Modification Form for Permit BIO-RRI-0032

Permit Holder: Paula Foster

Approved Personnel
(Please stroke out any personnel to be removed)

Mevan Perera

Vasiliki Economopoulos

Jennifer Noad

Shruti Krishnamoorthy

Yuhua Chen

Roja Rohani

Laura GonzalezLara

Jonathon Snir

Christiane Mallet

Catherine Ramsay

Emeline Ribot

Additional Personnel
(Please list additional personnel here)

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED.*

Classification: 2

Date of last Biohazardous Agents Registry Form: Dec 19, 2008

Signature of Permit Holder: Paula Foster

BioSafety Officer(s): _____

Chair,Biohazards Subcommittee: _____

Modification Form for Permit BIO-RRI-0032

Permit Holder: Paula Foster

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms		PC-3/M1 Cell line
Approved Cells	Human (established): THP-1, FaDu Luc2.11, MDA-MB-231PA, MDA-MB-231BR. Rodent (established): B16F10, Glioma-261, Glioma-261 RFP. C4-2 Cell line, C4-2B Cell Line	
Approved Use of Human Source Material		
Approved GMO	pCMV-DSRed Express, pGL4.14. Androgen independent cell line, clone of C4. Androgen Independent Cell line colony of C4	Human prostate tumor lines
Approved use of Animals	Nu/Nu (nude) mice, C57B1/6 mice AUS#: 2006-013-03, 2007-041-03, 2006-008-01	
Approved Toxin(s)	Pertussis	

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Classification: 2

Date of last Biohazardous Agents Registry Form: Dec 19, 2008

Signature of Permit Holder: Paula Foster

BioSafety Officer(s): _____

Chair,Biohazards Subcommittee: _____

Subject: Re: [Fwd: Re: [Fwd: Re: Containment Level request for C4-2B cell line]]
From: pfoster@imaging.robarts.ca
Date: Mon, 13 Jul 2009 20:02:49 -0400 (EDT)
To: Jennifer Stanley <jstanle2@uwo.ca>

Hi Jennifer

the cell line C4-2B does not contain human pathogens and has not been transformed with human pathogens.

-Paula

> -------- Original Message --------
> Subject: Re: [Fwd: Re: Containment Level request for C4-2B cell line]
> Date: Mon, 13 Jul 2009 15:48:46 -0400 (EDT)
> From: pgareau@imaging.robarts.ca
> To: Jennifer Stanley <jstanle2@uwo.ca>
> References: <4A5B7F7E.7010903@uwo.ca>
>
> Paula's new email is pfoster@imaging.robarts.ca
>
> > Hi Paula
> > Can you confirm that this cell line does not contain human pathogens and has not been transformed with human pathogens (see note below from PHAC - the Public Health Agency of Canada).
> > Jennifer
> >
> > -------- Original Message --------
> > Subject: Re: Containment Level request for C4-2B cell line
> > Date: Mon, 13 Jul 2009 13:35:58 -0400
> > From: Geneviève Lacroix <genevieve_lacroix@phac-aspc.gc.ca>
> > To: Jennifer Stanley <jstanle2@uwo.ca>
> >
> > Hi Jennifer,
> >
> > If the cell line does not contain human pathogens (i.e. EBV) or was not transformed with a human pathogens (i.e. SV40) then as far as it concerns
> > our office, it does not require a containment level 2 or more laboratory.
> > Also, you can refer to our LBGs, we have a general section on risk assessment and a more specific one on cell line more in our Laboratory Biosafety Guidelines (Chapter 2 and Chapter 7.3).
> >
> > Hope this helps.
> >
> > Genevieve Lacroix, M.Sc.
> > A/Head, Importation and Biosafety Programs
> > Chef intérimaire/Importation et servuce de biosécurité
> > Office of Laboratory Security / Bureau de la sécurité des laboratoires
> > Public Health Agency of Canada / Agence de la santé publique du Canada
> > 100 ch. Colonnade Rd. AL: 6201A, Ottawa, Ontario, Canada, K1A 0K9
> > Tel: (613) 946-6982
> > Fax: (613)941-0596
> > genevieve_lacroix@phac-aspc.gc.ca
> > http://www.phac-aspc.gc.ca/ols-bsl/index.html

Modification Form for Permit BIO-RRI-0032

Permit Holder: Paula Foster

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Classification: 2

Date of last Biohazardous Agents Registry Form: Dec 19, 2008

Signature of Permit Holder: Paula Foster

BioSafety Officer(s): V Hawley May 15/09

Chair,Biohazards Subcommittee: Stu Kilpatrick

Modification Form for Permit BIO-RRI-0032

Permit Holder: Paula Foster

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms		C4-2B Cell Line
Approved Cells	Human (established): THP-1, FaDu Luc2.11, MDA-MB-231PA, MDA-MB-231BR. Rodent (established): B16F10, Glioma-261, Glioma-261 RFP. C4-2 Cell line.	
Approved Use of Human Source Material		
Approved GMO	pCMV-DSRed Express, pGL4.14. Androgen independent cell line, clone of C4	Androgen independent Cell line. Colone of C4.
Approved use of Animals	Nu/Nu (nude) mice, C57B1/6 mice AUS#: 2006-013-03, 2007-041-03, 2006-008-01	
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Classification: 2

Date of last Biohazardous Agents Registry Form: Dec 19, 2008

Signature of Permit Holder: Paula Foster

BioSafety Officer(s): [illegible] May 15/09

Chair,Biohazards Subcommittee: [illegible]

To Whom it May Concern,

Dr. Foster, of the Robarts Research Institute at The University of Western Ontario, would like to purchase the C4-2B cell line. The cells will be labeled, by co-culture of transfection, with super paramagnetic iron oxide particles to permit their detection by magnetic resonance imaging. If the cell labeling is effective then the cells will be it is injected/implanted into mice for a cancer imaging project.

Mailing Address:
Dr. Paula Foster
Attn: Yuhua Chen/Cat Ramsay
P.O. Box 5015
100 Perth Dr
London ,ON
N6A 5K8

Proposed use of C4-2B Cells

These cells will be used to investigate a mouse model of prostate cancer in which cell and tumour progression will be monitored using magnetic resonance imaging. We will culture and label the cells with a contrast agent containing iron or gadolinium, in our tissue culture room which is approved for level 2 biohazards. Then, the cells will be injected into immune-compromised mice, which will be housed in a barrier facility at the University of Western Ontario. The mice will be imaged periodically with MRI at the Robarts Research Institute.

ViroMed Laboratories
A LabCorp Company

CHUNG cells

Human Prostatic Carcinoma Cell Lines

Catalog Number	Cell Line Abbreviation	Product Description	Price
12-100	LNCaP	Direct parental prostate cancer cell line	$ 210.00
12-101	C4	Androgen dependent cell line, clone of LNCap	367.50
12-102	C4-2	Androgen independent cell line, clone of C4	367.50
12-103	C4-2B	Androgen independent cell line, clone of C4, developed in bone	367.50

A materials transfer agreement (MTA) is required prior to obtaining these cell lines.

Shipping Cost: Please contact your ViroMed account manager for specific information regarding domestic and international shipping costs.

For further information, please contact ViroMed Client Services at 800-582-0077.

Source → Same procedure to obtain

www.viromed.com

Revised February 19, 2009

ViroMed Laboratories
6101 Blue Circle Drive
Minnetonka, MN 55343-9108
Telephone: 800-582-0077
www.ViroMed.com

Chung Cancer Cell Lines – Procedure to Obtain

Submit the following information via email:

1. Chief Investigator's Name P Foster
2. Intended usage statement
3. Exact shipping address (and billing, if different; we also accept Visa/MC for payment)

This information will then be forwarded and,

4. A Materials Transfer Agreement (MTA) will then be sent to you via email by our Sales Administration Dept. The above 3 items will be added to the MTA.

PLEASE NOTE: The terms of the MTA cannot be altered or changed. We can only accept the MTA as originally prepared. If terms of the MTA are altered, we will submit the changes to both our legal dept and MD Anderson Cancer Center for their approval. The approval process can take several weeks.

5. When the signed MTA (2 original copies) is received back, the ordering process can then proceed with verification of order and payment information.

For International shipments: Credit card or direct bank transfer is the preferred payment.

6. The order is then submitted to our production dept. We normally ship these cells out on a Monday or Tuesday; the orders may take up to several weeks to ship.

Reference Publications

1. LNCaP Human Prostrate Cancer Progression Model. Leland W.K. Chung, Ph.D. Urol Oncol 1996; 2:000-000
2. Androgen and retinoic acid interaction in LNCaP cells, effects on cell proliferation and expression of retinoic acid receptors and epidermal growth factor receptor. Ming-tang Li, Frank Richter, Chawnshang Chang, Robert J Irwin, Hosea FS Huang. BMC Cancer 2002 2:16.
3. Androgen-independent Cancer Progression and Bone Metastasis in the LNCaP Model of Human Prostrate Cancer. Cancer Research 54, 2577-2581, May 15, 1994.
4. Derivation of Androgen-Independent Human LNCaP Prostatic Cancer Cell Sublines: Role of Bone Stromal Cells. Int.J.Cancer 57, 406-412, 1994.

CELL-LINE SPECIFICATIONS

Name of Cell Line: C4-2

Origin: The human prostatic carcinoma cell line, *LNCaP* (1×10^6 cells; passage # 29) previously described [1] was co-inoculated into an athymic male nude mouse with (1×10^6) human fibroblasts derived from an osteosarcoma (cell line MS). The nude mouse host was castrated after 8 weeks incubation. A tumor specimen was excised after a total of 12 weeks. The *C4* cell line constitutes the *in vitro* cultured subline grown from the murine host's tumor [2]. When the *C4* sub-line was subsequently co-inoculated with MS osteosarcoma fibroblasts in a castrated athymic male nude mouse host for another 12 weeks by the same protocol described above. Prostatic epithelial cells cultured from the resultant tumor in this host constituted the *C4-2* subline[3].

Cell Line characteristics:
Morphology: fibroblast-like growth in tissue culture flasks; may form concentric zones of low cell growth upon reaching confluence.

Tumorigenicity & Osseous Metastasis: Orthotopic administration of 1×10^6 resuspended C4-2 cells in both intact and castrated athymic male nude mice yielded 100% tumorigenicity (20/20 and 14/14, respectively). Osseous prostate cancer metasteses were detected in both intact and castrated murine hosts (2/20 and 3/14, respectively) [3].

Soft-agar cloning: When 1×10^4 resuspended C4-2 cells were cloned on 0.3% soft agarose supplemented with 5% FBS and 2% TCM, colony formation was scored as 313 ± 15.3 relative to 12 ± 2.5 from the parental LNCaP cell line [2].

Androgen sensitivity: LNCaP sublines are androgen-independent.

Maintenance of C4-2, C4 and parental LNCaP cell lines:

Freezing Media: 10% DMSO + 90% T Media (containing 20% FBS)

Freezing Procedure: Freeze cells when cell layer becomes 90% confluent. Do not freeze at a confluency higher than 90%. Change media 1 day prior to freezing cells. Freeze at 1.5×10^6 cells per vial.

Frequency of Passage: Passage should be performed when confluency reaches 80-90%. If culture is allowed to remain too high of a confluency for more than a day or two, cells will begin to slough away from the monolayer. Over-grown passages can be difficult to re-plate as single cells. If cell number is too low when passing, cell growth tends to aggregate in isolated areas instead of growing and spreading across the flask.

Passing procedure: Cells are washed in PBS, pH 7.2. These cells are passed using a 0.05% trypsin/0.53mM EDTA solution. Apply trypsin/EDTA in the following volumes:

1.5 – 2ml/T25 flask
4 – 5 ml/T75 flask
5 – 6 ml/T175 flask

Leave trypsin/EDTA solution on cells for about 5 minutes, checking them frequently. Neutralize trypsinized cells with T media containing 5% FBS. Always centrifuge the trypsin/EDTA out of the culture and transfer cells to a new flask. Media volumes are as follows:

5ml media/T25 flask
15ml media/T75 flask
30ml media/175T flask

[1] Horoszewiez, JS et al. *Cancer Research* 43:1809-1818, 1983.

[2] Wu et al. Derivation of androgen-independent human LNCaP prostatic cell sublines: Role of bone stromal cells. *International Journal of Cancer* 57:406-412, 1994.

[3] Thalmann et al. Androgen-independent cancer progression and bone metastatis in the LNCaP model of human prostate cancer. *Cancer Research* 54:2577-2581, 1994.

CELL LINE THAWING & REVITALIZATION PROTOCOL

(It is recommended that the "C-4" series of LNCaP-derived cell lines be stored long-term in liquid nitrogen.)

1) Remove cell storage vial from liquid nitrogen. Thaw rapidly in a 37° C. water bath.
2) Immediately transfer thawed cell solution to a 15 ml centrifuge tube.
3) Slowly resuspend thawed cells in 10 ml of growth media.
4) Centrifuge at 4°C or room temperature for 7-8 minutes at 800 rpm.
5) Aspirate media from cell pellet and resuspend in 15 ml fresh media.
6) Transfer into T-75 tissue culture flask and place in 37°C (5% CO2) incubator.
7) Do NOT perform media exchange the day after thaw date. Perform media exchange only after cells have sufficiently attached to surface of flask. Because these cells may take 1-3 days to adhere to the flask surface, premature media exchange could result in the loss of unattached viable cells.

The LNCaP cell line may require 2-3 days for a sufficient amount of cells to attach to the surface of the flask.

The C4, C4-2 and C4-2Bone cell lines typically will adhere in less 2 days.

PRODUCT AND MEDIA INFORMATION FOR LNCAP, C4, C4-2 AND C4-2 BONE

T-Media:

DMEM	liquid with sodium bicarbonate	Vendor: Fisher Cat #: BW12-741F Package size: 500 ml
HAM'S F12	liquid with sodium bicarbonate	Vendor: Fisher Cat #: BW12-615F Package size: 500 ml
FBS	Premium Heat inactivated	Vendor: Fisher Cat #: BW14-503F Package size: 500 ml

T MEDIA SUPPLEMENTS: STORE at -20°C

1. Insulin

Aliquot concentration at 500x: 2.54 mg/ml in 0.1% BSA/PBS
Final concentration in 500 ml: 5 ug/ml

Vendor: Sigma
Cat #: 1-1882
Package size: 100 mg

2. T3 (TRIIODO-THYRONINE)

Aliquote concentration at 500x: 6.825 ng/ml in 0.1% BSA/PBS
Final concentration in 500 ml: 13.65pg/ml

Vendor: Sigma
Cat #: T-2877
Package size: 100 mg

3. apo-TRANSFERRIN Human

Aliquot concentration at 500x: 2.2 mg/ml
Final concentration in 500 ml: 4.4 ug/ml

Vendor: Sigma
Cat #: T-4382
Package size: 1 g

4. d-BIOTIN

Aliquote concentration at 500x: 0.122 mg/ml in 0.1% BSA/PBS

Vendor: Sigma
Package size: 1 g

Final concentration in 500 ml: 0.244 ug/ml

5. ADENIN

Vendor: Sigma
Cat #: A-3159
Package size: 5 g

Aliquot concentration at 500x: 6.25 mg/ml in 0.1% BSA/PBS
Final concentration in 500 ml: 12.5 ug/ml

6. Penicillin and Streptomycin

Vendor: Mediatech
Cat #: 30-002-C1
Package size: 100 ml

Concentration:
Pen 50,000 units/ml
Strep 10,000 g/ml

Final Concentration:
Pen 100 units/ml
Strep 100 g/ml

7. TRYPSIN-EDTA (10X)
Ready to use

Vendor: Fisher
Cat #: BW17-161E
Package size: 100ml

Media Preparation:
400 ml DMEM
100 ml Ham's F12
50 ml Heat inactivated FBS (final conc. 10%)
5 ml 10X Pen/Strep (final conc. 1%)
1 ml of above supplements

Mix well and use, media will be good for 30 days.

Materials for trypsinizing the cells:
10X Trypsin (Fisher Cat # BW17-161E)
1X PBS

Procedure:

1. Thaw frozen cells quickly at 37° water bath and transfer into 15 ml tissue culture tube with 9 ml media.
2. Centrifuge at 12 RPM for 5 minutes at room temp or 4°
3. Aspirate media and wash cells with 1X PBS and centrifuge same as before.
4. Aspirate PBS and resuspend in 2 ml media and transfer to tissue culture plate or flask. Incubate at 37° with 5% CO2. **Do not disturb cells for at least four days. These cells are very sensitive and they may not adhere if they are disturbed.**

Splitting cells:

Warm up all reagents to 37° before use.

1. Split cells 1:4 when they are about 75-85% confluent by trypsinizing them with 21 ml 1x trypsin-EDTA for 5 min. at most at 37° (1ml for 100 mm plates and 25 cm2 tissue culture flask, 2 ml for 75 cm^2 flask and 3.5 ml for 150 cm^2 flask).
2. Stop trypsin with 9 ml of media; transfer cells into tissue culture tube and spin for 5 min at 1200 RPM at Room temp.
3. Aspirate media and wash cells with 10 ml 1X PBS, Centrifuge same as before and aspirate PBS.
4. Resuspend the pellet in 10 ml media and transfer into T75 tissue culture flask and incubate at 37° with 5% CO2.

Do not disturb the flask for at least two days.

MAINTENANCE OF C4, C4-2, C4-2B AND LNCaP CELL LINES:

Freezing Media: 10% DMSO + 90% T Media (containing 20% FBS)

Freezing Procedure: Freeze cells when layer becomes 90% confluent. Do not freeze at confluency higher than 90%. Change media 1 day prior to freezing cells. Freeze at 1-1.5x10^6 cells per vial.

Frequency of Passage: Passage should be performed when confluency reaches 80-90%. If culture is allowed to remain at too high of a confluency for more than a day or two, cells will begin to slough away from the monolayer. Over-grown passages can be difficult to re-plate as single cells. If cell number is too low when passing, cell growth tends to aggregate in isolated areas instead of growing and spreading across the flask.

Passing procedure: Cells are washed in PBS, pH 7.2. These cells are passed using a 0.05% trypsin/0,53mM EDTA solution. Apply trypsin/EDTA in the following volumes:

1.5 ml / T25 flask
4.0 ml / T 75 flask
5-6 ml / T 175 flask

Leave trypsin/EDTA solution on cells for about 5 minutes, checking them frequently. Neutralize trypsinized cells with T media containing 5% FBS. Always centrifuge the trypsin/EDTA out of the culture and transfer cells to a new flask. The lowest dependable amount of cells that will yield an even spread is 30,000 cells/ml – this number will vary depending each technician's own ability. Media volumes are as follows:

5 ml / T25 flask
15 ml / T75 flask
30 ml/T175 flask

Source: Cheryl Savola, PhD, ViroMed/LabCorp 10/14/04 – this document is the original information provided from UroCor/Dianon to ViroMed .

Proposed use of PC-3M Cells

These cells will be used to investigate a mouse model of prostate cancer in which cell and tumour progression will be monitored using magnetic resonance imaging. We will culture and label the cells with a contrast agent containing iron or gadolinium, in our tissue culture room which is approved for level 2 biohazards. Then, the cells will be injected into immune-compromised mice, which will be housed in a barrier facility at the University of Western Ontario. The mice will be imaged periodically with MRI at the Robarts Research Institute.

NCI-FREDERICK CANCER
DCT TUMOR REPOSITORY

AVAILABLE INFORMATION ON TRANSPLANTABLE TUMORS OR CELL LINES

DESIGNATION:	**PC-3/M**
VIAL DESIGNATION:	0502392
SPECIES:	HUMAN
TUMOR ORIGIN:	PROSTATE
HISTOLOGIC TYPE:	CARCINOMA

CELL CULTURE INFORMATION:

GROWTH MEDIUM:	RPMI 1640 + 10%FBS + 2 mM L-Glutamine
MODE OF GROWTH:	Adherent
METHOD OF HARVEST:	Trypsin/EDTA
SPLIT RATIO:	1:20 Weekly
DOUBLING TIME:	60 Hours

TUMOR PROFILE INFORMATION:

HOST STRAIN:	ATHYMIC NUDE
TISSUE IMPLANTED:	N/A
ROUTE:	N/A
SUSPENDING MEDIUM:	N/A
OPTIMAL DAY:	N/A
HOST MEDIAN SURVIVAL TIME:	N/A
METASTATIC CHARACTERISTICS:	N/A
HISTOPATHOLOGY:	N/A

SOURCE:	Dr. Edward Kaighn
REFERENCE:	Ohnuki, Y., Marnell, M., Babcock, M., Lechner, J., Kaighn, E.; Chromosomal Analysis of Human Prostatic Adenocarcinoma Cell Lines; Cancer Research 40: 524-534; March 1980.
CRYOPRESERVED:	10/27/87
VIRAL PROFILE:	NEGATIVE
OTHER INFORMATION:	The subline, PC-3/M, had a similar karyotype and retained the parental PC-3 markers. PC-3/M had a more restricted chromosomal frequency distribution range. Nearly 73% of the PC-3/M cells examined had 60 or 61 chromosomes in contrast to the wide distribution seen in PC-3. Silver staining for nucleolus organizer regions indicated that the number of functional nucleolus organizer regions in PC-3 was proportional to the number of acrocentric chromosomes.

CARE AND USE OF TUMOR LINES

The transplantable tumors are distributed as frozen vials of tumor tissues or cell suspension. Transplantable tumors as well as cell culture lines are shipped as frozen vials of tissues on dry ice. Each tumor shipment includes an information sheet showing, among other items, the proper tumor designation; Cryopreserved date, In vivo host, etc.

Requested tumors are shipped in two to three weeks after the receipt of all completed paperwork. Shipments leave the Repository no later than Wednesday in order to reach their destination on weekdays. Before the shipment leaves the Repository, the Recipient is notified by email or fax of the waybill number and carrier, to allow time for the recipient to notify the Repository or make arrangements for receipt of cells, in the event that they will not be available to receive the shipment. An invoice for payment will follow and payment is due upon receipt. When vials are received they should be cultured right away, expanded and frozen down.

SUBMISSION OF TUMORS FOR CRYOPRESERVATION

Investigators who have unique and novel experimental tumor lines and are desirous of submitting their tumors to the Repository for cryopreservation and storage should write a letter of intent to the Project Officer. Upon acceptance, the Project Officer will inform the investigator in writing, and instructions on the procedure of shipment of the tumor materials to the Repository will be given. Tumor tissues or cells (frozen or ambient) are preferred over tumor-bearing animals.

At the Repository, the tumor line(s) will be tested for viral and bacterial contamination. When proven "clean," the line(s) will be expanded, in vivo or in vitro as appropriate, for large batch cryopreservation. Viability and growth of frozen tumors will be evaluated. The tumors will be included in the Repository's inventory, and upon joint approval of the submitting investigator and the Project Officer; they will be made available for distribution to the scientific community.

MOUSE TUMORS FROM THE JACKSON LABORATORY: These tumors formerly were maintained and distributed by the Jackson Laboratory. The list of available tumors begins on page 42 of the DCTD Tumor Repository Catalog. They were Cryopreserved at EG&G Mason Research Institute and are distributed only as vials of frozen tumor tissue. The required host animals for carrying the JAX tumors in serial transplantation may be obtained from:

Animal Resources
The Jackson Laboratory
600 Main Street
Bar Harbor, NE 04609
USA

T: 800.422.MICE
207.288.5845
F: 207.288.6150

FACTORS OF TRANSPLANTATION LOGISTICS: Since this inventory is concerned with transplantable animal and human tumor systems, the following information must be taken into consideration when planning studies involving tissues to be removed from cryopreservation:

a. Tumors have characteristic lag times (the time lapses between tissue implantation and the first palpable growths), which vary from several days to several months with different tumor systems.
b. Tumors also have characteristic rates of growth which markedly influence host survival, and which may vary from weeks to months with different tumor systems.
c. The above two factors are significantly prolonged in the first, and sometimes the second, transplant generation's post-freeze and thaw.
d. Histologically more complex tumors require two or three transplant generations, after thawing, before they return to normal histology and growth characteristics.

FREEZING PROCEDURE

Solid tumors are frozen as 2 x 2 x 2 mm fragments suspended in a freezing medium. Ascites or tissue-cultured cells are frozen as single cell suspension at a concentration of $10^6 - 10^7$ cells per ml. The freezing medium consists of appropriate tissue culture growth medium plus 10% DMSO and 10% fetal bovine serum.

Aseptically harvested ascites tumors are diluted in the freezing medium at a concentration of $10^6 - 10^7$ cells per ml. One ml suspension is pipetted into each 2 ml vial (Nunc cryotube). The vials are screw-capped tightly and labeled with a Repository number. Tissue cultured cells are prepared in a similar manner. For solid tumors, the aseptically excised tumor tissue is cut into 2 x 2 x 2 mm fragments after freeing it of necrotic materials. The fragments are placed in vials containing l.5 ml of freezing medium.

The processed tumors are frozen initially in a controlled slow-rate freezing apparatus at the rate of 0.5°C per minute to –20°C and 1°C per minute to -80°C. The frozen vials are stored in specific locations in the liquid nitrogen freezers in the Repository after the controlled freezing cycle.

RECOMMENDED THAWING PROCEDURE

Frozen tumor cells or tissues received from the Repository should be kept frozen at -70°C or lower until ready for use. For prolonged storage (more than two days), liquid nitrogen freezers are recommended.

The vials in which the cell lines are stored are reliable; however, they are very susceptible to contamination if thawed in a contaminated water bath. Thawing should be rapid, i.e., within 60-90 seconds. Place the vial in a warm water bath at 37-40°C and agitate vigorously to thaw. Immerse the vial in 70% ethanol before uncapping.

Implant immediately after thawing. The concentration of DMSO is not toxic to tissues and implantation may be made directly from the vial.

For tissue culture, transfer the contents of the vial into a petri dish or flask containing at least 10 volumes of the recommended culture medium and incubate. In order to remove the protective freezing additive (DMSO) from the culture medium, we suggest that the culture medium be changed 24 hours after seeding. If it is desired that DMSO be removed immediately, centrifuge the diluted suspension at approximately 125 X g for 10 minutes, discard the supernatant, and resuspend the cells in an appropriate volume of growth medium.

CAUTION: We strongly recommend wearing protective glasses or face shields when thawing tissues in glass vials.

TUMOR TRANSPLANTATION: It is recognized that transplantable tumor systems are experimental tools for investigators in scientific disciplines other than tumor biology or transplantation immunogenetics. Therefore, we encourage investigators with limited transplantation experience not to hesitate to contact the Tumor Bank for more detailed information on techniques. The following instructions may prove helpful.

TRANSPLANTATION TECHNIQUES: All equipment used should be sterile. Sacrifice the donor animal in a humane manner and immerse it totally in 0.1% Zephiran (Benzalkonium Chloride) or equivalent antiseptic solution.

A. **SUBCUTANEOUS TROCAR IMPLANTATION OF FRAGMENTS** – The tumors are implanted in the axillary region with puncture in the inguinal region. The sacrificed donor animal is pinned to a dissecting board, dorsal surface up. A midline incision is made through the skin, then horizontal incisions at the level of the fore and hind legs are made on the side, usually the right side, where the tumor was implanted. The flap of skin is pinned back, exposing the tumor mass. Free the tumor with scissors or forceps and transfer in to a sterile petri dish placed over ice. Free it of normal fascia and debride it of any necrotic material. Cut the tumor into cubes, usually about 2 x 2 x 2 mm. Thirteen gauge trocars are used to implant the tumors. Lubricate the Trocar by drawing up and expelling some sterile, balanced salt solution, e.g., Earle's, Tissue Culture Medium 199, Hank's etc. With forceps, place a fragment of tumor into the bevel end of the trocar. With a second person holding the animal into which the tumor will be implanted, swab the fur and skin in the area of implant with 70% ethanol. Pierce the skin in the inguinal area with the tip of the trocar, push the trocar forward to the axillary area and eject the tumor fragment from the trocar by pushing the plunger as far into the barrel as possible. In order to prevent the tumor fragment from being pulled back when the trocar is removed, grasp the tip of the trocar through the skin, and then remove the trocar. No more than 30 minutes should elapse from the time the tumor is removed from the donor until it is transplanted.

TROCARS FOR SUBCUTANEOUS IMPLANT OF FRAGMENTS ARE AVAILABLE FROM:

Popper and Sons, Inc.	NAME: Cancer Implant Needle Sets	
300 Denton Avenue	CATALOG NO:	7927
New Hyde Park, New York 11040	DESCRIPTION:	13 G X 3¼"
Telephone: (516) 248-0300	BRAND:	Perfektum

B. **PREPARATION OF TUMOR BREI** – Tumor brei is implanted with a syringe and 19-gauge or smaller needle. It may be implanted subcutaneously, intramuscularly, or intracerebrally. The tumor is excised, freed of normal fascia and debrided of necrotic tissue as described below. The tumor is minced with scissors and pressed through a sterile, 40-gauge stainless steel mesh into a sterile beaker in an ice bath. Add sterile, balanced salt solution, usually in an amount sufficient to prepare a 5% suspension. If specific numbers of cells are to be implanted, a sample of this brei can be counted in a hemocytometer and the brei diluted with sterile balanced salt solution as required. The usual inoculum size is o.2 or 0.3 ml per animal. No more than 30 minutes should elapse from tumor removal to transplantation in all recipient animals.

C. **ASCITES TUMORS** – These are implanted in the peritoneal cavity. Sacrifice and
Disinfect the donor animal as described above. Pin to a dissecting board, ventral surface up. Remove skin from the abdominal area. Withdraw fluid with a sterile syringe by inserting the needle through the abdominal wall. Transfer the fluid from the syringe to a sterile glass container in an ice bath. A cell count may be made, using a hemocytometer. Dilution is made with sterile, balanced salt solution. The usual mouse inoculum is 10^5 cells in 0.1 ml. It is injected intraperitoneally with a 23-gauge needle. No more than 60 minutes should elapse from the time fluid is taken from the donor and stored in the ice bath, and the diluted fluid is implanted in all the recipient animals.

BACTERIAL AND VIRAL MONITORING OF CRYOPRESERVED TISSUES: In addition to testing all freeze-runs for bacterial contamination, Cryopreserved tissues are tested for viral contamination by the MAP test. The viruses tested for are as follows: pneumonia virus of mice (PVM), reo virus-type 3 (Reo 3), Theiler's virus, Murine encephalitis (GD VII), polyoma (Poly), Sendia virus (Send), ectromelia, mouse pox (Ectro), lactic dehydrogenase virus (LDH).

IN VITRO ESTABLISHED CELL LINES:

A. **QUALITY CONTROL AND CHARACTERIZATION** – The quality control and characterization procedures for the incorporation of new cell lines into the Tumor Bank are as follows: Upon receipt, each cell line is immediately transferred to fresh antibiotic free medium and cultured for one week, after which it is tested for mycoplasma (PPLO) contamination. Standard culture

procedures under aerobic and anaerobic conditions, as well as the orcein staining procedure of Fogh, are used. The PPLO medium is extremely rich, and this procedure will also detect most bacterial and fungal contaminants.

B. **FREEZING AND STORAGE** – The cell cultures are frozen in ampules containing 1.0 ml of cell suspension at 2-6 x 10^6 cells/ml in fresh culture medium containing 10% DMSO. Freezing is performed as on page 2. Twenty-four hours after freezing, a representative ampule is removed, thawed, and viable cell count is performed, using the trypan blue dye exclusion procedure. The culture is also tested for its ability to initiate a heavy viable culture. Cell preparations, which show less than 50% viability or poor growth, are discarded and a new lot is prepared.

RECOMMENDED PROCEDURE FOR THAWING FROZEN CELL CULTURES

The vials in which the cell lines are stored are reliable; however, they are very susceptible to contamination if thawed in a contaminated water bath. The following procedures are provided to eliminate this problem.

Remove the ampule form the dry ice container and place it directly into a 37-40°C-water bath (or vessel) of freshly drawn water containing an effective concentration of disinfectant. The thawing should be vigorous and rapid (within 40-60 seconds). As soon as the thawing is complete, remove the ampule from the water bath and immerse in 70% ethanol at room temperature. All of the operations from this point should be carried out under strict aseptic conditions in a sterile room, cubicle, or hood.

Transfer the contents of the ampule (1 ml volume) into a 100 mm petri dish or 25-cm2 flask containing 8-10 ml of the recommended culture medium, and incubate at the appropriate temperature and carbon dioxide level.

In order to remove the protective freezing additive (DMSO) from the culture medium, we suggest that the culture medium be changed 24 hours after thawing. If it is desired that the freezing additive be removed immediately or that a more concentrated cell suspension be obtained, centrifuge the above diluted suspension at approximately 125 x g for 10 minutes, discard the fluid, and resuspend the cells in an appropriate volume of growth medium.