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**Revision History:**

<b>Version</b>	<b>Description</b>	<b>Review Date</b>
<b>1.0</b>	<b>Initial SOP</b>	<b>06/07/2017</b>
<b>1.1</b>	<b>Revised SOP</b>	<b>12/22/2017</b>
<b>1.2</b>	<b>Revised SOP</b>	<b>03/26/2018</b>
<b>2.0</b>	<b>Revised SOP</b>	<b>09/19/2018</b>
<b>2.1</b>	<b>Revised SOP</b>	<b>05/09/2019</b>

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## Purpose

This document provides the standard procedural guidelines for preparing for biospecimen collection from patients with NF1, distribution of tumor samples, generation of cell line and xenograft models, and preservation of tissue and blood fractions. All procedures described herein are in accordance with guidelines from the *NCI Best Practices for Biospecimen Resources*, v. March 2016.

## Scope

This standard operating procedure (SOP) applies to all specimens collected from NF1 patients having surgery for removal of tumors [including, but not limited to, cutaneous neurofibromas, plexiform neurofibromas (PN), malignant peripheral nerve sheath tumors (MPNST), GIST, breast, and gliomas]. Tumors will be collected, cryopreserved and made available for future research.

### 1. Authority and Responsibility


The Director/ Principal Investigator (or his/her designee), co-Principal Investigator, and the Research Specialist have the authority to establish this procedure. The Research Specialist is responsible for the implementation of this procedure and for ensuring that all personnel are appropriately trained. All personnel working within Dr. Christine Pratilas' laboratory are responsible for reading and understanding this SOP prior to performing the procedures described.

### 2. Introduction

**Processing:** Sample processing must be done in a clean, certified biosafety cabinet at all times.

Pipettes, pipet tips, conical vials, and cryovials used must be sterile. All sample processing must be done quickly, methodically, and in a sterile fashion.

**Storage:** All specimens must be stored in barcoded vials at  $-80^{\circ}\text{C}$  (flash frozen tissues) and at  $-180^{\circ}\text{C}$  (viably frozen tissues) according to currently recommended best practices for long-term storage. Freezers are monitored 24/7 by an alarm system.

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### **3. Definitions**

- 3.1 CTF = Children’s Tumor Foundation  
 CRF = Case report form  
 ICF = informed consent form  
 PN = plexiform neurofibroma  
 MPNST = malignant peripheral nerve sheath tumor

### **4. References**


- 4.1 10x Genomics Sample Preparation Demonstrated Protocol Rev B (CG000136)  
 4.2 10x Genomics Procedure for Dead Cell Removal and Washing (CG000093)  
 4.3 Case Report Form (CRF) for the “Nerve Sheath Tumor Bank from Patients with NF1”

### **5. Reagents and Materials**

- 5.1 RPMI cell growth medium (Gibco 11875-093)  
 5.2 Fetal Bovine Serum (Gemini Bioproducts 100-106)  
 5.3 Penicillin/Streptomycin (Gibco 15140-122)  
 5.4 Matrigel (Corning: CB-40230)  
 5.5 Ketamine (Controlled substance, RX only)  
 5.6 Xylazine 100mg/mL (MP Biomedicals MP215830701)  
 5.7 3M Vetbond (3M 1469SB)  
 5.8 10ml EDTA tubes (BD 367841)  
 5.9 6ml additive free red top tube (BD 367815)  
 5.10 Cool Cell (Corning 432001)  
 5.11 Tissue Culture Flask (Falcon 353136)  
 5.12 NOD SCID gamma (NSG) immunodeficient mice  
 5.13 Cell Freezing Medium (Sigma C6295)  
 5.14 Harvest medium (100ml of FBS and 10ml of pen-strep/glutamine added to a 500ml bottle of RPMI, expires 30 days from the date of creation)

### **6. Instrumentation**

- 6.1 Centrifuge  
 6.2 Biosafety cabinet


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### 6.3 gentleMACs Dissociator

## 7. Protocol

### 7.1 Sample Acquisition

- 7.1.1** The lead technician will review operating room (OR) schedules in advance to identify patients diagnosed with Neurofibromatosis Type 1 (NF1) who are scheduled for surgical removal of an NF1-associated tumor.
- 7.1.2** Once a patient is identified, the technician will contact the patient via phone to obtain verbal consent prior to the day of surgery.
- 7.1.3** The patient will sign a hard copy of the consent form (ICF) prior to surgery.
- 7.1.4** A patient identification number will be assigned, with candidates numbered sequentially in the format of a leading “2”, followed by a three-digit number starting with “001”, (eg. “2-001”).
- 7.1.5** The technician will contact the nurse in pre-op prior to surgery and request that 2 x 10ml EDTA tubes, 1 x 6ml red top be drawn either in pre-op or under anesthesia.
- 7.1.6** Once the blood specimens are available, the nurse will contact the technician for pickup of the specimens.
- 7.1.7** Pathology will receive the tumor specimen first and create H&E stained slides and save sufficient specimen for diagnostics. Dr. Fausto Rodriguez (or, in his absence, an appropriate designee) will then review any remaining tissue and provide pieces from multiple locations of the tumor whenever possible for banking.
- 7.1.8** When the tissue is picked up, the patient name for the sample received must be confirmed against the patient name for the expected surgery. Other information needed from pathology will be the anatomical location of the sample, and gross pathologic diagnosis of the sample.

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**7.1.9** Tissue will be maintained in a sterile collection container containing harvest media until brought to the lab for processing.

## **7.2** Specimen Handling and Processing

**7.2.1** Blood samples will be handled as follows:

**7.2.1.1** Two 10ml EDTA and one 6ml red top tubes will be processed by centrifuging the sample at 1500 x g for 15 minutes at room temperature.

**7.2.1.2** Plasma will be aliquoted into microcentrifuge tubes at 1,050µl. The microcentrifuge tubes will be spun again at 14,000RPM, for 10 minutes, at 4°C.

**7.2.1.3** The resulting ultra-pure plasma will be aliquoted into 5 x 1ml aliquots and 2-3 aliquots of buffy coat with ~500µL of buffy in each cryovial.


**7.2.1.4** Serum will be aliquoted into 3 vials at 750µL.

**7.2.1.5** These cryovials will be labeled appropriately prior to any specimen being aliquoted into them and frozen immediately at -80°C. Freeze time will be documented.

**7.2.2** The heterogeneity of the sample must be assessed and some of each histology/ tissue type for the tumor will be saved, where possible.

**7.2.2.1** For tissues that are not completely homogenous the tumor must be saved and identifier must be recorded on the cryovials of tissue to denote different types of tissue (i.e. nodular beige tissue may be denoted as “beige”, soft purple tissue containing blood vessels denoted with the term “purple portion” etc.).

**7.2.3** Tissue for the NTAP Biobank must first have tissue saved snap frozen, then viably frozen, and finally formalin fixed.

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**7.2.4** The tissue pieces to be flash frozen will be cut into 4mm pieces at the smallest (depending on total amount of tissue received), and tissue pieces must be placed into cryovials, and the cryovials placed on dry ice. The aliquots will be labeled with tumor/tissue type, patient ID, date, and preservation method prior to adding tumor to the vial.

**7.2.5** Other tissue for dissociation (cell line creation and viably frozen single cell suspension), xenografting and for formalin fixation will be maintained in 15ml conicals of stock media on ice, in the biosafety cabinet, until after the procedure in step 7.2.9 is completed.

**7.2.6** Another priority will be to establish a xenograft. Only laboratory members trained in animal handling and experienced in xenografting must perform this portion of the procedure. Those who are not familiar with animal handling must request a trained laboratory member to perform the procedures described in this section and skip ahead to continue the procedures noted in section 7.2.8.


**7.2.6.1** A tumor chunk will be cut into 2-3mm diameter pieces for implantation into the mouse flank (5-7 pieces for xenografting is ideal).

**7.2.6.2** Tumor pieces will be maintained on ice and an aliquot of Matrigel (also maintained on ice) will be taken to the mouse room G20 in the basement of CRB I.

**7.2.6.3** Two to ten NSG mice will be selected from a cage of unused mice in room G20 in CRB I.

**7.2.6.4** The mice will be anesthetized by injecting the animal's intraperitoneal (IP) space with 200 $\mu$ L of the ketamine/xylazine mix, which is maintained in the locker in room 262.

**7.2.6.5** At least two mice (and up to ten) will have tumor fragments placed subcutaneously on the flank.

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**7.2.7** Representative tumor fragments will be placed into vials of formalin for the bank.

**7.2.8** Sixty to seventy-five percent of the provided tumor fragments will be cut into 2-4mm pieces and aliquoted into barcoded cryovials. These tissues will be snap frozen immediately on liquid nitrogen or a dry ice/ethanol bath and placed in the -80°C freezer. Freeze time will be documented.

**7.2.9** A single 5-10mm tumor fragment will be saved for dissociation and the creation of a cell line (**for MPNST and PN tumors only**).

**7.2.9.1** The gentleMACS Dissociator will be used in tandem with the Tumor Dissociation Kit (Miltenyi) with the “SCS” program selected to create a viable single cell suspension.


**7.2.9.2** The isolated cells will be washed in 10ml of RPMI.

**7.2.9.3** Red blood cells will be removed from the suspension by adding 1ml of chilled 1x Red Blood Cell Removal Solution and then incubating for 10 minutes at 4°C.

**7.2.9.4** Lysis buffer will be washed off of the cell pellet using 10ml of cold Wash Buffer and centrifuging the specimen at 300 x g for 10 minutes at 4°C.

**7.2.9.5** Cells will be counted and checked for viability. If the viable percentage is below 70%, the 10X procedure for Dead Cell Removal and Washing will be followed.

**7.2.10** Two 4mm pieces of each tumor core will be placed into tumor freezing media. The aliquots will be labeled with tumor/tissue type, patient ID, date, and preservation method prior to adding any tumor to the vial. The samples must then be placed into a “CoolCell” rate-controlled freezing chamber and frozen at -80°C. After 24 hours, these aliquots will be transferred to liquid nitrogen for long-term storage.

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- 7.2.11** Any lab personnel substituting for the lead technician must place all frozen samples into the freezer into the Temp Box in the Prtilas lab freezer. The lead technician will then sort, add specimens to the clinical database, and box them appropriately.
- 7.2.12** All specimens will then be logged into the “NTAP/ JHU Banked Specimens” database with their respective freezer locations and sample information.
- 7.2.13** Specimens will also be annotated in a Microsoft Access database by collecting the data listed on the NF1 Bank CRF curated from the patient’s medical records.
- 7.2.14** MPNST cell lines and xenografts created from these banking efforts will be shipped in batches to Dr. Angela Hirbe’s laboratory (Washington University, St. Louis) based upon a mutual agreement aimed to consolidate genomic and biological characterization of available MPNST models.



