

SOP50101: Patient Tumor Implantation (Subcutaneous) and Monitoring		
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Change History

Revision	Description
4/17/2017	New Document for PDMR Public Website
6/28/2018	Separated Patient tissue tumor implantation and PDX fragment passaging and cryopreservation into two separate SOPs (SOP50101 and SOP50102, respectively). Updated recommendations on implantation from cryopreserved material, updated company/catalog numbers
6/25/2020	Add additional basement membrane extract options for implantation. Updated syringe-type details.

RELATED DOCUMENTS

Available on the PDMR website: <https://pdmr.cancer.gov/sops>

SOP20101: Fresh Tumor Collection and Handling for Generation of Patient-Derived Models
SOP50102: PDX Implantation, Expansion and Cryopreservation (Subcutaneous)
SOP50103: Histopathological Assessment of Patient-Derived Xenografts

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1.0 PURPOSE/SCOPE

This Standing Operating Procedure (SOP) describes the procedures for tissue preparation and procedures for subcutaneous implantation to generate patient-derived xenografts (PDXs) and cryopreservation of material under BSL-2 safety criteria. This SOP is used/performed by the Biological Testing Branch (BTB) at NCI-Frederick, Frederick National Laboratory for Cancer Research.

2.0 SAFETY

BTB treats all patient tissue and mice carrying patient tumors as a possible health threat as the human tissue could still retain human pathogenic agents. Mice are housed in barrier facilities using full micro-isolator technique with all work, including husbandry, provided by experienced technical staff. The primary mouse strain used is the NOD.*Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ* (NSG) which are highly susceptible to infection due to their profound immunodeficiency. All materials coming into the barrier facilities are decontaminated by autoclaving, or chemical means including the use of chlorine dioxide gas, Clorox Healthcare Hydrogen Peroxide spray/wipes, and Virkon® dip tanks for non-autoclavable items.

3.0 MATERIALS & EQUIPMENT

Review all equipment based on method of interest

3.1 Implant Materials & Equipment

- 3.1.1 200 µL pipettor and sterile tips
- 3.1.2 Sterile scalpel and forceps
- 3.1.3 Sterile petri dishes
- 3.1.4 Matrigel® (BD BioSciences, Bedford, MA.) or Cultrex Basement Membrane Extract Type 3 (BME3), Pathclear, (R&D Systems, Cat#: 3632-001-02)
 - IMPORTANT: All basement membrane extract purchases should be submitted specifying mouse rodent pathogen screened LDEV-Negative material. If not, there is a possibility of LDEV contamination which can result in LDEV+ tumors as a consequence.
- 3.1.5 11-gauge Trocar (tissue implant needle), sterile (pre-chilled)
- 3.1.6 1-cc low dead space syringe with luer-lock hub, sterile (pre-chilled at -20°C) (Exel Cat#: 26048-1)
- 3.1.7 Bone rongeur, sterilized
- 3.1.8 Rituximab (Rituxan® 10 mg/mL; Genentech)
- 3.1.9 RPMI-1640 containing primocin (working concentration: 100 µg/mL)
- 3.1.10 NOD.*Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ* (NSG) mice, sex-matched to human patient
- 3.1.11 Isoflurane and anesthesia machine for delivery
- 3.1.12 Marcaine solution, 0.25% (Bupivacaine HCl)

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- 3.1.13** Nolvasan® (disinfectant for animal surgery)
- 3.1.14** Clorox Healthcare® Hydrogen Peroxide Spray, Bleach (5.25% Hypochlorite) diluted 1:10, 2% Virkon®, or similar disinfectant
- 3.2** Alternate Implant Methodology: Additional Materials
 - 3.2.1** Estradiol pellets (Innovative Research; 3.0-mm, 90-day release, 0.18 mg)
 - 3.2.2** Testosterone pellets (Innovative Research; 3.0-mm, 90-day release, 12.5 mg)
 - 3.2.3** 10X PBS, sterile
 - 3.2.4** Sterile water
 - 3.2.5** 10 mm cloning ring, sterile (Sigma Catalog# C2059-1EA)
 - 3.2.6** 6-well plate, sterile
 - 3.2.7** Advanced DMEM/F12 containing 5% fetal bovine serum (FBS) and primocin (working concentration: 100 µg/mL)
 - 3.2.8** Wound closure: sterile staples or sutures

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4.0 FRESH TISSUE PREPARATION

- Be sure animals are ready for implantation **immediately following** tissue preparation.
- At all patient tissue preparation steps, whenever possible some residual tissue is kept for pathology review.
- All tissue preparation should be performed in a Biological Safety Cabinet (BSC) using sterile instruments and technique. Maintain sterility at all times.

4.1 Tumor Fragments and 18-g Needle Biopsies

- 4.1.1** Tumor tissue should be implanted as soon as practical post-collection. The tumor should be maintained at 4°C in an appropriate storage medium (e.g., CO₂ independent media containing primocin; see SOP20101 on the PDMR website: <https://pdmr.cancer.gov/sops>) until implantation. The PDMR has detected viable tumor material up to 96-hr post-collection using our established shipping and handling procedures, but implantation within 24 hours is desired.
- 4.1.2** Transfer the tumor material from the shipping/transport media into a sterile petri dish along with a small volume of transport media adequate to keep the tissue wet.
- 4.1.3** Using a sterile scalpel cut the tissue into 1-2 mm³ fragments (~30 mg). Evaluate the material prior to cutting to ensure tumor tissue is being implanted as non-tumor tissue (e.g., cartilage, muscle) can provide supporting cells but should not be implanted separately. Grossly necrotic areas are avoided.
- From resected tissue, generally 5-10 P0 mice (first passage in mouse) can be implanted. 18-gauge biopsy material is generally sufficient for 1-2 P0 mice.
 - In addition, to avoid loss of viable tumor cells, the transport media is centrifuged to pellet any shed tumor material and this is also implanted or cultured in vitro (SOP Step 5.1.2).
- 4.1.4** Add Rituximab to the media containing the tumor fragments to a final concentration of 1 mg/mL. Mix well and incubate 30 minutes at room temperature.
- 4.1.5** Place one tumor fragment into the end of a pre-chilled 11-gauge Trocar needle and add a drop of Matrigel®/BME3 (~50-100 µL).
- 4.1.6** Keep the Trocar on ice to prevent the Matrigel®/BME3 from polymerizing until implantation.
- 4.1.7** Transfer the transport media to a 50-mL conical-bottomed tube and centrifuge at 200-300xg for 5 minutes. (The PDMR always assumes there are shed viable cells in the shipping media following overnight shipments.)
- 4.1.8** Disinfect the outside of the tube. Remove all but 30-50 µL of the media without disturbing the pellet – a pellet may not be visible.
- 4.1.9** Recap tube and gently flick to re-suspend cells in the remaining media.

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- 4.1.10** Using a 1-cc syringe with 22-gauge needle (both pre-chilled), draw up 100 μ L Matrigel®/BME3 (kept on ice to prevent polymerizing) followed by the re-suspended cells. Avoid air bubbles in the process.
- 4.1.11** Keep syringe/needle on ice to prevent Matrigel®/BME3 from polymerizing until implantation.
- 4.2** Malignant effusions (e.g., pleural, peritoneal, etc.)
- 4.2.1** Same-day receipt of up to 1-L volumes of malignant effusions. Sterile container(s) containing effusion should be packed on ice for transport.
- 4.2.2** Use sterile technique to transfer effusion to 50-mL conical tubes or 250-ml bottles and pellet cells at 200-300xg for 5 minutes.
- 4.2.3** Remove supernatant without disturbing the cells and resuspend in the residual volume.
- If significant red blood cells are present, use hypotonic shock to lyse them as follows: (1) Fill one pipette with 9-mL of sterile water and a second pipette with 1-mL 10X PBS (or other 10X physiological salt solution). (2) Add the 9-mL sterile water to the tube containing the cells and immediately add the 1-mL of 10X salt solution – mix well.
- 4.2.3.1** Pool cells from all centrifuge tubes into a single conical tube and pellet the cells by centrifugation at 200xg for 5 minutes and resuspend cells in residual volume (generally 30-50 μ L). Transfer the resulting cells to a conical tip microfuge tube.
- 4.2.4** Add Rituxan stock solution to the cell suspension to a final concentration of 1 mg/mL. Incubate for 30 minutes at room temperature.
- 4.2.5** Using a 1-cc syringe with 22-gauge needle (both pre-chilled), draw up 100 μ L Matrigel®/BME3 (kept on ice to prevent polymerization) followed by the re-suspended cells. Avoid air bubbles in the process.
- 4.2.6** Keep syringe/needle on ice to prevent Matrigel®/BME3 from polymerizing until implantation.
- 4.3** Bony tumor tissue (osteosarcomas, bony metastases)
- 4.3.1** Transfer the tumor material to a sterile petri dish along with some of the transport material.
- 4.3.2** Use a sterile bone rongeur and forceps to break-up the bony components of the tumor tissue.
- 4.3.3** Follow the same mouse preparation procedures as that used for SC implantation.
- Note: Bony components can be implanted subcutaneously without difficulty although they may not fit into a trocar, they can be inserted using forceps.

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5.0 IMPLANTATION

NCI PDMR nomenclature for counting of passaged material: Patient material is referred to as Originator and the PDX grown in the first implanted host NSG mouse is called passage 0 (P0). Subsequent passages are iterative. Each PDX/host mouse identifier is a unique identifier which includes the parental identifiers so that the full passage lineage can be traced back to the Originator material.

Protocol for use of tumor growth supplements and alternate implant methodologies (mammary fat pad [breast cancer], thigh muscle [sarcomas]) can be found in [Appendix 1](#).

5.1 Subcutaneous Implantation Procedure

- 5.1.1 Shave the implantation site prior to anesthesia.
- 5.1.2 Anesthetize a sex-matched NSG mouse by isoflurane inhalation until non-responsive to toe-pinch. Keep mouse warm and monitor breathing during procedure.
- 5.1.3 Disinfect implantation site with Nolvasan®. We recommend to NOT use alcohol, as this can chill the animal during the procedure.
- 5.1.4 Using scissors, make a small nick incision in the inguinal area (for standard SC implants placed in the posterior axillary area) and insert the Trocar through the skin opening.
- 5.1.5 Slide the Trocar (or 1-cc syringe with 22-gauge needle) craniodorsally to about 0.5 cm caudal to the axillary area; the Trocar should slide smoothly under the skin.
 - 5.1.5.1 Notes on Implantation Using Trocar/ Matrigel®/BME3 Method
 - Expel the tumor fragment into the axillary area. This may require twisting the Trocar slightly to ensure the tumor fragment does not stick to the Trocar and then gently pull out the Trocar. Forceps can be used over the skin to hold the fragment in place during trocar withdrawal.
 - 5.1.5.2 Notes on Implantation Using 1-cc Syringe/ Matrigel®/BME3 Method
 - Inject the cell/ Matrigel®/BME3 inoculum into the subcutaneous space on the lateral body wall just posterior to the axillary area. Leave the needle in place in the mouse for a few seconds once injected to allow the expelled Matrigel®/BME3 to solidify; this will prevent the solution from flowing out of the needle track.
- 5.1.6 The entry site will heal well and does not require a wound clip, although one may be applied if preferred.
- 5.1.7 Apply 1-2 drops 0.25% Marcaine to the nick incision to relieve pain/discomfort.
- 5.1.8 Keep mice warm and monitor breathing until awakened from anesthesia.

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6.0 GROWTH ASSESSMENT

6.1 Observation

6.1.1 Mice are weighed 1-3 times per week as a gauge of animal health and more frequently if clinical signs are noted.

6.1.2 Mice are observed at least once daily and tumor growth is monitored with caliper measurements at least once weekly. The tumor weight (mg) is calculated using the following formula [3]:

$$Tumor\ weight\ (mg) = \frac{(tumor\ length) \times (tumor\ width)^2}{2}$$

6.1.3 P0 (first passage in mouse) PDX host mice are monitored for up to 300 days after implantation at the NCI PDMMR. Mice not showing progressive tumor growth by 300 days post-implant are euthanized.

6.1.4 Antemortem determination for harvesting tumor tissues is based upon tumor size as well as clinical signs. Animals without palpable tumors are evaluated clinically and by monitoring body weights.

6.2 Tumor Assessment and Humane Euthanasia

6.2.1 Tumors are harvested immediately following euthanasia or under terminal isoflurane anesthesia.

6.2.2 Palpable tumors are evaluated 2-3 times per week and efforts are made to identify animals that may experience adverse health effects before the next observation (and euthanize accordingly).

6.2.2.1 It is recommended to harvest PDX tumors in the 1000-2000 mg size range as there is typically less necrotic tissue present. (Individual institutional guidelines for maximum allowable tumor burden should be followed)

6.2.2.2 An animal with a tumor that grows in a manner that limits their mobility, impedes access to food and water, or has a tumor that is showing the onset of skin necrosis is euthanized.

6.2.2.3 Tumors may be red to purple in color when highly vascularized and this appearance does not warrant euthanasia. When the skin overlying the tumor shows evidence of necrosis or other compromise, euthanasia is warranted.

6.2.2.4 Animals with tumors arising at sites other than the implantation site will be euthanized based mainly upon gross observations such as weight loss or gain, lethargy, respiratory distress, inactivity, ruffled hair coat, decreased body temperature, inappetence, etc.

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6.2.2.5 Some internal tumors (prostate, spleen, renal) may be palpable and will be checked regularly so that the tumor does not become large and debilitating.

6.3 Static and No-Growth Monitoring

6.3.1 Animals with no signs of tumor growth are euthanized at 300 days post-implant.

6.3.2 NCI has observed numerous tumors that we have categorized as “Static” tumors.

6.3.2.1 These tumors reach a steady-state tumor burden and appear to stop growing in size; possibly due to walling off of the tumor in the animal.

6.3.2.2 These mice are euthanized and fragments of the tumor are passaged to determine if tumor growth will re-initiate.

6.4 Proceed to SOP50102 if PDX growth is observed

7.0 CLEAN-UP

7.1.1 All materials coming into contact with patient tissue as well as the mice carrying patient tumor samples are treated as a potential health threat (BSL-2 precautions) since the human tissues could retain human pathogenic agents even if they do not replicate in mouse cells (e.g., HIV, HPV, etc).

7.1.2 Flush/soak any items (e.g., tubes, syringes, petri dishes, lab mats, etc) that were in contact with human tissue with disinfectant (e.g., 10% bleach, commercial hydrogen peroxide disinfectant, 2% Virkon®) for a minimum of 10 minutes before disposal in biohazard waste or sharps containers (follow institutional guidelines and manufacturer’s recommendations).

7.1.3 For items that can’t be rinsed (e.g., micropipettors), wipe down thoroughly with Clorox Healthcare® Hydrogen Peroxide Spray or other appropriate disinfectants.

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APPENDIX 1: TUMOR GROWTH SUPPLEMENTS AND ALTERNATE IMPLANT METHODOLOGIES

1. Tumor Growth Supplements

- A. Tumor types that may be dependent upon exogenously administered hormones (e.g., breast, prostate, ovarian, testicular) should receive subcutaneously implanted hormone pellets to aid in tumor survival and growth.
- B. Estradiol pellets (Innovative Research; 3.0-mm, 90-day release, 0.18 mg) should be implanted subcutaneous into mice receiving tissue implants originating from female patients with cancer of the Breast, Ovary, Uterus, Fallopian tube, or Cervix unless contra-indicated in patient diagnoses record.
 - a. A single pellet (or 2 if desired) used in an NSG mouse should be adequate to provide the needed support for tumor growth and survival while avoiding estrogen toxicosis. Only one pellet should be used in athymic nude mice due to their sensitivity to estrogen toxicosis.
 - b. Injectable estradiol cypionate is avoided if possible as it is more likely to result in estrogen toxicosis before the tumor is able to grow.
 - c. Implant pellet at a SC site distant to the PDX implant. The pellet should be replaced every 90 days.
- C. Testosterone pellets (Innovative Research; 3.0-mm, 90-day release, 12.5 mg) should be implanted subcutaneously into mice receiving tissue implants originating from male patients with cancer of the testicles, prostate, vas deferens, and seminal vesicles unless contra-indicated in patient diagnoses record.
 - a. A single pellet in an NSG mouse should be adequate to provide the needed support for tumor growth and survival.
 - b. Implant pellet at a site distant to the PDX implant. The pellet should be replaced every 90 days.

2. Common PDMR Alternate Implant Methodologies

PDMR implants essentially all tumor types subcutaneously (SC) per the method above except breast tumors which are implanted in the mammary fat pad (MFP).

Adult Soft Tissue Sarcomas are implanted using the “Sarcoma Plug” method below as well as SC (if sufficient material) at P0. P1 tumors are then implanted at the alternate site as well as SC to determine if SC growth is supported. For adult soft tissue sarcomas, the PDMR noted a low take-rate with direct SC implantation of material. The “Sarcoma Plug” method has increased take-rate from <5% to 35%-40% across all sarcoma histologies.

Other alternate implant methodologies are used less frequently and will be posted as a separate SOP to the PDMR website. After successful growth in the alternate implant site, PDMR may try growth SC and if successful do subsequent passages there.

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- A. “Sarcoma Plug” Implant [1, 2]
- a. The Day Before Implantation:
- i) Using a sterile forceps, press a sterile 10-mm cloning ring, greased side down, in the center of a well in a sterile 6-well plate to create a seal between the plate and the ring.
 - ii) Quickly layer ~100 μ L of Matrigel®/BME3 into the cloning ring followed by addition of a small tumor fragment. Cover the fragment with an additional~ 100 μ L of Matrigel®/BME3.
 - iii) Add adequate media (Advanced DMEM/F12 with 5% FBS and primocin) to fill the cloning ring and incubate overnight in a 37°C CO₂ incubator. If the ring must be incubated longer than 24-h, then the entire well should be filled with media such that the cloning ring is covered to prevent dessication.
- b. Day of Implantation
- i) Using a sterile forceps, remove the cloning ring from the 6-well plate and sterilely remove the “Sarcoma Plug” from the cloning ring. We have found using the end of a sterile 1-cc plunger works well to expel the plug from the cloning ring.
 - ii) Follow the same mouse preparation procedures as that used for SC implantation with the following changes:
 - Make a blunt dissection just cranial to the rear leg for access to the #4 (#9) MFP.
 - Use a small spatula to “scoop” up the “Sarcoma Plug” and place it into the subcutis of a surgically anesthetized mouse, near but not within the MFP.
 - iii) Close the skin and staple (or suture if preferred) the overlying skin
 - iv) Apply 1-2 drops 0.25% Marcaine to the nick incision to relieve pain/discomfort.
 - v) Keep mice warm and monitor breathing until awakened from anesthesia.
- B. Mammary Fat Pad (MFP) Implant
- a. Follow the same mouse preparation procedures as that used for SC implantation with the following changes:
- i) Make a small incision just cranial to the rear leg for visualization of the #4 (#9) MFP.
 - ii) Gently lift the MFP with forceps, incise the gland creating a small pocket. Slide the Trocar into this space and expel the prepared tumor fragment into the pocket.

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- iii) Return the gland to its proper position, close and staple (or suture if preferred) the overlying skin.
- iv) Apply 1-2 drops 0.25% Marcaine to the nick incision to relieve pain/discomfort.
- v) Keep mice warm and monitor breathing until awakened from anesthesia.

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