Modification Form for Permit BIO-LRCC-0014

Permit Holder: Frederick Dick

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

Jasmyne Carnevale

Matt Cecchini

Srikanth Talluri

Courtney Coschi

Additional Personnel
(Please list additional personnel here)

Daniel Passos

Mehdi Amiri

Charles Ishak

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. Give the full name - do not abbreviate.
Approved Microorganisms	E. coli, DH5 alpha, BL21, BS1365, and derivates of these strains	
Approved Primary and Established Cells	Rodent (primary): embroyos and organs, Human (established), C33A, Saos2, U20S, HeLa, H1299, IMR90, Phoenix -Eco Rodent (established), NIH 3T3, other 3T3s	primary human fibroblasts (1)
Approved Use of Human Source Material	blood (DNA extracted from cells), Human tissues (unpreserved)	
Approved Genetic Modifications (Plasmids/Vectors)	Oncogenes, Ras, E1A, E7, Adenovirus, Ecotropic retrovirus, pBABE, pBSK, pGEx, pcDNA, pCMV-neo-Bam, pET, pGL, pCR, BAC, pScodon, pEGFP PSP, pLMP, pGEM, pUC. SEE LIST of PLASMIDS. [Vectors]:	lentivirus, pLKO (other new vectors are pLKO based, or are CMV based vectors in the list already) (3)
Approved Use of Animals	Mice (2007-058)	
Approved Biological Toxin(s)		cholera toxin (2)

① primary human fibroblasts
② cholera toxin

③ lentivirus, pLKO (other new vectors are pLKO based or are CMV based vectors in the list already)

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 STORED, USED AND DISPOSED OF.

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca.

Signature of Permit Holder: _____

Current Classification: 2 **Containment Level for Added Biohazards:** 2+

Date of Last Biohazardous Agents Registry Form: Jan 24, 2011

Date of Last Modification (if applicable): _____

BioSafety Officer(s): Dave Ryder MARCH 16, 2011

Chair, Biohazards Subcommittee: _____ **Date:** _____

Appendix 1: Safety precautions and culture methods for primary human fibroblasts

Appendix 2: Protocols and safety procedures for lentivirus production

Appendix 3: MSDS and safety precautions for use of cholera toxin

Minimum Safety Guidelines
Recommended for Working with Human and Animal Cell Cultures

BIOSAFETY LEVEL

It is recommended that all cell cultures distributed by Coriell Cell Repositories be handled according to appropriate Biosafety Level guidelines established by U.S. Department of Health and Human Service, Centers for Disease Control and Prevention and the National Institutes of Health. The HHS publication *Biosafety in Microbiological and Biomedical Laboratories* (BMBL) 5th Edition, contains details of practices and schedules of pathogens according to Biosafety Level.

Coriell Cell Repositories recommends that (unless otherwise indicated) all CCR human and animal cell lines be handled at **BioSafety Level 2**.

SUPERVISION

Administrative Responsibilities
Responsibility of Management: Management should establish a biohazards committee to institute and enforce a health and safety policy which includes a specific safety program for work involving human cell lines. The program should meet applicable federal, state, and local regulations and include safety training, maintenance of accident records, and provisions for emergency treatment.

Responsibility of the Principal Investigator: The principal investigator is responsible for the preparation of safety protocols for the research program under his or her direction. The protocols should include appropriate procedures for use, storage, decontamination, disposal, and emergency treatment. The protocols should be approved by the biohazard committee and discussed with the research staff before starting the research program.

Medical Surveillance and Screening:
Physical Examinations: Appropriate pre-employment and periodic medical examinations are desirable for persons working with human cell lines.

Work Restrictions: Persons having reduced immunologic competency should be restricted from working with these human cell lines.

Serum Collection and Banking: Serum should be collected at the time of employment to establish a baseline reference. Serum should be collected immediately after accidental injection or ingestion and at an appropriate interval thereafter. For additional information, see CDC publication *Biosafety in Microbiological and Biomedical Laboratories* (BMBL) 5th Edition.

Laboratory Access
Access to the cell culture area should be restricted to persons working directly with the cell lines, or by specific authorization by the principal investigator or director of the laboratory.

PERSONNEL PRACTICES

Pipetting: Mechanical pipetting aids rather than mouth pipetting should be used for all pipetting procedures.

Eating, Drinking, and Smoking: Eating, drinking, and smoking should not be allowed in the same areas where cell lines are under study.

Protective Clothing: It is recognized that the criteria for protective clothing may vary according to the physical situation of the laboratory and the agents handled. Ideally, adequate protective clothing such as a fully fastened laboratory coat should be worn. This clothing should not be worn outside the work area once the work area has been entered.

PHYSICAL CONTROL PRACTICES

Recommended for all cell lines, but required for long-term lymphoid lines and their derivatives.

Ventilated Safety Cabinets or Hoods: Ventilated safety cabinets and hoods and other safety apparatus should be employed and should be tested at least annually to certify correct containment and operation. A list of specifications for satisfactory hoods and instructional materials may be obtained from the Office of Biohazards, NCI.

Housekeeping: Appropriate housekeeping procedures which suppress the formation of aerosols should be used. Work surfaces should be wiped down with an appropriate disinfectant before and after work with each cell culture and at the end of the workday.

Decontamination and Disposal: Contaminated glassware and similar materials should be appropriately decontaminated or stored for decontamination before removal from the work area for recycling or disposal. Liquid wastes should be decontaminated either chemically or by heat before being discharged to the community sanitary sewer system.

Protection of Vacuum Lines: Vacuum services, if used, should be protected with disposable absolute air filters and liquid traps. The effluent should be collected in liquid traps containing concentrated disinfectant.

Support

- Ordering
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- FAQs
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    - DNA Samples
  - Fibroblast Cultures
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  - Quick Search
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  - Quality Control
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- Miscellaneous
  - Feedback
  - Links
  - Contact Us
  - Cell Collections

Frequently Asked Questions about Fibroblast Cell Cultures
(Click on the question to be linked with the answer.)

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1. What medium should be used for culturing CCR fibroblasts?

MEDIA EQUIVALENTS

(Coriell provides the following information for comparative purposes only and does not recommend any particular manufacturer.)

Manufacturer	Catalog Number
BioWhittaker	12-662 (with sodium pyruvate)
GIBCO	10370-021
Sigma	M-5650

Alternative media for fibroblasts include: alpha-MEM and Dulbecco's modified MEM.

We add L-glutamine to a final concentration of 2 mM just before use. If long-term storage of cell culture medium is not an issue, commercially prepared medium containing L-glutamine can be used.

CCR does not use antibiotics or antifungals because of the danger of a cryptic infection in a cell repository. An investigator can add antibiotics if desired.

If a cell culture is growing slower than expected, our first approach is to switch to a different lot of pre-tested serum and/or to alter the serum concentration by 5%. Other causes of slow growth include: microbial contamination, too frequent subculture, too low density seeding at subculture, senescence of cell line, change in medium composition, incubator inadequacy in regulating temperature, humidity or CO2.

2. How should a newly received fibroblast cell culture be handled?

See the Web Catalog for details of the culture medium for individual cell lines.

Procedure

1. Wipe culture flasks with a disinfecting solution and place in a 37C incubator overnight with the cell sheet down. Do not remove medium (contains only 5% FBS to slow growth during transport). Observe cell sheet for confluency, morphology of cells and signs of contamination.
2. The next day the flask should examined as above and depending on the confluency of new culture's, the flask may be fed by withdrawing the shipping medium and covering the cells with growth medium (10 - 15% FBS) to a depth of 2mm or subcultured according to the cell count (see: Subculturing Fibroblast Cultures).
3. When subculturing a newly received fibroblast culture, the correct passage number must be determined. If the passage number is noted on the submission sheet or flask, the subcultured flasks should receive the next consecutive passage number.

3. How is a fibroblast cell line subcultured?

Supplies

- 0.53 mM EDTA in HBSS
- 0.04% trypsin/0.53 mM EDTA in HBSS
- Fibroblast Growth medium

Procedure

1. Prepare appropriate volumes of growth medium, EDTA, trypsin and "stop medium" (growth medium with FBS) flasks according to chart.
2. Dispense growth medium into flasks.

Subculturing Fibroblast Chart -- Mls of Solution Needed				
Flask Size	Growth Medium	EDTA	EDTA/Trypsin	"Stop" Medium
T12.5	4.5	4	1-2	2-3
T25	5-8	5	2-3	3-5
T75	20	8-10	4-5	5-7
T175	50	15	10	10

1. Remove medium by aspiration.
2. Add EDTA solution to the flask without dislodging the cell sheet and lay the flask cell side down. Cells should be watched closely through an inverted microscope for up to 10 minutes. If the cells begin to round or lift off the flask, the EDTA solution should be removed immediately.
3. Replace the EDTA solution with the EDTA/trypsin solution. Incubate the flasks at 37C for 4 to 7 minutes. Examine the flasks microscopically to make sure the cells begin to round up. The cells should have lifted off the surface after seven minutes. If the cells do not become detached after seven minutes, incubate an additional 1 to 2 minutes.
4. Tighten cap and lightly tap the side of the flask to lift the remaining cells from the flask. Wash the sides of the flask with

growth medium (Stop Medium) to inactivate the trypsin. Gently mix cells and medium. Remove an aliquot for a cell count.
5. Seed the flasks according to the following chart:

Flask Size	Flask Seeding Ranges	Final Volume
T12.5	1.0 - 2.5 x 10^5	4.5 ml
T25	2.0 - 6.0 x 10^5	5-8 ml
T75	9.0 - 15.0 x 10^5	15-30 ml
T175	1.9 - 2.4 x 10^6	45-60 ml

Note: Basic seeding rule is 1.0 - 1.4 x 10^4 cells/cm^2

1. Place flasks in 37C, 5% CO2 incubator, loosen caps if not vented. Check the cultures after a few hours for cell attachment and pH. The time between subcultures will depend on the incubation temperature, cell line, and serum and medium. The majority of mammalian cell lines require subculturing every 3-7 days. If the duration is longer than 5 days, change the culture medium every 3-4 days.

4. How are fibroblast cultures frozen for cryogenic storage?

Supplies

- 0.53mM EDTA in HBSS
- 0.04% trypsin/0.53 mM EDTA in HBSS
- Fibroblast Growth medium
- Fibroblast Freeze medium (growth medium with 10% glycerol or 5% DMSO)

Procedure

1. If cells are to be frozen in 10% glycerol, complete freeze medium may be kept at room temperature until used. Freeze medium prepared with DMSO should be kept refrigerated until used.
2. Each flask that is to be pooled for the freeze (freeze pool) should be examined microscopically for contamination and any unusual growth pattern. One flask should be maintained as a "backup" flask until the viability of the freeze can be checked.
3. Aspirate the growth medium from each flask. Add EDTA to each flask without dislodging cells and incubate at room temperature for 10 minutes. If cells begin to round or the edges of the cell sheet constrict, remove EDTA immediately.
4. Replace the EDTA solution with the EDTA/trypsin. Incubate the flasks at 37C for 4 to 7 minutes. Examine the flasks microscopically to make sure the cells begin to round. The cells should have lifted after seven minutes. If the cells do not become detached after seven minutes, incubate an additional 1 to 2 minutes.
5. Once the cells have lifted, add an equal or greater amount of growth medium to each flask to inactivate the trypsin. Gently triturate and then transfer cell suspension from all flasks and pool cells in a centrifuge bottle. Maintain the centrifuge bottle in ice while pooling flasks.
6. Remove an aliquot of the freeze pool, count the cells and calculate the total viable cells in the freeze pool. Centrifuge the freeze pool at 60-100 x g for 10 minutes at 8-10C.
7. Remove the supernatant and resuspend the cell pellet using gentle trituration in freeze medium at a final concentration of at least 5 x 10^5 viable cells per ml.
8. Distribute one-ml aliquots of the cell suspension into glass ampoules or plastic cryovials.
9. Seal glass ampules using an oxygen-propane flame. Check each glass ampule for pinholes or glass bubbles formed during sealing by immersion in a methylene blue/ethanol solution at 4C.
10. Freeze the ampules or cryovials at a rate of 1C per minute.
11. Frozen cell stocks are stored in the liquid nitrogen tanks. Glass ampules are submerged in liquid; plastic cryovials are stored in the vapor phase.
12. One ampule or cryovial from every freeze is recovered and cultured to check for viability and sterility.

5. How should fibroblast cell cultures be recovered from cryogenic storage?

Procedure

1. Prepare appropriate recovery medium (see Shipping Sheets for individual cell line).
2. Remove one ampule or cryovial from frozen storage and place immediately in a 37C water bath and agitate vigorously.
3. Once completely thawed, wipe ampule or cryovial with a 70% alcohol sponge. Score the neck of a glass ampule and open utilizing an ampule opener.
4. Remove the contents of the ampule or cryovial using a sterile transfer pipette and place in a T25 tissue culture flask containing 5 ml of the appropriate fresh growth medium for fibroblast cultures.
5. If a cell count is required, mix the contents of the flask gently with a 1 ml pipette and remove 0.2 ml for a 1:5 diluted cell count. Place the flask in the 37C incubator lying cell surface down. Gently swirl the flask to distribute the cell suspension evenly over the flask surface. Adjust the cap to allow appropriate gas exchange (depending on buffering system of the medium). Fibroblast cultures should be refed with fresh medium the day after recovery.
6. Some cell lines recover better if all traces of cryoprotectant are removed by washing and centrifugation. Transfer the contents of ampule or cryovial to a 15-ml centrifuge tube with 3 ml of growth medium. Centrifuge for 5 min at 60-100xg and 10C. Remove supernatant, resuspend pellet, and transfer to a T25 flask with a final volume of 5 ml.
7. Culture as described for subculturing fibroblasts. If cells fail to proliferate after 1-2 weeks, expand the backup flask for a second freeze.

-------- Original Message --------
Subject: Re: Biohazard Modification (Dick)
Date: Mon, 21 Mar 2011 10:57:13 -0400
From: Fred Dick <fdick@uwo.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>

Lentivirus

Jennifer,

Sorry for not responding sooner. My daughter is out of daycare sick and it's taken all my time to look after her. The vectors that we intend to use for packaging lentiviruses are in the modification. The shRNA vector is called pLKO. I've attached the protocol from the Open Biosystems website. We will use 293 cells for packaging. I'll add them to the hard copy of the protocol modification and send it. I've also filled out your table and included it below.

Fred

Dr. Golding – Mann Lab
Modified 3/14/2011

VIRUS ROOM OPERATING PROCEDURES
LHSC ROOM A4-820A

The virus room is designed as a biohazard level 2+ (BSL-2+) room. All work conducted in the room must be carried out using BSL-2+ procedures, at the minimum. These procedures are designed to limit the physical contact with the material used. The viral agents used in this room require special handling procedures, due to their greater inherent risks, and should be handled using these modified BSL-3 procedures, (commonly referred to as BSL-2+), which are designed to limit exposure to aerosolized virus. Examples of BSL-2 viruses are ecotropic retrovirus, bacculovirus, non-oncogenic amphotropic retrovirus, lentivirus and adenovirus. Examples of BL-3 viruses are oncogenic amphotropic retrovirus, lentivirus or adenovirus, herpesvirus, or any of the human infecting viruses expressing unknown genes (as in a library). BSL-2+ procedures are included at the end of each subsection and should be followed **in addition** to the standard BSL-2 protocols.

A. General Operating Procedures -

1. Access in and out of the virus room should be permitted to authorized individuals (properly trained) only to prevent inadvertent contamination of personnel or cross contamination of cell lines. Containment Laboratory must be kept locked at all times.
2. Experiments should be carefully reviewed for all reagents and supplies necessary before initiating any work to minimize trips into the room.
3. Only items used in the virus room should be stored in the virus room.
4. No organisms used in the virus room may be used or transferred to any other tissue culture facility within the building.
5. No food/drink/cosmetic should be brought into the room.

B. Training

1. All personnel using the virus room must receive training from a qualified researcher, with experience using the specific class of viral agent to be used prior to initiating any experiments. (Approved users will be posted next to the entrance door).
2. All individuals using the virus room should be trained in sterile technique, and standard tissue culture practice as well as spill containment procedures. (Complete with proper usage of an N95 filter mask)

C. Personal Protective Equipment & Hygiene

1. Disposable solid front lab coats and gloves must be worn whenever working in the virus room and disposed of as regulated medical waste (RMW) before leaving the room.
2. Hands should be thoroughly washed before leaving the virus room.

BSL-2+ Safety glasses should be worn whenever performing BSL-2+ work practices.

D. Incubator Use

1. Incubators must be labeled with the specific organism(s) used along with the user name.
2. If an incubator becomes contaminated the procedures posted in the virus room for decontaminating an incubator must be followed.

E. Use of Biological Safety Cabinets

1. Biological safety cabinets (BSC) should be used following the operating instructions posted in the room.
2. At the beginning of each session a fresh batch of 10% bleach should be made and a small aliquot placed in the bottom of each of the double waste traps.
3. BSC should be decontaminated before and after use. 70% ethanol, wescodine® or another suitable decontamination agent. Bleach in any concentration should be avoided because over time this can corrode stainless steel surfaces.
4. All material must be surface decontaminated before removing from BSC.
5. If a BSC is not working properly it should be reported to the Responsible Official immediately.
6. Check the liquid waste traps before starting work. The traps should be empty before starting work. Check that all rubber vacuum lines are attached and that the inline HEPA filter is in place. Ensure that there is approximately 2 inches of a 10% bleach solution in each of the trap flasks. This allows disinfections of the elements to start as they are suctioned into the traps.
7. When empting traps pour the contents of the trap into a 4-liter plastic beaker that has been placed in the sink. Prior to pouring off the traps ensure that there is at least 1 liter of 10 % bleach solution in the plastic beaker. Gently pour the waste solution from the flask into the beaker taking great care not to splash the material. PPE should be worn when performing this operation (Safely Glasses, disposable gloves, and lab coat).

F. Refrigerator and Freezer Use

1. Refrigerators are only for reagents, stock solutions, and media used in the virus room.
2. All stock solutions and media must be properly labeled with the chemical name, user name, and date prepared or purchased.
3. All reagents, stock solutions, and media no longer in use must be properly discarded.

G. Microscope Use

1. The compound microscope is dedicated to work in the virus room only.
2. If the microscope is not working properly contact the Responsible Official.

H. Centrifuge Use

1. Material centrifuged outside the virus room must be transported and labeled in accordance with sections I and K of these procedures.
2. Centrifugation must be carried out in sealed centrifuge cups/rotors, which are to be unloaded in BSC.

BSL-2+ Materials should not be centrifuged outside the virus room.
BSL-2+ Viruses should not be "spinocculated" in or out of the virus room.

I. Labeling

1. All containers (boxes or plastic bags containing eppendorf tubes) used to store samples should be labeled with a biohazard symbol and the name of the organism.
2. All doors, to the virus room, incubators, freezers and refrigerators should be labeled with a biohazard label containing the name of the organisms present, user name, and the biohazard level.
   All transport containers used to carry material outside the virus room should be labeled with a biohazard label containing the name of the organism and the user name.

J. Waste Disposal

1. All disposable lab ware and personal protective equipment generated in the BL2+ virus room should be disposed of in a RMW box or autoclave in the virus room.
2. All liquids contaminated with virus material must be treated with a 10% bleach solution before disposal down the sink.
3. All culture dishes and culture flasks containing liquids can be disposed in a RMW box provided they are properly sealed in an impervious plastic bag.

BSL-2+ all glass and plastic pipet tips and pasteur pipets should be bleached with 10% bleach solution **in the hood** prior to placing them in either a sharps container or dirty pipet bucket. The use of nedles, syringes and other sharps should be strictly limited.

K. Material Transport

1. Always transport biohazardous material outside the virus room in an unbreakable well-scaled primary container placed inside a leakproof,

closed, and unbreakable secondary container (plastic cooler, bio-specimen pack, etc.). The container should be labeled with the biohazard symbol.

BSL-2+ BSL-2+virus should never be transported out of the virus room.

L. Spills

1. If a spill occurs in or outside the virus room, in an incubator, centrifuge or other type of equipment, follow the appropriate procedures posted for a biological spill.

BSL-2+ Spills larger than a paper towel should be immediately reported to the safety office.

M. Personal Contamination

1. If an exposure occurs to the skin wash the affected area thoroughly with using antimicrobial soap and report the incident to OH&S.
2. If an exposure occurs to the eyes immediately with running water for 15 minutes using an eyewash and forcibly hold eye(s) open to ensure effective wash behind eyelids. Report the incident to OH&S.
3. If an exposure occurs via a needle stick or puncture wound wash the affected area thoroughly using antimicrobial soap for five 5 minutes and report the incident to OH&S. If the incident occurs after hours, proceed to the Hospital Emergency Room.
4. If an exposure is suspected via inhalation report the incident to OH&S.

Spill Inside an Incubator

If you notice there has been a leak of tissue culture material inside an incubator follow the procedures written below. Always have a complete biological spill kit ready before starting any clean up.

- Immediately close the door to the incubator when a spill is first discovered.
- If it is a large volume spill (>10 mL) call OH&S for assistance.
- Clear the area of all personnel and post a warning sign "Do Not Enter-Biological Spill".
- Wait 30 minutes for aerosols to settle before attempting to clean up the spill. Be sure to wear the N95 filter mask from the spill kit when attempting to clean up the spill.
- Wear a lab coat, safety goggles and gloves during the clean up.
- Very slowly open the door to the incubator and spray disinfectant (Wescodyne or 10% bleach solution) into the incubator compartment. Be sure to spray all interior surfaces of the incubator.
- Close the incubator door and wait 10 minutes.
- Slowly open the incubator and wipe the shelves and walls with disinfectant. Add Wescodyne solution (approximately 5 mls.) to the water in the bottom of the incubator and let stand for 30 minutes.
- Remove the water bath tray from the bottom of the incubator and discard the water to the sanitary sewer. Replace the discarded water in the incubator.
- Place all contaminated debris and protective clothing into biohazard bag(s), seal the bag(s) and run through the autoclave located in the BSL-2+ room.
- Wash hands using antimicrobial soap.

Spill Inside a Centrifuge

If you notice there has been a leak outside the safety bucket or rotor of a centrifuge assume a spill has occurred and follow the procedures written below. Always have a complete biological spill kit ready before starting any clean up.

Clean-up of BSL-1 or BSL-2 Spill

- Immediately close the lid to the centrifuge when a spill is first discovered.
- Clear the area of all personnel and post a warning sign "Do Not Enter-Biological Spill".
- Wait 30 minutes for aerosols to settle before attempting to clean up the spill. Be sure to wear the N95 filter mask included in the spill kit.
- Wear a lab coat, safety goggles and gloves during the clean up.
- Spray the outside lid and handle of the centrifuge with disinfectant (10% bleach), wait 10 minutes, and remove disinfectant using paper towels. Reapply as needed.
- Very slowly open the lid to the centrifuge and spray disinfectant into the centrifuge compartment. Be sure to spray the safety bucket and rotor.
- Remove safety buckets and rotors and place in a bio-safety bag then carry the closed bag to a biological safety cabinet located in the virus room and thoroughly disinfect.
- Place all contaminated debris and protective clothing into biohazard bag(s), seal the bag(s) and place in a regulated medical waste (RMW) box.
- Wash hands using antimicrobial soap.

Clean-up of BSL-2+ Spill

- Follow the same clean-up procedure for BSL-1 or BSL-2 spills and:

    - If it is a large volume spill (>10 mL) call OH&S for assistance.
    - Dispose of all contaminated debris and protective clothing using the autoclave.

Spill Inside a Biological Safety Cabinet

Always have a complete biological spill kit ready BEFORE starting any clean up. – make sure it includes:

N95 filter mask
Universal Spill pads
Universal Spill sock
Mercury sponge
A bag of universal sorbent
Latex gloves
Rubber gloves
Dust pan and brush
Paint scraper
Roll of paper towel
Garbage bags

- Notify your Supervisor Immediately
- Notify others in the area that a spill has occurred.
- Wear a lab coat, safety glasses and gloves during clean-up
- Allow biological safety cabinet (BSC) to run during clean-up
- Soak up spilled material with disposable paper towels (work surfaces and drain basin) and apply disinfectant (10% bleach) with a minimum of 10 minutes of contact time. Reapply if it evaporates before the 10 minute period is up.
- Wipe up spillage and disinfectant with disposable paper towels.
- Wipe the walls, work surfaces and any equipment in the BSC with a disinfectant soaked paper towel.
- Discard contaminated disposable materials in biohazard bag(s), seal the bags, and place in a regulated medical waste (RMW) box.
- Place contaminated reusable items in biohazard bags, or heat resistant pans or containers with lids before autoclaving and further clean up.
- Expose non- autoclavable materials to disinfectant, 10-minute contact time, before removal from the BSC. Reapply as needed.
- Remove protective clothing used during clean up and place in a RMW box.
- Run the BSC for at least 10 minutes after clean up before resuming work.
- Inform all users of the BSC that a spill occurred and it was properly cleaned up.

Personal Contamination

Decontamination Procedure

Skin exposure:

- If exposure occurs to the skin wash the affected area thoroughly using antimircobial soap and report the incident to Occupational Health and Safety.

Splash to eyes:

- If an exposure occurs to the eyes immediately flush with running water for 15 minutes using eyewash and forcibly hold eye(s) open to ensure effective wash behind the eyelids. Report the incident to Occupational Health and Safety.

Needle stick or puncture:

- If an exposure occurs due to a needle stick or puncture wound wash affected area thoroughly using antimicrobial soap for 5 minutes and report the incident to Occupational Health and Safety.

Inhalation:

- If an exposure is suspected via inhalation report the incident to Occupational Health and Safety.

- **Safety Office Emergency phone extension**

55555

Operating Procedures for Class II Biological Safety Cabinets

- Turn off UV light if in use and turn on fluorescent light and blower. Ensure that sash is in proper position. Hold tissue at the middle of edge to ensure intake of air.
- Clean work surfaces by wiping with 70% alcohol or Wescodyne solution. Wipe off each item you need for your procedures before placing inside the biological safety cabinet (BSC).
- DO NOT place any objects over the front air intake grille. DO NOT block the rear exhaust grille. DO NOT store a objects in the Biological Safety Cabinets. When preparing for session places all needed objects in the hood and mark them as contaminated with a red sticker. At the end of the session remove all the objects and store in your designate space.
- Segregate contaminated and clean items. Work from "clean to dirty." Enter and exit cabinet straight on.
- Place a covered tray with disinfectant and/or sharps container inside the BSC near rear for pipette discard. DO NOT use vertical pipette discard canisters on the floor outside the BSC.
- It is not necessary to flame items. This can create air turbulence in airflow and will compromise sterility. Heat build up could damage the HEPA filter. A buildup in gas can cause an explosion.
- Move arms slowly when moving or introducing new items into a BSC.
- If you use a piece of equipment that creates air turbulence in the BSC, such as a microfuge or blender, place the equipment in the back 1/3 of the cabinet. Stop all other work while the equipment is operating.
- Wait 3 – 5 minutes for contaminants to purge from work area.
- Protect the building vacuum system from biohazards by placing a cartridge filter between the vacuum trap and the source valve in the BSC. Use a double flask trap to prevent liquid escape and empty the primary flask when it reaches the halfway mark.
- Clean up spills immediately. Wait 10 minutes before resuming work.
- When work is finished, close/cover containers, remove all material and wipe all interior surfaces with 70% alcohol or Wescodyne solution.
- Turn off fluorescent light and blower.

- Remove lab coat, gloves and other protective equipment (PPE) and wash hands thoroughly before leaving the laboratory.

Will genetic modification(s) of bacteria and/or cells involving viral vectors be made?

O YES, complete table below O NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results from transduction
Lentivirus	*pLKO*	*Openbiosystems, Addgene, other investigators*	*Various shRNAs*	*We will target growth regulating genes, so the result will be cell proliferation when cells would normally stop.*

* Please attach a Material Safety Data Sheet or equivalent.

Technical Manual

Thermo Scientific Open Biosystems Expression Arrest - The RNAi Consortium (TRC) Lentiviral shRNA

Product Description

The Open Biosystems Expression Arrest™ TRC library is the result of a collaborative research effort based at the Broad Institute of MIT and Harvard, and includes six MIT and Harvard associated research institutions and five international life sciences organizations. The goal of TRC is to create lentiviral shRNA libraries targeting 15,000 human and 15,000 mouse annotated genes with multiple constructs per gene. We have partnered with the TRC to make these shRNA libraries available to researchers worldwide.

Shipping And Storage

Individual constructs are shipped as bacterial cultures of *E. coli* (DH5α) in LB-Lennox (low salt) broth with 8% glycerol, 100 µg/ml carbenicillin. Individual constructs are shipped on wet ice. Collections are shipped in 96-well plate format on dry ice. Individual constructs and collections should be stored at -80°C.

All cultures are checked for growth prior to shipment.

To allow any CO_2 that may have dissolved into the media from the dry ice in shipping to dissipate, please store plates at –80°C for at least 48 hours before thawing.

Important Safety Note

Please follow the safety guidelines for use and production of vector-based lentivirus as set by your institution's biosafety committee. In general, the NIH Office of Biotechnology BSL2 or BSL2+ guidelines should be followed.

Design Information

The TRC Library Design

The shRNA constructs were designed to include a hairpin of 21 base pair sense and antisense stem and a 6 base pair loop. Each hairpin sequence was cloned into the lentiviral vector (pLKO.1) and sequence verified. Multiple constructs (4-5) were created per gene to ensure adequate coverage of the target gene. The TRC predicts that 1 or 2 out of the 4-5 constructs offered per gene are expected to give at least 70% knockdown.

Features of the TRC shRNA library include:

- Rules-based shRNA design for efficient gene knockdown
- Already cloned into lentiviral vectors
- Amenable to *in vitro* and *in vivo* applications such as the creation of stable cell lines
- Lentiviral vector enables transduction of primary and non-dividing cell lines
- Broad coverage: 4-5 constructs per gene

The TRC Hairpin Design

Stem: 21 bases
Loop: 6 bases, *Xho*I restriction site: CTCGAG
Flanking = 5' CCGG overhang for *Age*I
3' TTTTT termination for Pol III and AATT overhang for *Eco*RI

Vector Information

The pLKO.1 HIV-based lentiviral vector (Figures 1-2, Table 1) allows for transient and stable transfection of shRNA and also the production of viral particles using lentiviral packaging cell lines. Stable cell lines can be selected using the puromycin selectable marker.

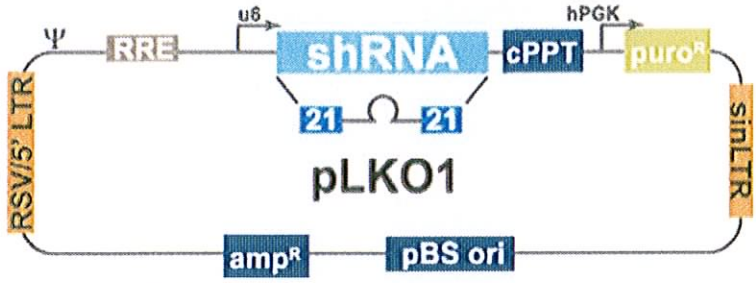

Figure 1. The pLKO.1 vector

Table 1. Features pLKO.1 vector

Vector Element	Utility
Human U6 Promoter	RNA generated with four uridine overhangs at each 3' end
hPGK	Human phosphoglycerate kinase promoter
PuroR	Puromycin mammalian selectable marker
3' SIN LTR	3' self inactivating long terminal repeat (Shimada, *et al.* 1995)
f1 ori	f1 origin of replication
AmpR	Ampicillin bacterial selectable marker
5'LTR	5' long terminal repeat
RRE	Rev response element
cPPT	Central polypurine tract

Vector Map

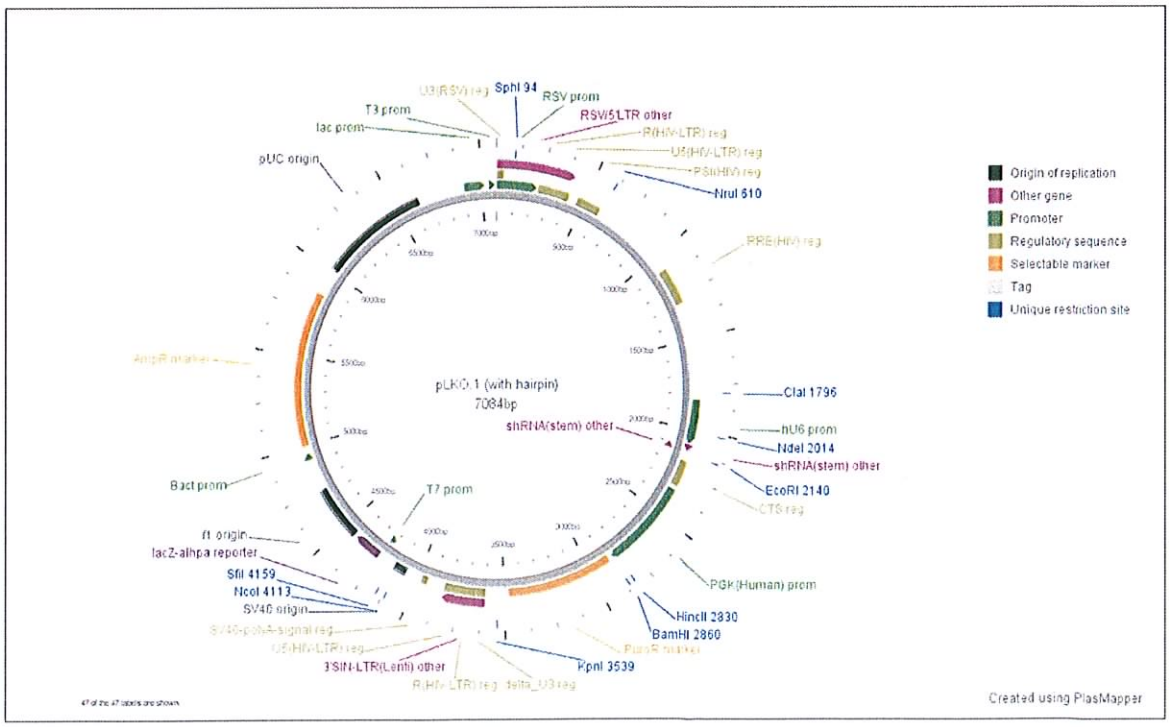

Figure 2. Map of the pLKO.1 vector

Antibiotic Resistance

pLKO.1 contains 2 antibiotic resistance markers (Table 2). The TRC recommends the use of carbenicillin instead of ampicillin for the growth and maintenance of pLKO.1.

Table 2. Antibiotic resistances conveyed by pLKO.1

Antibiotic	Concentration	Utility
Ampicillin (carbencillin)	100 µg/ml	Bacterial selection marker (outside LTRs)
Puromycin	variable	Mammalian selectable marker

Protocols

There are protocols recommended by the TRC for culturing, plasmid prep, virus production and transduction of TRC lentiviral shRNA constructs. These protocols can be accessed from the following link: http://www.broad.mit.edu/genome_bio/trc/publicProtocols.html

Culturing Protocols and Maintenance of pLKO.1

The Expression Arrest TRC shRNA Library is constructed in the pLKO.1 vector. This vector allows for both transient and stable gene knockdown via the mechanism of RNA interference. The vector is capable of producing self-inactivating lentiviral particles when used in conjunction with lentiviral packaging lines.

In order to obtain a good yield of cells in a short period of incubation, rich media containing carbenicillin and 8% glycerol should be used to culture pLKO.1 constructs. The TRC recommends the use of carbenicillin instead of ampicillin. An incubation period of 14-20 hours at 37°C with aeration is sufficient. It is recommended that the cultures remain frozen at –80°C when not in use. Freeze/thaw cycles do not seem to have any detrimental effect providing the cultures are not incubated at room temperature or higher, for long periods of time.

Protocol I - Replication

Table 3. Materials for plate replication

Item	Vendor	Catalog #
LB-Lennox Broth (low salt)	VWR	EM1.00547.0500
Peptone, granulated, 2 kg - Difco	VWR	90000-368
Yeast Extract, 500 g, granulated	VWR	EM1.03753.0500
NaCl	Sigma	S-3014
Glycerol	VWR	EM-2200 or 80030-956
Carbenicillin	Novagen	69101-3
Puromycin	Cellgro	61-385-RA
96-well microplates	Nunc	260860
Aluminum seals	Nunc	276014
Disposable replicators	Genetix	X5054
Disposable replicators	Scinomix	SCI-5010-OS

2X LB broth (low-salt) media preparation

LB-Broth-Lennox	20 g/l
Peptone	10 g/l
Yeast Extract	5 g/l
Appropriate antibiotic(s) at recommended concentration(s)	
*Glycerol	8% for long term storage

**LB media can be used instead of 2X LB*

***Glycerol can be omitted from the media if you are culturing for plasmid preparation. If making copies of the constructs for long term storage at –80°C, 8% glycerol is required.*

Replication of Plates

Prepare target plates by dispensing ~160 µl of LB media supplemented with 8% glycerol and appropriate antibiotic (100 µg/ml of carbenicillin).

Prepare Source Plates

1. Remove foil seals while the source plates are still frozen. This minimizes cross-contamination.
2. Thaw the source plates with the lid on. Wipe any condensation underneath the lid with a paper wipe soaked in ethanol.

Replicate

1. Gently place a disposable replicator in the thawed source plate and lightly move the replicator around inside the well to mix the culture. Make sure to scrape the bottom of the plate of the well.
2. Gently remove the replicator from the source plate and gently place in the target plate and mix in the same manner to transfer cells.
3. Dispose of the replicator.
4. Place the lids back on the source plates and target plates.
5. Repeat steps 1-4 until all plates have been replicated.
6. Return the source plates to the -80°C freezer.
7. Place the inoculated target plates in a 37°C incubator for 14-20 hours.

Note: Due to the tendency of all viral vectors to recombine, we recommend keeping the incubation times as short as possible and avoid subculturing. Return to your glycerol stock for each plasmid preparation.

Protocol II - Plasmid Preparation

Culture Conditions For Individual Plasmid Preparations

Most plasmid mini-prep kits recommend a culture volume of 1–10 ml for good yield. For shRNA constructs, 5 ml of culture can be used for one mini-prep generally producing from 5–20 µg of plasmid DNA.

1. Upon receiving your glycerol stock(s) containing the shRNAmir of interest store at -80°C until ready to begin.
2. To prepare plasmid DNA first thaw your glycerol stock culture and pulse vortex to resuspend any *E. coli* that may have settled to the bottom of the tube.
3. Using a sterile loop or a pipette tip, streak the shRNA culture onto a LB agar plate containing 100 µg/ml carbenicillin. Incubate the plate overnight at 37°C. Return the glycerol stock(s) to –80°C.
4. The following day, pick 1 to 3 colonies from the agar plate and inoculate 6 ml of the 2X LB. Incubate at 37°C for 16-20 hrs with vigorous shaking (300 rpm).
5. The following day remove 1 ml of the culture and place in a sterile 2 ml sterile microcentrifuge tube. Place this tube at 4°C until the plasmid DNA from the remaining culture has been analyzed. Pellet the remaining 5ml culture and begin preparation of plasmid DNA. We recommend preparing Ultra-pure DNA to ensure both high-purity and low endotoxin levels (Qiagen Catalog #12123) as required for transfection into eukaryotic cells.

   If you wish to continue at a later time cell pellets can be kept frozen at –20°C overnight.
6. Run 3-5 µl of the plasmid DNA on a 1% agarose gel. The uncut pLKO.1 shRNA constructs run at about 7-10 kb. Prepare an 8% glycerol stock culture using the 1 ml of culture you removed prior to plasmid preparation. This culture can be used for future plasmid preparations but it is still recommended you streak isolate and work from a fresh colony. Store at –80°C.

   Note: Due to the tendency of all viral vectors to recombine we recommend keeping the incubation times as short as possible and avoid subculturing. Return to your original glycerol stock or the colony glycerol stock for each plasmid preparation.

Gel images of plasmid isolated from cultures grown under the above conditions are shown below (Figure 3).

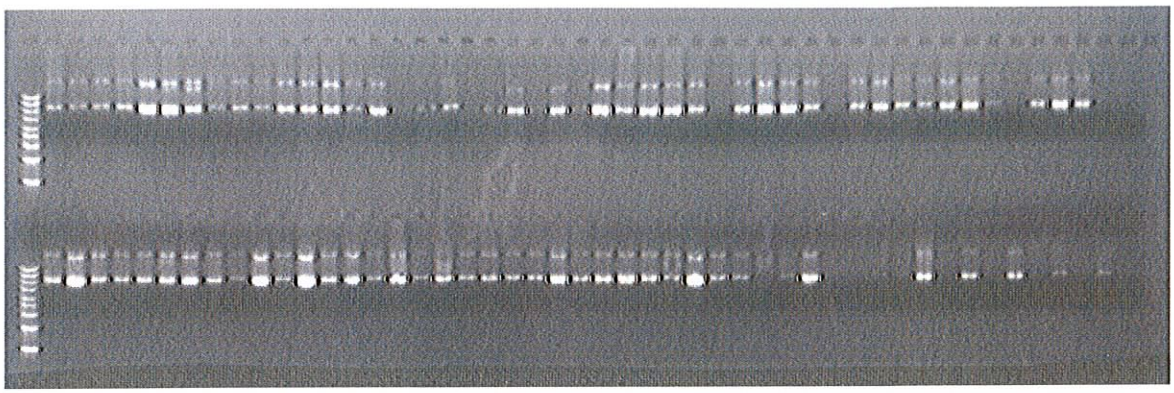

Figure 3. 1.5 ml cultures of 92 different shRNA constructs after 20 hours of incubation at 37°C with shaking (~170 rpm). 2X LB media (low-salt) with 8% glycerol was used for culturing.

Protocol III - Restriction Digest

You may wish to restriction digest a sample of your plasmid DNA following plasmid DNA preparation. The following is a protocol for dual restriction enzyme digestion using *Bam*HI and *Nde*I for quality control of pLKO.1 vectors.

1. Using filtered pipette tips and sterile conditions add the following components, in the order stated, to a sterile PCR thin-wall tube.

Sterile, nuclease-free water	14.8 µl
Restriction enzyme *Bam*HI	1.0 µl
Restriction enzyme *Bam*HI 10X buffer	2.0 µl
BSA (10X, 10 mg/ml)	0.2 µl
DNA sample 1 µg, in water or TE buffer	1.0 µl
Restriction enzyme *Nde*I 20U	1.0 µl
Final volume	20.0 µl

2. Mix gently by pipetting.
3. Incubate in a thermalcycler at 37°C for 2.5 hours to digest then at 70°C for 20 minutes to kill the enzyme.
4. Add 4 µl of 6X Loading Dye (or another appropriate DNA loading buffer), and proceed to gel analysis.
5. Load the gel with 20 µl of the digested samples on a 1% agarose gel. Also run 1 µl (1 µg) of the uncut sample combined with 16 µl of water and 3µl of 6X dye alongside the digested samples.
6. The digest will produce two fragments one approximately 6.3 kb band and a 794 bp band.

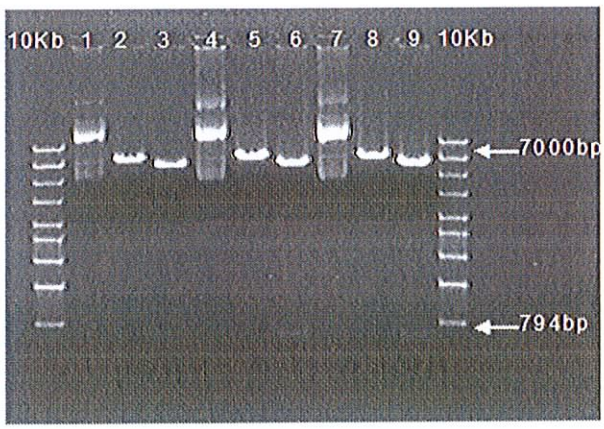

Figure 4. The 1% agarose gel above contains -10 kb ladder followed by undigested sample and restriction digests of three TRC shRNA clones (lanes 2-9), The lanes are loaded as follows: 1 - Clone E1 Uncut plasmid. 2 - Clone E1 Cut with *Bam*HI. Expected to linearize at 7032 bp. 3 - Clone E1 Cut with *Bam*HI and *Nde*I. Band sizes of 6238 bp and 794 bp expected. 4-6 Repeat of 1-3 only with clone E2. 7-9 Repeat of 1-3 only with clone F1.

Protocol IV-Transfection

The protocol below is optimized for transfection of the shRNA plasmid DNA into HEK293T cells in a 24-well plate using serum-free media. If a different culture dish is used, adjust the number of cells, volumes and reagent quantities in proportion to the change in surface area (Table 4).

It is preferable that transfections be carried out in medium that is serum-free and antibiotic-free. A reduction in transfection efficiency occurs in the presence of serum, however it is possible to carry out successful transfections with serum present (see Transfection Optimization).

Warm Thermo Scientific Open Biosystems Arrest-In to ambient temperature (approximately 20 minutes at room temperature) prior to use. Always mix well by vortex or inversion prior to use.

Maintain sterile working conditions with the DNA and Open Biosystems Arrest-In™ mixtures as they will be added to the cells.

Table 4. Suggested amounts of DNA, medium and Arrest-In reagent for transfection of shRNA plasmid DNA into adherent cells.

Tissue Culture Dish	Surface Area per Plate or Well (cm^2)	Total Serum-Free Media Volume per Well (ml)	Plasmid DNA (µg)*	Arrest-In (µg)**
60 mm	20.0	2.0	4.0	21.0
35 mm	8.0	1.0	2.0	10.0
6-well	9.4	1.0	2.0	10.0
12-well	3.8	0.5	1.0	5.0
24-well	1.9	0.25	0.5	2.5
96-well	0.3	0.1	0.1 - 0.2	0.5 - 1.0

*Recommended starting amount of DNA. May need to be optimized for the highest efficiency.
**Recommended starting amount of Arrest-In reagent. See Transfection Optimization.

1. The day before transfection (day 0), plate the cells at a density of 5 x 10^4 cells per well of a 24-well plate.
   Full medium (i.e. with serum and antibiotics) will be used at this stage.
2. On the day of transfection, form the DNA/Arrest-In transfection complexes.
   The principle is to prepare the shRNA plasmid DNA and transfection reagent dilutions in an equal amount of serum-free medium in two separate tubes. These two mixtures (i.e. the DNA and the Arrest-In) will be added to each other and incubated for 20 minutes prior to addition to the cells. This enables the DNA/Arrest-In complexes to form.
   a. For each well to be transfected, dilute 500 ng shRNA plasmid DNA into 50 µl (total volume) of serum-free medium in a microfuge tube.
   b. For each well to be transfected, dilute 2.5 µg (2.5 µl) of Arrest-In into 50 µl (total volume) serum-free medium into a separate microfuge tube.
   c. Add the diluted DNA (step a) to the diluted Arrest-In reagent (step b), mix rapidly then incubate for 20 minutes at room temperature.
      This will give a 1:5 DNA:Arrest-In ratio which is recommended for optimal transfection into HEK293T cells. Your total volume will be 100 µl at this stage.
   d. Set up all desired experiments and controls in a similar fashion as outlined in Table 5. It is also advisable to set up an Arrest-In only control.

Table 5. Quantities of DNA for transfection experiments.

Type of Transfection Experiment	shRNA Plasmid DNA (ng)	Reporter* (ng)	Carrier DNA** (ng)	Serum-Free Medium (final volume in µl)
shRNA plasmid DNA	500 – hairpin to gene of interest	0	0	50
Transfection efficiency	0	500	0	50
Knockdown efficiency of reporter	450-500 – hairpin to reporter	50	0	50
Control for knockdown efficiency	0	50	450-500	50
Non-silencing control	500 – scramble hairpin	0	0	50

*It is not necessary to transfect a reporter into cells if you are using a construct which already has a reporter for convenient estimation of transfection efficiency. Recommended reporters for other vectors include GFP, luciferase, and/or β-gal (X-gal staining and/or ONPG assays).
**Carrier DNA is required to increase the total DNA quantity for the formation of adequate DNA/Arrest-In complexes. Recommended carriers are pUC19 or pBluescript plasmids.

3. Aspirate the growth medium from the cells. Add an additional 150 µl of serum-free medium to each of the tubes containing transfection complexes and mix gently. Add the 250 µl DNA/Arrest-In complex mixture to the cells and incubate for 3-6 hours in a CO_2 incubator at 37°C.

   Your total volume will be 250 µl at this stage.

4. Following the 3-6 hour incubation, add an equal volume of growth medium (250 µl) containing twice the amount of normal serum to the cells (i.e. to bring the overall concentration of serum to what is typical for your cell line). Alternatively, the transfection medium can be aspirated and replaced with the standard culture medium (see note). Return the cells to the CO_2 incubator at 37°C.

   Note – Arrest-In has displayed low toxicity in the cell lines tested, therefore removal of transfection reagent is not required for many cell lines. In our experience, higher transfection efficiencies have been achieved if the transfection medium is **not** *removed. However, if toxicity is a problem, aspirate the transfection mixture after 5-6 hours and replace with fresh growth medium. Additionally, fresh growth medium should be replenished as required for continued cell growth.*

5. After 48-96 hours of incubation, examine the cells microscopically for the presence of reporter expression where applicable as this will be your first indication as to the efficiency of your transfection. Then assay cells for reduction in gene or reporter activity by quantitative/real-time RT-PCR, western blot or other appropriate functional assay; compare to untreated, reporter alone, non-silencing shRNA or other negative controls.

   Optimal length of incubation from the start of transfection to analysis is dependent on cell type, gene of interest, and the stability of the mRNA and/or protein being analyzed. Quantitative/real-time RT-PCR generally gives the best indication of expression knock-down. The use of western blots to determine knock-down is very dependent on quantity and quality of the protein, its half-life, and the sensitivity of the antibody and detection systems used.

6. If selecting for stably transfected cells (optional), transfer the cells to medium containing puromycin for selection. It is important to wait at least 48 hours before beginning selection.

   The working concentration of puromycin needed varies between cell lines. We recommend you determine the optimal concentration of puromycin required to kill your host cell line prior to selection for stable shRNA transfectants. Typically, the working concentration ranges from 1-10 µg/ml. You should use the lowest concentration that kills 100% of the cells in 3-5 days from the start of puromycin selection.

Cells Grown In Suspension

Transfection of cells in suspension would follow all the above principles and the protocol would largely remain the same, except that the DNA/Arrest-In mixture should be added to cells (post 20 minute incubation for complex formation) to a total volume of 250 µl serum-free medium or to a total volume of 250 µl of medium with serum (no antibiotics).

Transfection Optimization using Arrest-In

It is essential to optimize transfection conditions to achieve the highest transfection efficiencies and lowest toxicity with your cells. The most important parameters for optimization are DNA to transfection reagent ratio, DNA concentrations and cell confluency. We recommend that you initially begin with the Arrest-In and DNA amount indicated in Table 4 and extrapolate the number of cells needed for your vessel size from the number of cells used in a well of a 24-well plate as listed in step 1 of the protocol for delivery of plasmid DNA.

Determining Puromycin Dose-Response

In order to generate stable cell lines expressing the shRNA of interest, it is important to determine the minimum amount of puromycin required to kill non-transfected cells. A simple procedure to quickly test this is as follows:

1. Plate cells at a 25% confluency in 14 wells of a 24-well plate. Allow them to incubate overnight under proper conditions for your cells.
2. Label the wells to reflect the concentration of antibiotic to be applied (in duplicate). Prepare medium containing 0, 1, 2, 4, 6, 8, 10 µg/ml puromycin.
3. Aspirate the growth medium from the cells.
4. Apply the medium containing the dilutions of the antibiotic to the appropriate well.
5. Return the plate to the proper conditions for your cells.
6. Every 3 days aspirate the old medium and replace with freshly prepared selective medium.
7. Monitor the cells daily and observe the percentage of surviving cells. Optimum effectiveness should be reached in 3–10 days with puromycin.

8. The minimum antibiotic concentration to use is the lowest concentration that kills 100% of the cells in 5–10 days from the start of antibiotic selection.

Transfection Optimization using Arrest-In

It is essential to optimize transfection conditions to achieve the highest transfection efficiencies and lowest toxicity with your cells. The most important parameters for optimization are transfection reagent to DNA ratio, DNA concentrations and cell confluency. We recommend that you initially begin with 5–8 x 10^4 cells/well of a 24-well plate, and with the Arrest-In and DNA amount indicated in Table 5.

Additional Factors Influencing Successful Transfection:

1. **Concentration and purity of nucleic acids** – Determine the concentration of your DNA using 260 nm absorbance. Avoid cytotoxic effects by using pure preparations of nucleic acids.
2. **Transfection in serum containing or serum-free medium** – Our studies indicate that Arrest-In/DNA complexes should always be formed in the absence of serum. In the cell lines tested we found that the highest transfection efficiencies can be obtained if the cells are exposed to the transfection complexes in serum-free conditions followed by the addition of medium containing twice the amount of normal serum to the complex medium 3–6 hours post transfection (leaving the complexes on the cells). However, the transfection medium can be replaced with normal growth medium if high toxicity is observed.
3. **Presence of antibiotics in transfection medium** – The presence of antibiotics can adversely affect the transfection efficiency and lead to increased toxicity levels in some cell types. It is recommended that these additives be initially excluded until optimized conditions are achieved, then these components can be added, and the cells can be monitored for any changes in the transfection results.
4. **Cell history, density, and passage number** – It is very important to use healthy cells that are regularly passaged and in growth phase. The highest transfection efficiencies are achieved if cells are plated the day before. However, adequate time should be given to allow the cells to recover from the passaging (generally >12 hours). Plate cells at a consistent density to minimize experimental variation. If transfection efficiencies are low or reduction occurs over time, thawing a new batch of cells or using cells with a lower passage number may improve the results..

Validated Controls

The TRC eGFP shRNA (Catalog #RHS4459) is a positive control designed against the enhanced GFP reporter (BD Biosciences Clontech Catalog #6085-1; GenBank Accession #pEGFP U476561). This construct has been validated by the TRC to produce knockdown of GFP fluorescence at all MOIs tested. The TRC eGFP shRNA sequence is provided in pLKO.1, an HIV-based lentiviral vector and is expressed under the control of the U6 promoter.

The empty pLKO.1 vector (Catalog #RHS4080) contains a 18 bp stuffer sequence between the *Age*I and *Eco*RI restriction sites.

Table 6. Related reagents

Reagent	Vendor	Catalog #
TRC Lentiviral eGFP shRNA Positive Control	Thermo Scientific Open Biosystems	RHS4459
pLKO.1 Empty Vector	Thermo Scientific Open Biosystems	RHS4080
Arrest-In Transfection Reagent 0.5 ml-10 mls*	Thermo Scientific Open Biosystems	ATR1740-1743
TransLenti Viral shRNA Packaging System	Thermo Scientific Open Biosystems	TLP4614
TransLenti Viral shRNA Packaging System (contains cell line)	Thermo Scientific Open Biosystems	TLP4615

What Clones Are Part Of My Collection?

A CD containing the data for this collection will be shipped with each collection. This file contains the location and accession number for each construct in the collection. This data file can be downloaded from the lentiviral pLKO.1 product page at www.openbiosystems.com.

Where Can I Find The Sequence Of An Individual shRNAmir Construct?

If you are looking for the sequence an individual shRNA construct, you can use the gene search. Just enter the catalog number or clone ID of your hairpin into the gene search, hit submit and then click on the query result. If you then click on the oligo ID (the TRC number) and then click on the word "sequence" in the details grid, the hairpin sequence is listed with the target sequence annotated.

If you are looking for the sequence of several shRNA constructs, you can access this information in the data file of the collection. This data file can be downloaded from the Lentiviral pLKO.1 product page at www.openbiosystems.com.

Can I Use Ampicillin Instead Of Carbenicillin?

No. The TRC and the Broad Institute suggest that carbenicillin be used with the pLKO.1 vector. Constructs grown in ampicillin tend to not produce high plasmid yield.

Should I Use A Second Or Third Generation Packaging Cell Line For Packaging TRC Constructs?

The pLKO.1 vector contains a chimeric 5' LTR, so this vector can be packaged using either second or third generation packaging systems, however second generation packaging is recommended and will result in higher titers than third generation. The Broad Institute and the TRC recommend use second generation packaging to make viral particles.

What Restriction Sites Were Used To Clone The Hairpins Clone Into The pLKO.1 Vector?

The hairpins were cloned in at *Age*I and *Eco*RI, but the *Eco*RI site is usually destroyed upon ligation.

What Is The Sequencing Primer For The pLKO.1 Vector?

The pLKO.1 sequencing primer is:
5' AAACCCAGGGCTGCCTTGGAAAAG 3' 1540 R

Using this primer the hairpin will show up somewhere in the frame of 180-260 bp into the read. Notice it is reading in the reverse orientation (Figure 8).

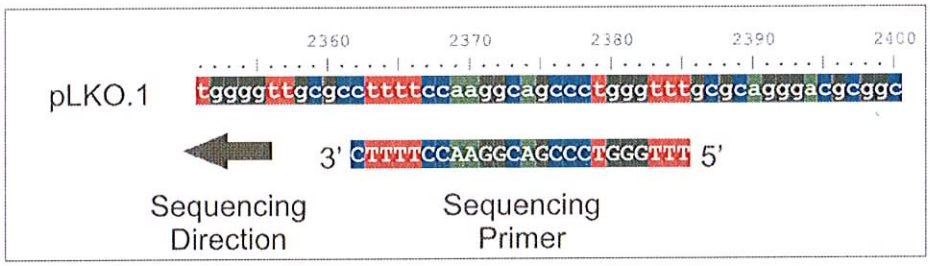

Figure 8. TRC sequencing primer

Troubleshooting

For help with transfection or transduction of your retroviral constructs, please email technical support at info@openbiosystems.com with the answers to the questions below, your sales order or purchase order number and the catalog number or clone ID of the construct with which you are having trouble.

1. Are you using direct transfection or transduction into your cell line?
2. What did the uncut and restriction digested DNA look like on a gel?
3. What was the transfection efficiency if you used direct transfection? What transfection reagent was used?
4. Were positive and negative knockdown controls used (i.e. the empty vector or the eGFP shRNA positive control)?
5. What were the results of the controlled experiments?
6. How was knockdown measured (i.e. real-time RT-PCR or western blot)?
7. What is the abundance and the half-life of the protein? Does the protein have many isoforms?
8. What packaging cell line was used if you are using infection rather than transfection?
9. What was your viral titer?
10. What was your MOI?
11. Did you maintain the cells on puromycin after transfection or transduction?
12. How much time elapsed from transfection/transduction to puromycin selection?

If Transfection Into Your Cell Line Is Unsuccessful, You May Need To Consider The Following List Of Factors Influencing Successful Transfection:

1. Concentration and purity of plasmid DNA and nucleic acids – Determine the concentration of your DNA using 260 nm absorbance. Avoid cytotoxic effects by using pure preparations of nucleic acids.
2. Insufficient mixing of transfection reagent or transfection complexes.
3. Transfection in serum containing or serum-free media – Our studies indicate that Arrest-In/DNA complexes should preferably be formed in the absence of serum. In the cell lines tested we found that the highest transfection efficiencies can be obtained if the cells are exposed to the transfection complexes in serum-free conditions followed by the addition of medium containing twice the amount of normal serum to the complex medium 3-6 hours post transfection (leaving the complexes on the cells). However, the serum-free transfection medium can be replaced with normal growth medium if high toxicity is observed.
4. Presence of antibiotics in transfection medium – The presence of antibiotics can adversely affect the transfection efficiency and lead to increased toxicity levels in some cell types. It is recommended that antibiotics be excluded until transfection has mostly occurred (3-6 hours) and then be added together with the full medium.
5. High protein expression levels – Some proteins when expressed at high levels can be cytotoxic; this effect can also be cell line specific.
6. Cell history, density, and passage number – It is very important to use healthy cells that are regularly passaged and in growth phase. The highest transfection efficiencies are achieved if cells are plated the day before, however, adequate time should be given to allow the cells to recover from the passaging (generally >12 hours). Plate cells at a consistent density to minimize experimental variation. If transfection efficiencies are low or reduction occurs over time, thawing a new batch of cells or using cells with a lower passage number may improve the results.

If Arrest-In seems to be toxic to a particular cell line, try reducing the DNA:Arrest-In ratio.

References

Kappes J.C., Wu X. Safety considerations in vector development. *Somat Cell Mol Genet.* 26(1-6):147-58. (2001).

Shimada, T., *et. al.* Development of Vectors Utilized for Gene Therapy for AIDS. *AIDS* 4.

Stewart, S.A., *et al.* Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA*, 9, 493-501 (2003).

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Zufferey R, *et al.*, Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery, J Virol. 72, 9873-80 (1998).

FAQS/Troubleshooting

For answers to questions that are not addressed here, please email technical support at openbiosystems@thermofisher.com with your question, your sales order or purchase order number and the catalog number or clone ID of the construct or collection with which you are having trouble.

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Contact Information
Technical Support
Tel: 1.888.412.2225
Fax: 1.256.704.4849
openbiosystems@
thermofisher.com

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? X YES NO If no, please proceed to Section 9.0

8.2 If YES, please name the toxin(s)_____Cholera Toxin_____
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

8.3 What is the LD_{50} (specify species) of the toxin____not known_____

8.4 How much of the toxin is handled at one time*? __0.5 mg_____

8.5 How much of the toxin is stored*? __2 mg_____

8.6 Will any biological toxins be used in live animals? ☐ YES, Please provide details:_____
X NO

Cholera toxin will used as a cell culture additive. It will be stored in a locked freezer in a locked room adjoining our laboratory, A4-126.

Material Safety Data Sheet

Version 4.2
Revision Date 11/05/2010
Print Date 02/09/2011

1. PRODUCT AND COMPANY IDENTIFICATION

Product name : Cholera Toxin Vibrio cholerae

Product Number : C8052
Brand : Sigma
Product Use : For laboratory research purposes.

Supplier : Sigma-Aldrich Canada, Ltd
2149 Winston Park Drive
OAKVILLE ON L6H 6J8
CANADA

Manufacturer : Sigma-Aldrich Corporation
3050 Spruce St.
St. Louis, Missouri 63103
USA

Telephone : +19058299500
Fax : +19058299292
Emergency Phone # (For both supplier and manufacturer) : 1-800-424-9300
Preparation Information : Sigma-Aldrich Corporation
Product Safety - Americas Region
1-800-521-8956

2. HAZARDS IDENTIFICATION

Emergency Overview

Target Organs

Bowel

WHMIS Classification

D2B Toxic Material Causing Other Toxic Effects — Moderate skin irritant; Moderate eye irritant

GHS Classification

Acute toxicity, Oral (Category 5)
Skin irritation (Category 2)
Eye irritation (Category 2A)
Specific target organ toxicity - single exposure (Category 3)

GHS Label elements, including precautionary statements

Pictogram

Signal word: Warning

Hazard statement(s)

H303	May be harmful if swallowed.
H315	Causes skin irritation.
H319	Causes serious eye irritation.
H335	May cause respiratory irritation.

Precautionary statement(s)

P261	Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
P305 + P351 + P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

HMIS Classification

Health hazard: 2

Chronic Health Hazard: *
Flammability: 0
Physical hazards: 0

Potential Health Effects

Inhalation	May be harmful if inhaled. Causes respiratory tract irritation.
Skin	Harmful if absorbed through skin. Causes skin irritation.
Eyes	Causes eye irritation.
Ingestion	Harmful if swallowed.

3. COMPOSITION/INFORMATION ON INGREDIENTS

Synonyms : Cholera enterotoxin
Cholergen

CAS-No.	EC-No.	Index-No.	Concentration
Tris (hydroxymethyl) aminomethane			
77-86-1	201-064-4	-	>= 5.82 - <= 5.94 %
2-Amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride			
1185-53-1	214-684-5	-	>= 31.3 - <= 31.9 %
Sodium chloride			
7647-14-5	231-598-3	-	>= 57.6 - <= 58.8 %
Exotoxin, vibrio cholerae			
9012-63-9	-	-	>= 0.5 - <= 2.5 %
Edetate disodium dihydrate			
6381-92-6	205-358-3	-	>= 0.96 - <= 0.98 %

4. FIRST AID MEASURES

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.Move out of dangerous area.

If inhaled
If breathed in, move person into fresh air. If not breathing, give artificial respiration. Consult a physician.

In case of skin contact
Wash off with soap and plenty of water. Consult a physician.

In case of eye contact
Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

5. FIRE-FIGHTING MEASURES

Conditions of flammability
Not flammable or combustible.

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for fire-fighters
Wear self contained breathing apparatus for fire fighting if necessary.

Hazardous combustion products
Hazardous decomposition products formed under fire conditions. - Nature of decomposition products not known.

Explosion data - sensitivity to mechanical impact
no data available

Explosion data - sensitivity to static discharge
no data available

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Use personal protective equipment. Avoid dust formation. Avoid breathing vapors, mist or gas. Ensure adequate ventilation. Evacuate personnel to safe areas. Avoid breathing dust.

Environmental precautions
Do not let product enter drains.

Methods and materials for containment and cleaning up
Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Precautions for safe handling
Avoid contact with skin and eyes. Avoid formation of dust and aerosols.
Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection.

Conditions for safe storage
Keep container tightly closed in a dry and well-ventilated place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment

Respiratory protection
For nuisance exposures use type P95 (US) or type P1 (EU EN 143) particle respirator.For higher level protection use type OV/AG/P99 (US) or type ABEK-P2 (EU EN 143) respirator cartridges. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

Eye protection
Safety glasses with side-shields conforming to EN166 Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

Skin and body protection
impervious clothing, The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Hygiene measures
Handle in accordance with good industrial hygiene and safety practice. Wash hands before breaks and at the end of workday.

Specific engineering controls
Use mechanical exhaust or laboratory fumehood to avoid exposure.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	solid
Colour	no data available

Safety data

pH	no data available
Melting/freezing	no data available

point	
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Autoignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Vapour pressure	no data available
Density	no data available
Water solubility	no data available
Partition coefficient: n-octanol/water	no data available
Relative vapour density	no data available
Odour	no data available
Odour Threshold	no data available
Evaporation rate	no data available

10. STABILITY AND REACTIVITY

Chemical stability
Stable under recommended storage conditions.

Possibility of hazardous reactions
no data available

Conditions to avoid
no data available

Materials to avoid
Dimethyl sulfate, Acid chlorides, Halogenated hydrocarbon, Metals, Acids

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Nature of decomposition products not known.

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Oral LD50
no data available

Inhalation LC50
no data available

Dermal LD50
no data available

Other information on acute toxicity
no data available

Skin corrosion/irritation
no data available

Serious eye damage/eye irritation
Eyes: no data available

Respiratory or skin sensitization
no data available

Germ cell mutagenicity
no data available

Carcinogenicity

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.

ACGIH: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH.

Reproductive toxicity

no data available

Teratogenicity

no data available

Specific target organ toxicity - single exposure (Globally Harmonized System)
no data available

Specific target organ toxicity - repeated exposure (Globally Harmonized System)
no data available

Aspiration hazard
no data available

Potential health effects

Inhalation	May be harmful if inhaled. Causes respiratory tract irritation.
Ingestion	Harmful if swallowed.
Skin	Harmful if absorbed through skin. Causes skin irritation.
Eyes	Causes eye irritation.

Synergistic effects
no data available

Additional Information
RTECS: Not available

12. ECOLOGICAL INFORMATION

Toxicity

no data available

Persistence and degradability
no data available

Bioaccumulative potential
no data available

Mobility in soil
no data available

PBT and vPvB assessment
no data available

Other adverse effects

no data available

13. DISPOSAL CONSIDERATIONS

Product
Offer surplus and non-recyclable solutions to a licensed disposal company. Contact a licensed professional waste disposal service to dispose of this material.

Contaminated packaging
Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)
Not dangerous goods

IMDG
Not dangerous goods

IATA
Not dangerous goods

15. REGULATORY INFORMATION

DSL Status
This product contains the following components that are not on the Canadian DSL nor NDSL lists.

	CAS-No.
Exotoxin, vibrio cholerae	9012-63-9

WHMIS Classification

D2B	Toxic Material Causing Other Toxic Effects	Moderate skin irritant Moderate eye irritant

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all the information required by the Controlled Products Regulations.

16. OTHER INFORMATION

Further information

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Co., shall not be held liable for any damage resulting from handling or from contact with the above product. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	Cholera toxin
Proposed Use Dose:	500 µg
Proposed Storage Dose:	**2000** µg
LD_{50} (species):	250 µg

Calculation:

250 µg/kg x 50 kg/person

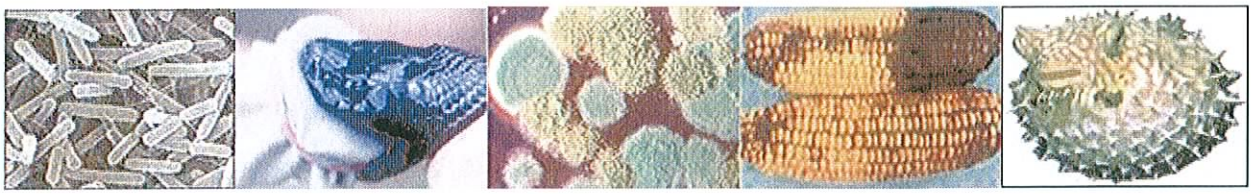
Dose per person based on LD_{50} in µg = 12500

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = ***1250***

Comments/Recommendations:

Proposed storage dose is over the limit. Please store in two different locations.

Toxins of Biological Origin



Biological toxins are produced by certain bacteria, fungi, protozoa, plants, reptiles, amphibians, fish, echinoderma (spiny urchins and starfish), mollusks, and insects.

The EH&S Biosafety Office regulates the **possession, use, and transfer of unfractionated mixtures and purified preparations of biological toxins with a mammalian LD_{50} of ≤ 100 ug/kg body weight, as well as the organisms, both natural and recombinant, which produce these biological toxins.** These are called "Acute Toxins". Registration forms can be found at http://www.ehs.ufl.edu/Bio/default.asp

The following table from the UF EH&S Biological Safety Manual lists LD_{50} values for some biological toxins. Toxins not on this list may still require registration. For more information, please contact the Biosafety Office at 392-1591.

Toxin	LD50 (ug/kg)*
Abrin	0.7
Aerolysin	7.0
Botulinin toxin A	0.0012
Botulinin toxin B	0.0012
Botulinin toxin C1	0.0011
Botulinin toxin C2	0.0012
Botulinin toxin D	0.0004
Botulinin toxin E	0.0011
Botulinin toxin F	0.0025
b-bungarotoxin	14.0
Caeruleotoxin	53
Cereolysin	40-80
Cholera toxin	250
Clostridium difficile enterotoxin A	0.5
Clostridium difficile cytotoxin B	220
Clostridium perfringens lecithinase	3
Clostridium perfringens kappa toxin	1500
Clostridium perfringens perfringolysin O	13-16
Clostridium perfringens enterotoxin	81
Clostridium perfringens beta toxin	0.4
Clostridium perfringens delta toxin	5
Clostridium perfringens epsilon toxin	0.1
Conotoxin	12-30
Crotoxin	82
Diphtheria toxin	0.1
Listeriolysin	3-12
Leucocidin	50