Specimen ID refers to the unique identifier of the fresh sample. Fill out the Specimen ID on the label of the vial using a black or blue ballpoint pen. Enter the sample information on the Requisition Form being sent with the sample. Make sure the specimen ID on the vial matches exactly with the information entered on the requisition form. It is critical to follow given instructions to enter each field correctly or the sample may not be accepted and sample processing may be delayed.

A long-sleeved lab coat, gloves, face mask and safety goggles should be worn when handling human tissue or laboratory specimens for both biosafety precautions and prevention of contamination of the specimens. Clean the grossing board with nuclease wipe or HAC solution. Disposal of materials used in samples collection should be performed according to Good Laboratory Practices.

Upon arrival at CellCarta, the sample’s fixation duration will be checked, and an optimal post-fixation protocol will be carried out. At CellCarta, biopsies/specimens are classified into three classes: Resection, Punch and Needle. The size of the sample will determine the type of tissue processing program that is selected for further tissue processing. The tissue (optimally) fixed in 10% NBF will be processed using Tissue-TEK VIP-6 Vacuum Infiltration Processor. The tissue is dehydrated in a series of alcohols and xylene, followed by infiltration of melted paraffin at no more than 60°C (± 2°C). The in-house method used for different tissue sizes on the Sakura VIP-6 tissue processor is shown in Table 1.

				Needle		Punch		Resection	
Station	Solution	P/V	Mix	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)
1	Ethanol 70%	P/V	OFF	0:05	37	0:10	39	0:10	39
2	Ethanol 80%	P/V	SLOW	0:30	37	1:00	39	1:00	39
3	Ethanol 90%	P/V	SLOW	0:30	37	1:00	39	1:00	39
4	Ethanol 100%	P/V	SLOW	0:30	37	1:00	39	1:00	39
5	Ethanol 100%	P/V	SLOW	0:30	37	1:00	39	1:00	39
6	Ethanol 100%	P/V	SLOW	0:30	37	1:00	39	1:00	39
7	Ethanol 100%	P/V	SLOW	0:30	37	1:00	39	2:00	39
8	Xylene	P/V	SLOW	0:30	37	0:45	39	1:00	39
9	Xylene	P/V	SLOW	0:30	37	0:45	39	1:00	39
10	Xylene	P/V	SLOW	0:30	37	1:00	39	1:30	39
11	Paraffin	P/V	SLOW	0:30	58	1:30	58	1:00	58
12	Paraffin	P/V	SLOW	0:30	58	1:30	58	1:00	58
13	Paraffin	P/V	SLOW	0:30	58	1:30	58	2:00	58
Total Time				6h5min		13h 10min		14h 40min	

Table 1: Needle, Punch and Resection CellCarta VIP-6 Programs

4.2 FFPE Tumor Block

Fresh biopsies, with a documented histopathological diagnosis of interest, containing a representative sample of the tumor, if applicable, can be processed into a FFPE block if the tissue was sufficiently fixed and site has the necessary capacity and infrastructure. Follow the site's procedures to process these fresh samples. The block must be fixed in formalin (preferably 10% NBF, e.g. VWR cat. No. FOR0150AF59001); alcohol based fixatives are not acceptable. The recommended fixation time is 24-48 hours at RT in formalin. Tissue should not be fixed for less than 24 hours or more than 48 hours in formalin. For bone samples requiring decalcification, use of a soft (e.g. EDTA-based) decalcifier is advised. While mineral acids offer a faster decalcification rate, rapidly processed specimens are not optimal for molecular studies.

Ideally, the tissue (optimally) fixed in 10% NBF is processed using a Tissue-TEK VIP-6 Vacuum Infiltration Processor as described above. The tissue is dehydrated in a series of alcohols and xylol (Xylene), followed by infiltration of melted paraffin at no more than 60°C (+- 2). The in-house CellCarta method for different tissue sizes on the Sakura VIP-6 processor is shown in table 1. There might be deviations to the beforementioned protocol. In this case, a comment should be mentioned on the Oncology Request Form for diagnostic samples.

If sending a core biopsy, preferably 3-5 cores are aligned and embedded into a single block. The surface area preferably reaches at least 25mm2 and the sample volume at least 1 mm3 with a total depth of at least 40 microns. Ideally, the tumor sample contains at least 30,000 cells but preferably 75,000 to 150,000 cells with at least 80% of cells being nucleated and at least 20% of cells being malignant.

The paraffin embedded tissue should be fully surrounded by paraffin, firmly mounted onto a cassette fitting into a microtome cassette clamp. No air bubbles should be present between tissue and paraffin or in the paraffin in general. The paraffin/tissue should not show ruptures, splits or cracks and the tissue should not erupt from the paraffin or be collapsed. The paraffin block thickness ideally reaches minimally 0.5cm and maximally 1cm to be easily cut on the microtome. If one of the criteria described above is not met, the paraffin block can be melted and re-embedded according to the CellCarta procedure. The above criteria are however not imperative/binding. If the laboratory technologist who performs the sectioning notices any deviation (not mentioned above) regarding the quality of the paraffin block, the SH or HLT of the CellCarta Preanaltyics unit are consulted to decide whether re-

embedding is advisory. If re-embedding is performed, this is documented in pre- and post-embedding photographs.

When multiple blocks are submitted for a given patient of which only one needs to be tested, preferably one section is created from each block, HE stained and evaluated by a certified CellCarta pathologist for selection of the best block for further testing.

Specimen ID refers to the unique identifier of the block. If there is no identifier on the block, label the block with a unique Specimen ID (for example, 001-023) specifically assigned for the Study. The bag that holds the block should be labeled with the same Specimen ID. The specimen ID on the block must match the information entered on the Requisition form being sent with the sample. It is critical to follow given instructions to enter each field correctly or the sample may not be accepted and sample processing may be delayed.

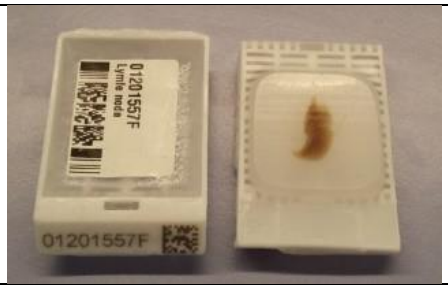

Figure 2: FFPE tumor block in labelled cassette

Preferably the block is handled according to the method and timelines mentioned above, however, deviations will be accepted. In this case, a comment should be mentioned on the Oncology Request Form for diagnostic samples.

4.3 Slides of FFPE Tissue

If a paraffin embedded tissue cannot be submitted, unstained and freshly cut slides may be submitted (number of slides as specified per test) serially sectioned from an FFPE tissue. The sections should be 4 ± 1 µm thick (or as specified per test) and cut on a microtome. Serial sectioning and corresponding labeling is advised. If paper labels are utilized, they must be xylene-resistant. If the slides are marked by writing directly on the slide, a xylene resistant marker or pencil must be used. Only one section should be collected per glass slide. The date of sectioning of the unstained slides should be documented. If the slides are used for molecular analysis, nuclease-free sectioning is required. All materials used for sectioning are cleaned between every sample, this includes the microtome (i.e. knife holder and handwheel), work surfaces, water bath, petri dish, spatula, lancet, labels and slide boxes. Water used should also be replaced by nuclease-free water. A long-sleeved lab coat and gloves should be worn during sectioning.

Specimen ID refers to the unique identifier on each slide. If there is no identifier on the slides, label each slide with a unique Specimen ID (for example, 001-023-001, 001-023-002,…) specifically assigned for the Study. Place the slides, sequentially as sectioned, in a slide mailer and close the mailer securely. The slide mailer that holds the sections should be labeled with the Specimen ID. The specimen ID on the slide mailer and on the sections must match the information entered on the Requisition form being sent with the sample. It is critical to follow given instructions to enter each field correctly or the sample may not be accepted and sample processing may be delayed.

Preferably use Superfrost Plus coated glass slides (VWR, cat.no. J1800AMNZ). If not available, other glass slides may be used, but make sure that these slides have been treated in order to provide a positively charged glass surface as this is necessary for proper adhesion of the tissue section. Pre-label the frosted area with a unique identification code. When small specimens (needle-core and punch) are to be collected on the glass slides or analytical testing involves fluorescent immunostaining, SUPERFROST PLUS GOLD slides (VWR, cat. no. K5800AMNZ72) are advised.

Figure 3: Glass Slide Types

Collect sections in a petri dish filled with water at ambient and float the sections one by one on a water bath at 48°C ±2°C keeping track of the order in which the sections were cut. The petri dish and water bath should contain distilled water without gelatin or other protein-rich products, as the latter damage the adhesive coating of the coated glass slides.

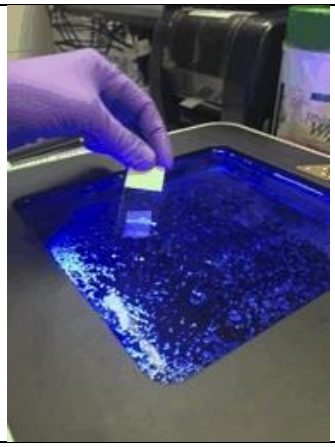

Figure 4: Tissue Section in a Water Bath

Mount each section on the coated side of an appropriately labelled slide ensuring that the Specimen ID from the block and the Specimen ID on the slide label match. Mount the section at the opposite end to the frosted area, about 1/3 from the edge. For larger specimens mount the sample from this point towards the frosted area (in the direction of the arrow), see diagram for illustration. For small specimens, it is advised to always collect consecutive sections on consecutively labelled microscope slides and not to mount a series of sections on one glass.

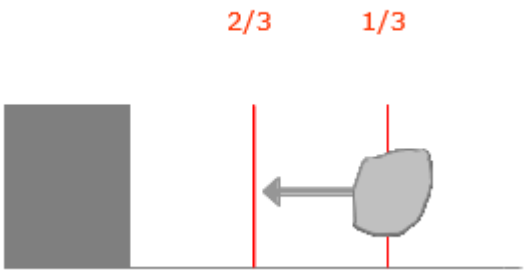

Figure 5: Diagram Illustrating Mounting a tissue section on a slide

Place the glass slide with mounted tissue section between two pre-wetted filter papers (Warning: sections can stick to the filter paper when too wet/dry) and allow sections to attach to the glass slide by gently pressing and rolling your fist over the filter paper. Place microscope slides in an appropriately labelled slide box.

Preferably, do not bake slides prior to the shipment. If needed, place slide racks/boxes in the oven as requested in the appropriate procedures or study protocol. CellCarta' standard recommendations are to bake at 60°C ± 2°C with ventilation (no humidification) during 0.5-2 hours depending on the staining applications.

Deviations will be accepted. In this case, a comment should be mentioned on the Oncology Request Form for diagnostic samples. For samples submitted for clinical trial testing, a comment should be included in the document that accompanies the sample upon shipment to CellCarta. The type of the document accompanying the shipment should be specified in the work order.

4.4 Liquid Biopsies

4.4.1 Blood

Blood collection must only be performed by phlebotomy trained personnel. Before sample collection initiation, purpose-specific collection tubes, as specified per test, should be at hand. Always refer to the study specific lab manual. Blood stability times, i.e. time between blood collection and processing at CellCarta, is for some tubes/purposes limited. Therefore, it is advised to ship for receipt at CellCarta by Friday noon at the latest. Place tubes in the appropriate storage condition, as specified per test, for shipment on the next business day, taking care to remain within the sample stability.

4.4.2 Plasma

Plasma isolated from whole blood may also be submitted for testing at CellCarta. Below are two suggested protocols for plasma isolation from whole blood.

•Starting from blood collected in Cell-Free DNA BCT Streck Tubes:

- Gently invert the tube 10 times after blood collection.
- Centrifuge at room temperature at 800 x g (RCF) for 20 minutes.
- Note: If centrifuge uses rpm (revolutions per minute) refer to the centrifuge manual for conversion to RCF.
- Transfer the top plasma layer carefully, to approximately 5mm above the buffy coat without disturbing the buffy coat, into a labeled centrifuge tube.
- Centrifuge, preferably in a pre-cooled centrifuge at 4°C, at 5250 x g (RCF) for 27 minutes to remove residual cells.

- Aliquot the plasma into appropriately labeled cryovials (e.g. Corning, cat. No. 430662).
- Discard the tube containing any residual cell pellets.
- Freeze plasma at <-70°C.

•Starting from blood collected in 10mL K2 EDTA tubes (Becton Dickinson, cat. No. 36725 or equivalent):

- Gently invert the tube 10 times after blood collection.
- Centrifuge at room temperature at 1600 x g (RCF) for 10 minutes.
- Note: If centrifuge uses rpm (revolutions per minute) refer to the centrifuge manual for conversion to RCF.
- Transfer the top plasma layer carefully, to approximately 5mm above the buffy coat without disturbing the buffy coat, into a labeled centrifuge tube.
- Centrifuge at 3000 x g (RCF) for 10 minutes to remove residual cells.
- Aliquot the plasma into appropriately labeled cryovials (e.g. Corning, cat. No. 430662).
- Discard the tube containing any residual cell pellets.
- Freeze plasma at <-70°C.

Note: Downstream analyses (e.g. PCR) may be affected by hemolysis of the sample (> 100mg/dL Hgb, Figure 6).

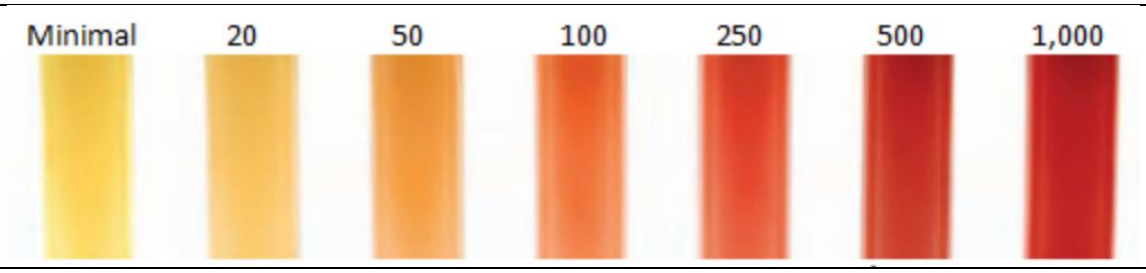

Figure 6: Approximate hemoglobin concentration in mg/dL

Ship plasma frozen to CellCarta.

4.5 Completion of Oncology Request Form

Submission of samples for diagnostic testing must always be accompanied with the Oncology Request Form. Guidelines for completion of this form can be found in Instructions for completion of Oncology Request Form. As stated in these guidelines, primary samples lacking proper identification shall not be accepted or processed by the laboratory. In addition, verbal requests for sample examination will not be accepted. Only tests requested in the Oncology Request form will be performed. Additional tests can only be performed if an additional Oncology Request Form is submitted. Retesting of samples can be done without submission of a new Oncology Request form only if the initial test failed due to analytical problems.

4.6 Logistics

Both tissue blocks and slides can be shipped at room temperature, however, shipment may also be done between 2-8°C. Fresh tissue biopsies are preferably shipped at cold in 10% formalin. Blood shipment temperature is purpose dependent, as specified per test. Plasma is to be shipped frozen.

Preferably, place the specimens (fresh biopsy vial, FFPE block or slide mailer) into a zip lock biohazard bag with gel wrap or other appropriate protection (e.g., absorbance pads, paper towels, etc.) and ensure the bag is securely sealed. For fresh biopsy specimens in Formalin at cold temperature, ship overnight with cooling packs. For diagnostic testing, the shipped samples must always be accompanied with the correctly completed Oncology Request Form. If not, samples cannot be accepted. Ship specimens to the addresses listed below.

CellCarta
Attn. Sample Reception Team – Pxxx
Sint-Bavostraat 78
2610 Wilrijk
Belgium, Europe
Email: trials@histogenex.com
Tel: +32 3 502 0620

4.7 Sample Receipt

Shipments can be sent for receipt at CellCarta on Monday through Friday between the hours of 07:30 – 16:30. For those samples that arrive after 14.00 hrs, accessioning will be initiated the day after delivery. **Ship fresh samples and liquid biopsies so that they are received at CellCarta before noon on Fridays.**

Samples that do not meet the acceptance criteria set in this work instruction can not be accepted for processing and testing in the laboratory. However, if compromised samples are accepted, this should be clearly mentioned in the sample reception log and the final report. Furthermore, caution should be taken while interpreting the results.

Samples will be processed according to fixed turnaround times as specified per test.

Updated on 11 June 2019