The University *of* Western Ontario
BIOLOGICAL AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: October 14, 2011
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario (UWO) or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biological agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biological agents being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Electronically completed forms are to be submitted to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190 or to jstanle2@uwo.ca) for distribution to the Biohazards Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety.

Please ensure that all questions are fully and clearly answered. Failure to do so will lead to the form being returned, which will cause delays in your approval and frustration for you and your colleagues on the Committee.

If you are re-submitting this form as requested by the Biohazards Subcommittee, please make modifications to the form in bold print, highlighted in yellow. Please re-submit forms electronically.

PRINCIPAL INVESTIGATOR:	**Mellissa RW Mann**
DEPARTMENT:	**Obstetrics & Gynecology and Biochemistry**
ADDRESS:	**4th Floor VRL, 800 Commissioners RD E**
PHONE NUMBER:	**519-685-8500 x55648**
EMERGENCY PHONE NUMBER(S):	**home 519-652-7264, cell 519-933-7264**
EMAIL:	**mmann22@uwo.ca**

Location of experimental work to be carried out :

Building :	**VRL**	Room(s):	**A4-128 (Level 2)**
Building :	**LRCP**	Room(s):	**A4-822 (Lenti room)**
Building :		Room(s):	

***For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 15.0, Approvals).**

FUNDING AGENCY/AGENCIES: **CIHR, UWO AEF**

GRANT TITLE(S): **Molecular Analysis of Genomic Imprint Maintenance During Embryogenesis**
Effects of Vitrification on Genomic Imprinting
The Role of Nucleoporin 107 in Imprinted Gene Regulation

UNDERGRADUATE COURSE NAME(IF APPLICABLE):

List all personnel working under Principal Investigators supervision in this location:

Name	UWO E-mail Address	Date of Biosafety Training
Liyue Zhnag	**lyzhang59@yahoo.com**	**Nov 18/11**
Michelle Denomme	**mdenomm2@uwo.ca**	**Nov 22/11**
Lauren Magri	**lmagri@uwo.ca**	**Nov 22/11**

William MacDonald	wmacdon7@uwo.ca	Nov 22/11
Saqib Sachani	ssachani@uwo.ca	Nov 22/11
Carlee White	cwhite67@uwo.ca	Nov 22/11
Analise Hofman	ahofman4@uwo.ca	Sept 29/11

Please explain how the biological agents are used in your project and how they are stored and disposed of. The BARF without this description will not be reviewed.

Nonpathogenic bacteria are used for cloning. Bacteria are stored as glycerol stocks in the -80°C freezer. They are disposed by bleaching.

Lentivirus is used to generate transgenic mouse stems cells (embryonic, trophectoderm and extraembryonic stem cells) with a short hairpin RNA (shRNA). The shRNAs are used to deplete RNAs for factor involved in chromatin regulation. Virus is not stored. They are disposed by autoclaving.

MSCV is used to infect mouse cells lines to study the factos involved in retroviral silencing. Virus is not stored. It is disposed by bleaching.

Primary cells and cell lines used from transfection, and gene and protein studies. They are stored in -150°C freezer. They are disposed by bleaching.

Hormones are used to superovulate mice. They are stored in -20°C freezer. They are stored until used and not disposed.

Changes to this page

Please include a ONE page research summary or teaching protocol in lay terms.
Forms with summaries more than one page will not be reviewed.

My research focuses on epigenetic mechanisms that control gene expression, specifically on genomic imprinting. Genomic imprinting is defined as a mechanism of transcriptional regulation that restricts expression to one parental allele. Imprinting may be envisaged as a multi-step process that begins in the gametes, where epigenetic modifications differentially mark the parental alleles. These marks must then be stably maintained in the developing embryo where they are translated into parental-specific expression. Errors in any of these stages can lead to genomic imprinting disorders, such as Beckwith-Wiedemann Syndrome, Angelman Syndrome, and Silver-Russell Syndrome.

The five main projects in Dr. Mann's lab are

1. Assisted Reproduction: Molecular and Developmental Effects of Gamete and Embryo Manipulation on Genomic Imprinting. Using a mouse model system, superovulation, embryo culture and vitrification are examined for their effects on genomic imprinting and preimplantation development.

2. The Role of Kcnq1ot1 Noncoding RNA in Imprinted Gene Regulation. The role of the Kcnq1ot1 noncoding RNA in imprinted gene regulation will be examined in extraembryonic endoderm, embryonic and trophectoderm stem cells and in preimplantation embryos.

3. Functional Genomic Screen for Epigenetic Modifiers of Genomic Imprinting or Retroviral Extinction. The role of candidate epigenetic factors will be examined in extraembryonic endoderm, embryonic and trophectoderm stem cells.

4. Identification and Evolutionary Comparison of Imprinting Centers. New imprinting centers will be identified using a novel protocol to isolate methylated sequences in preimplantation embryos. Genes adjacent to novel imprinting centers will be examined for their imprint status to identify new imprinted domains

5. Epigenetic Changes in the Nutrient Restricted Placenta. Global methylation and expression changes were identified in mouse nutrient-restricted placenta. Individual candidates will be examined as a potential underlying mechanism for fetal growth restriction.

The first three are directed towards understanding the regulation of genomic imprinting and how this relates to the development of genomic imprinting disorders. The fourth project will lead to the identification of novel imprinting centres and imprinted domains. The fifth project will identify epigenetic perturbations that arise due to fetal growth restriction. This research will have a direct impact on the improvement of current assisted reproductive technologies as it related to reducing risk and incidence of acquired genomic imprinting disorders, will provide insight into the cause of known imprinting disorders, such as Prader-Willi, Angelman, Beckwith-Wiedemann, and Silver-Russell Syndromes, as well as other complex diseases that have yet to be recognized as imprinting disorders, and will identify novel epigenetic mechanisms leading to low birth weight, spontaneous abortions, and possibly such conditions as preeclampsia.

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ☒ YES ☐ NO
(non-pathogenic and pathogenic biological agents including but not limited to bacteria and other microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)? If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? ☐ YES ☒ NO
If YES, please give the name of the species ______________________
What is the origin of the microorganism(s)? ______________________

Please describe the risk (if any) of escape and how this will be mitigated:

Changes on this page

...ase attach the CFIA permit.

1.2 Please complete the table below:

Full Scientific Name of Biological Agent(s)* (Be specific)	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
E. coli DH5 a	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	**2 L**	**Invitrogen**	☐ 1 ☒ 2 ☐ 2+ ☐ 3
Lentivirus	☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	**0.025 L**	**Open Biosystems**	☐ 1 ☐ 2 ☒ 2+ ☐ 3
MSCV	☒ Yes ☐ No	☒ Yes ☐ No	☐ Yes ☒ No	**0.025 L**	**Clontech**	☐ 1 ☒ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3

****Please attach a Material Safety Data Sheet or equivalent from the supplier if the bacterium used is not on this link:***
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

Additional Comments: ______________________

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ☒ YES ☐ NO
(If NO, please proceed to Section 3.0)

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture:

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	☐ Yes ☒ No		Not applicable
Rodent	☒ Yes ☐ No	**Preimplantation mouse embryos**	**2009-100**
Non-human primate	☐ Yes ☒ No		
Other (specify)	☐ Yes ☒ No		

2.3 Please indicate the type of established cells that will be grown in culture in:

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Containment Level of each cell line	Supplier / Source of cell line(s)
Human	☒ Yes ☐ No	**HEK 293**	**2**	**ATCC**
Rodent	☒ Yes ☐ No	**NIH 3T3**	**1**	**ATCC**
Non-human primate	☐ Yes ☒ No			
Other (specify)	☐ Yes ☒ No			

Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required ☐ 1 ☒ 2 ☐ 2+ ☐ 3

Additional Comments: _____

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ☐ YES ☒ NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Infected With An Infectious Agent? YES/UNKNOWN**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Blood (fraction) or other Body Fluid		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Organs or Tissues (unpreserved)		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Organs or Tissues (preserved)		Not Applicable		Not Applicable

Additional Comments: _____

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? ☒ YES ☐ NO If **NO**, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? ☒ YES, complete table below ☐ NO

Bacteria Used for Cloning *	Plasmid(s) **	Source of Plasmid	Gene Transformed or Transfected	Will there be a change due to transformation of the bacteria?	Will there be a change in the pathogenicity of the bacteria after the genetic modification?	What are the consequences due to the transformation of the bacteria?
E. coli DH5alpha	**BlueScript pCDNA3 pUC 19 pGEM T easy**	**Strategene Invitrogen Invitrogen Promega**	**Neomycin, Puromycin GFP, DSRed, Luciferase, H19, Snrpn, Peg1, Peg3, Kcnq1ot1**	**Antibiotic resistance Fluorescence None**	**No**	**Antibiotic resistance Fluorescence None**

**** Please attach a Material Safety Data Sheet or equivalent if available.***
***** Please attach a plasmid map.***
******No Material Safety Data Sheet is required for the following strains of E. coli:***
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

4.3 Will genetic modification(s) of bacteria and/or cells involving viral vectors be made?
☒ YES, complete table below ☐ NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results from transduction
Lentivirus MSCV	**GIPZ shRNAmir MSCV puro**	**Open biosystems Clontech**	**See attached list Puromycin**	**Decreased target gene expression Puro resistance**

**** Please attach a Material Safety Data Sheet or equivalent.***

4.3.1 Will virus be replication defective? ☒ YES ☐ NO

4.3.2 Will virus be infectious to humans or animals? ☒ YES ☐ NO

4.3.3 Will this be expected to increase the containment level required? ☐ YES ☒ NO

5.0 Will genetic sequences from the following be involved?

- HIV ☐ NO ☒ YES, specify **HIV enhancer**
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens ☒ NO ☐ YES, specify
- SV 40 Large T antigen ☐ NO ☒ YES
- E1A oncogene ☐ NO ☒ YES
- Known oncogenes ☒ NO ☐ YES, specify
- Other human or animal pathogen and or their toxins ☒ NO ☐ YES, specify

5.1 Is any work being conducted with prions or prion sequences? ☒ NO ☐ YES

Additional Comments: _____

6.0 Human Gene Therapy Trials

6.1 Will human clinical trials be conducted involving a biological agent? ☐ YES ☒ NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 7.0

6.2 If YES, please specify which biological agent will be used:
Please attach a full description of the biological agent.

6.3 Will the biological agent be able to replicate in the host? ☐ YES ☐ NO

6.4 How will the biological agent be administered?

6.5 Please give the Health Care Facility where the clinical trial will be conducted:

6.6 Has human ethics approval been obtained? ☐ YES, number: ☐ NO ☐ PENDING

7.0 Animal Experiments

7.1 Will live animals be used? ☒ YES ☐ NO If **NO**, please proceed to section 8.0

7.2 Name of animal species to be used **mice**

7.3 AUS protocol # **2009-100**

7.4 List the location(s) for the animal experimentation and housing. **VRL vivarium, LRCP**

7.5 Will any of the agents listed in section 4.0 be used in live animals
☒ NO ☐ YES, specify:

Changes to 7.5

7.6 Will the agent(s) be shed by the animal:
☐ YES ☒ NO, please justify:

8.0 Use of Animal species with Zoonotic Hazards

8.1 Will any animals with zoonotic hazards or their organs, tissues, lavages or other body fluids including blood be used (see list below)? ☐ YES ☒ NO - If **NO**, please proceed to section 9.0

8.2 Will live animals be used? ☐ YES ☐ NO

8.3 If **YES**, please specify the animal(s) used:

♦ Pound source dogs	☐ YES	☐ NO
♦ Pound source cats	☐ YES	☐ NO
♦ Cattle, sheep or goats	☐ YES, species	☐ NO
♦ Non-human primates	☐ YES, species	☐ NO
♦ Wild caught animals	☐ YES, species & colony #	☐ NO
♦ Birds	☐ YES, species	☐ NO
♦ Others (wild or domestic)	☐ YES, specify	☐ NO

8.4 If no live animals are used, please specify the source of the specimens:

9.0 Biological Toxins and Hormones

9.1 Will toxins or hormones of biological origin be used? ☒ YES ☐ NO If **NO**, please proceed to Section 10.0

9.2 If YES, please name the toxin(s) or hormones(s) **human and equine chorionic gonadotropin (hCG, eCG)**
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

9.3 What is the LD_{50} (specify species) of the toxin or hormone **unknown**

9.4 How much of the toxin or hormone is handled at one time*? **20 IU/ml**

9.5 How much of the toxin or hormone is stored*? **10000 IU**

9.6 Will any biological toxins or hormones be used in live animals? ☒ YES ☐ NO
If **YES**, Please provide details: **hormones are given by interperitoneal injection to mice**

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

Additional Comments: _____ Changes to 9.5

10.0 Insects

10.1 Do you use insects? ☐ YES ☒ NO - If **NO**, please proceed to Section 11.0

10.2 If YES, please give the name of the species.

10.3 What is the origin of the insect?

10.4 What is the life stage of the insect?

10.5 What is your intention? ☐ Initiate and maintain colony, give location:
☐ "One-time" use, give location:

10.6 Please describe the risk (if any) of escape and how this will be mitigated:

10.7 Do you use insects that require a permit from the CFIA permit? ☐ YES ☐ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

11.0 Plants

11.1 Do you use plants? ☐ YES ☒ NO - If **NO**, please proceed to Section 12.0

11.2 If YES, please give the name of the species.

11.3 What is the origin of the plant?

11.4 What is the form of the plant (seed, seedling, plant, tree…)?

11.5 What is your intention? ☐ Grow and maintain a crop ☐ "One-time" use

11.6 Do you do any modifications to the plant? ☐ YES ☐ NO
If yes, please describe:

11.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:

11.8 Is the CFIA permit attached? ☐ YES ☐ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

12.0 Import Requirements

12.1 Will any of the above agents be imported? ☐ YES, country of origin ☒ NO
If **NO**, please proceed to Section 13.0

12.2 Has an Import Permit been obtained from HC for human pathogens? ☐ YES ☐ NO

12.3 Has an import permit been obtained from CFIA for animal or plant pathogens? ☐ YES ☐ NO

12.4 Has the import permit been sent to OHS? ☐ YES, please provide permit # ☐ NO

13.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biological agents in Sections 1.0 to 9.0 have been trained.

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name Mellissa Mann ***Date:*** Nov 25/11

14.0 Containment Levels

14.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. ☐ 1 ☐ 2 ☒ 2+ ☐ 3

14.2 Has the facility been certified by OHS for this level of containment?
☒ YES, location and date of most recent biosafety inspection: **A4-822, A4-128, r-06-00599, Dec 10, 2010**
☐ NO, please certify
☐ NOT REQUIRED for Level 1 containment

14.3 Please indicate permit number (not applicable for first time applicants): **BIO-LHRI-0055**

15.0 Procedures to be Followed

15.1 Are additional risk reduction measures necessary beyond containment level 1, 2, 2+ or 3 measures that are unique to these agents? ☐ YES ☒ NO
If **YES** please describe:

15.2 Please outline what will be done if there is an exposure to the biological agents listed such as a needlestick injury or an accidental splash:
Skin exposure: Wash the affected area thoroughly using antimicrobial soap and report incident to OHS.

Splash to eyes: Immediately flush eyes with running water for 15 minutes using eyewash and forcibly hold eye(s) open to ensure effective wash behind the eyelids. Report incident to OHS

Needle stick: Wash affected area thoroughly using antimicrobial soap for 5 minutes and report incident to OHS.

15.3 As the Principal Investigator, 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.shs.uwo.ca/workplace/workplacehealth.html

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name **Mellissa Mann** ***Date:*** **Nov 25/11**

15.4 Additional Comments: _____

16.0 Approvals

1) UWO Biohazards Subcommittee:
SIGNATURE: _____
Date: _____

2) Safety Officer for the University of Western Ontario
SIGNATURE: _____
Date: _____

3) Safety Officer for Institution where experiments will take place (if not UWO):
SIGNATURE: Jane Ryder
Date: January 12, 2012

Approval Number: _____ Expiry Date (3 years from Approval): _____

Special Conditions of Approval:

-------- Original Message --------
Subject: Re: Biological Agents Registry Form: Mann
Date: Mon, 26 Mar 2012 18:19:34 -0400
From: Mellissa Mann <mmann22@uwo.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>

Please find attached the biohazard form with the following changes:
1. Amended to *E. coli* DH5 α
2. Correct to "gycerol stocks"
3. 7.5 States "no"
4. 9.5 Corrected to amount of hormone stored.

Liyue Zhang does not have a uwo email address. He is employed through the hospital and I have given the address at which he receives all his email.

Thanks
Mellissa

New Info

Canadian Food Inspection Agency | Agence canadienne d'inspection des aliments

Office of Biohazard Containment and Safety
Science Branch, CFIA
59 Camelot Drive, Ottawa, Ontario K1A 0Y9
Tel: (613) 221-7068 Fax: (613) 228-6129
Email: ImportZoopath@inspection.gc.ca

Bureau du confinement des biorisques et sécurité
Direction générale des sciences, ACIA
59 promenade Camelot, Ottawa, Ontario K1A 0Y9
Tél: (613) 221-7068 Téléc: (613) 228-6129
Courriel: ImportZoopath@inspection.gc.ca

October 20th, 2009

Ms. Shamila Survery / Mr. Michael Decosimo
Cedarlane Laboratories Ltd
4410 Paletta Court
Burlington, Ontario L7L 5R2

By Facsimile: (289) 288-0020

SUBJECT: Importation of *Escherichia coli* strains

Dear Ms. Survery / Mr. Decosimo:

Our office received your query about the importation of *Escherichia coli* from the American Type Culture Collection (ATCC) located in Manassas, Virginia, United States. The following *Escherichia coli* strains are consider to be level 1 animal pathogens:

- 5K
- 58
- 58-161
- 679
- 1532
- AB284
- AB311
- AB1157
- AB1206
- AG1
- B
- BB4
- BD792
- BL21
- BL21 (DE3)
- BM25.8
- C
- C-1a
- C-3000
- C25
- C41 (DE3)
- C43 (DE3)
- C600
- Cavalli Hfr
- CIE85
- DH1
- DH10 GOLD
- DH10B
- DH5
- DH5-alpha
- DP50
- DY145
- DY380
- E11
- EJ183
- EL250
- EMG2
- EPI 300
- EZ10
- FDA Seattle 1946
- Fusion-Blue
- H1443
- HF4714
- HB101
- HS(PFAMP)R
- Hfr3000
- Hfr3000 X74
- HMS174
- J52
- J53
- JC3272
- JC7661
- JC9387
- JF1504
- JF1508
- JF1509
- JJ055
- JM83
- JM101
- JM109
- K12
- KC8
- KA802
- KAM32
- KAM33
- KAM43
- LE450
- LE451
- LE452
- MB408
- MBX1928
- MC1061
- MC4100 (MuLac)
- MG1655
- MM294
- MS101
- NC-7
- Nissle 1917
- One Shot STBL3
- OP50
- P678
- PA309
- PK-5
- PMC103
- PR13
- Rri
- RV308
- S17-1λ -PIR
- SCS1
- SMR10
- SOLR
- SuperchargeEZ10
- SURE
- TOP10
- TG1
- U5/41
- W208
- W945
- W1485
- W3104
- W3110
- WA704
- WP2
- X1854
- X2160T
- X2541
- X2547T
- XL1-BLUE
- XL1-BLUE-MRF
- XLOLR
- Y10
- Y1090 (1090)
- YN2980
- W3110
- WG1
- WG439
- WG443
- WG445

The Office of Biohazard Containment and Safety (BCS) of the Canadian Food Inspection Agency (CFIA) only issues import permits for microorganisms that are pathogenic to animals, or parts of microorganisms that are pathogenic to animals. As the products listed above are not considered pathogenic to animals, the Office of BCS does not have any regulatory requirements for their importation.

Please note that other legislation may apply. You may wish to contact the Public Health Agency of Canada's (PHAC) Office of Laboratory Security at (613) 957-1779.

Note: Microorganisms pathogenic to animals and veterinary biologics require an import permit from the CFIA.

Sincerely,

Cinthia Labrie
Head, Animal Pathogen Importation Program
Office of Biohazard Containment & Safety

Canada

Expression Arrest™ GIPZ lentiviral shRNAmir-Glycerol Stocks

APPLICABLE CATALOG NUMBERS

Catalog Numbers	Description
RHS4430	Human GIPZ lentiviral shRNAmir individual clone
RMM4431	Mouse GIPZ lentiviral shRNAmir individual clone
RHS4346	Non-silencing-GIPZ lentiviral shRNAmir control-Glycerol stock
RHS4349	pGIPZ lentiviral empty vector - Glycerol stock
RHS4371	GAPDH-GIPZ lentiviral shRNAmir positive control-Glycerol stock
RHS4480	EG5-GIPZ lentiviral shRNAmir positive control-Glycerol stock
RHS4477	Human GIPZ lentiviral shRNAmir library subscription
RMM4501	Mouse GIPZ lentiviral shRNAmir library subscription

PRODUCT DESCRIPTION

The GIPZ lentiviral shRNAmir library was developed by Open Biosystems in collaboration with Dr. Greg Hannon (CSHL) and Dr. Steve Elledge (Harvard). This library combines the design advantages of microRNA-adapted shRNA (shRNAmir) with the pGIPZ lentiviral vector to create a powerful RNAi trigger capable of producing RNAi in most cell types including primary and non-dividing cells.

QUALITY CONTROL

Open Biosystems checks all cultures for growth prior to shipment.

SHIPPING AND STORAGE

Individual constructs are shipped as bacterial cultures of *E. coli* (Prime Plus) in LB-Lennox (low salt) broth with 8% glycerol, 100µg/ml carbenicillin and 25µg/ml zeocin. 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.

TO ALLOW ANY CO_2 THAT MAY HAVE DISSOLVED INTO THE MEDIA FROM THE DRY ICE IN SHIPPING TO DISSIPATE, PLEASE STORE CONSTRUCTS AT –80°C FOR AT LEAST 48 HOURS BEFORE THAWING.

Important Safety Note:

Follow NIH guidelines regarding lentiviral production and transduction; follow Biosafety Level 2 (BL2) or BL2+ laboratory criteria.
NIH Agent Summary Statement: http://bmbl.od.nih.gov/viral2.htm#retro
NIH Biosafety Level 2 Description: http://bmbl.od.nih.gov/sect3bsl2.htm
NIH/RAC "Guidance on Biosafety Considerations for Research with Lentiviral Vectors":
http://www4.od.nih.gov/oba/RAC/Guidance/LentiVirus_Containment/pdf/Lenti_Containment_Guidance.pdf

Please note that GIPZ vectors are not compatible with third generation packaging systems such as ViraPower from Invitrogen. We recommend the TransLentiviral Packaging system for use with our vectors.

PRODUCT INFORMATION

The product manual for the Expression Arrest pGIPZ lentiviral shRNAmir collection is available for download using the following link:
https://www.openbiosystems.com/collateral/rnai/pi/pGIPZ-manual.pdf

Expression Arrest™ GIPZ lentiviral shRNAmir

CONTENTS

Important Safety Note:
Follow NIH guidelines regarding lentiviral production and transduction; follow Biosafety Level 2 (BL2) or BL2+ laboratory criteria.
NIH Agent Summary Statement: http://bmbl.od.nih.gov/viral2.htm#retro
NIH Biosafety Level 2 Description: http://bmbl.od.nih.gov/sect3bsl2.htm
NIH/RAC "Guidance on Biosafety Considerations for Research with Lentiviral Vectors":
http://www4.od.nih.gov/oba/RAC/Guidance/LentiVirus_Containment/pdf/Lenti_Containment_Guidance.pdf

Please note that GIPZ vectors are not compatible with third generation packaging systems such as ViraPower from Invitrogen. We recommend the Trans-Lentiviral Packaging system for use with our vectors.

PRODUCT DESCRIPTION

The GIPZ lentiviral shRNAmir library was developed by Open Biosystems in collaboration with Dr. Greg Hannon (CSHL) and Dr. Steve Elledge (Harvard). This library combines the design advantages of microRNA-adapted shRNA (shRNAmir) with the pGIPZ lentiviral vector to create a powerful RNAi trigger capable of producing RNAi in most cell types including primary and non-dividing cells.

DESIGN INFORMATION

Unique microRNA-30 based hairpin design

Expression Arrest™ short hairpin RNA constructs are expressed as human microRNA-30 (miR30) primary transcripts (Figure 1). This design adds a Drosha processing site to the hairpin construct and has been shown to greatly increase knockdown efficiency (Boden, Pusch et al. 2004). The hairpin stem consists of 22nt of dsRNA and a 19nt loop from human miR30. Adding the miR30 loop and 125nt of miR30 flanking sequence on either side of the hairpin results in

greater than 10-fold increase in Drosha and Dicer processing of the expressed hairpins when compared with conventional shRNA designs without microRNA (Silva, Li et al. 2005). Increased Drosha and Dicer processing translates into greater siRNA/miRNA production and greater potency for expressed hairpins.

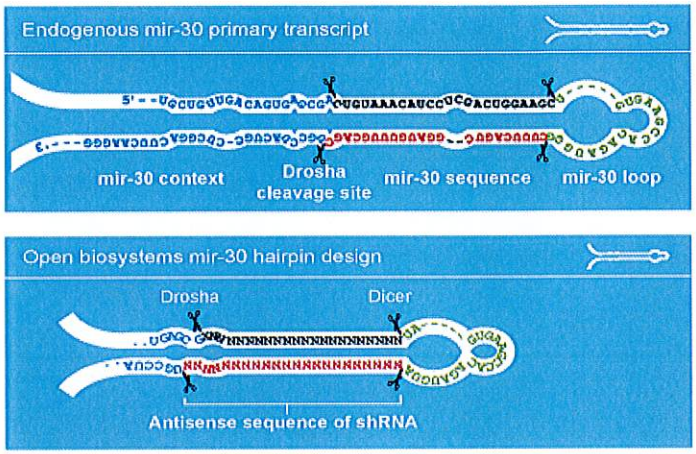

Figure 1. Expression Arrest shRNA are expressed as miR30 primary transcripts

Use of the miR30 design also allowed the use of **'rules-based' designs** for target sequence selection. One such rule is the destabilizing of the 5' end of the antisense strand which results in strand specific incorporation of miRNAs into RISC.

The proprietary design algorithm targets sequences in coding regions and the 3'UTR with the additional requirement that they contain greater than 3 mismatches to any other sequence in the human or mouse genomes.

Each shRNA construct has been sequence verified to ensure a match to the target gene. To assure you the highest possibility of modulating the gene expression level, each gene is represented by multiple shRNA constructs, each covering a unique region of the target gene.

VECTOR INFORMATION

Versatile vector design

Features of the pGIPZ™ lentiviral vector (Figure 2-3, Table 1) that make it a versatile tool for RNAi studies include:

- Ability to perform transfections or transductions using the replication incompetent lentivirus (Shimada, et al. 1995)
- TurboGFP and shRNAmir are part of a bicistronic transcript allowing the visual marking of shRNAmir expressing cells
- Amenable to *in vitro* and *in vivo* applications
- Puromycin drug resistance marker for selecting stable cell lines
- Molecular barcodes enable multiplexed screening in pools

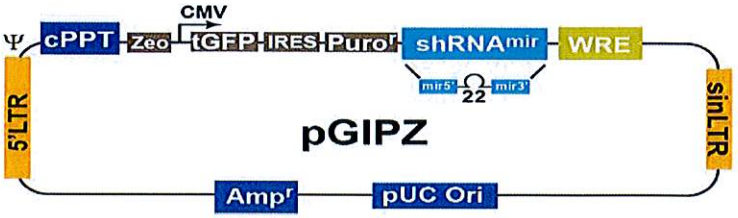

Figure 2: pGIPZ lentiviral vector

Table 1. Features of the pGIPZ vector

Vector Element	Utility
CMV Promoter	RNA Polymerase II promoter
cPPT	Central Polypurine tract helps translocation into the nucleus of non-dividing cells
WRE	Enhances the stability and translation of transcripts
TurboGFP	Marker to track shRNAmir expression
IRES-puro resistance	Mammalian selectable marker
Amp resistance	Ampicillin (carbenicillin) bacterial selectable marker
5'LTR	5' long terminal repeat
pUC ori	High copy replication and maintenance of plasmid in *E. coli*
SIN-LTR	3' Self inactivating long terminal repeat (Shimada, et al. 1995)
RRE	Rev response element
Zeo resistance	Bacterial selectable marker

VECTOR MAP

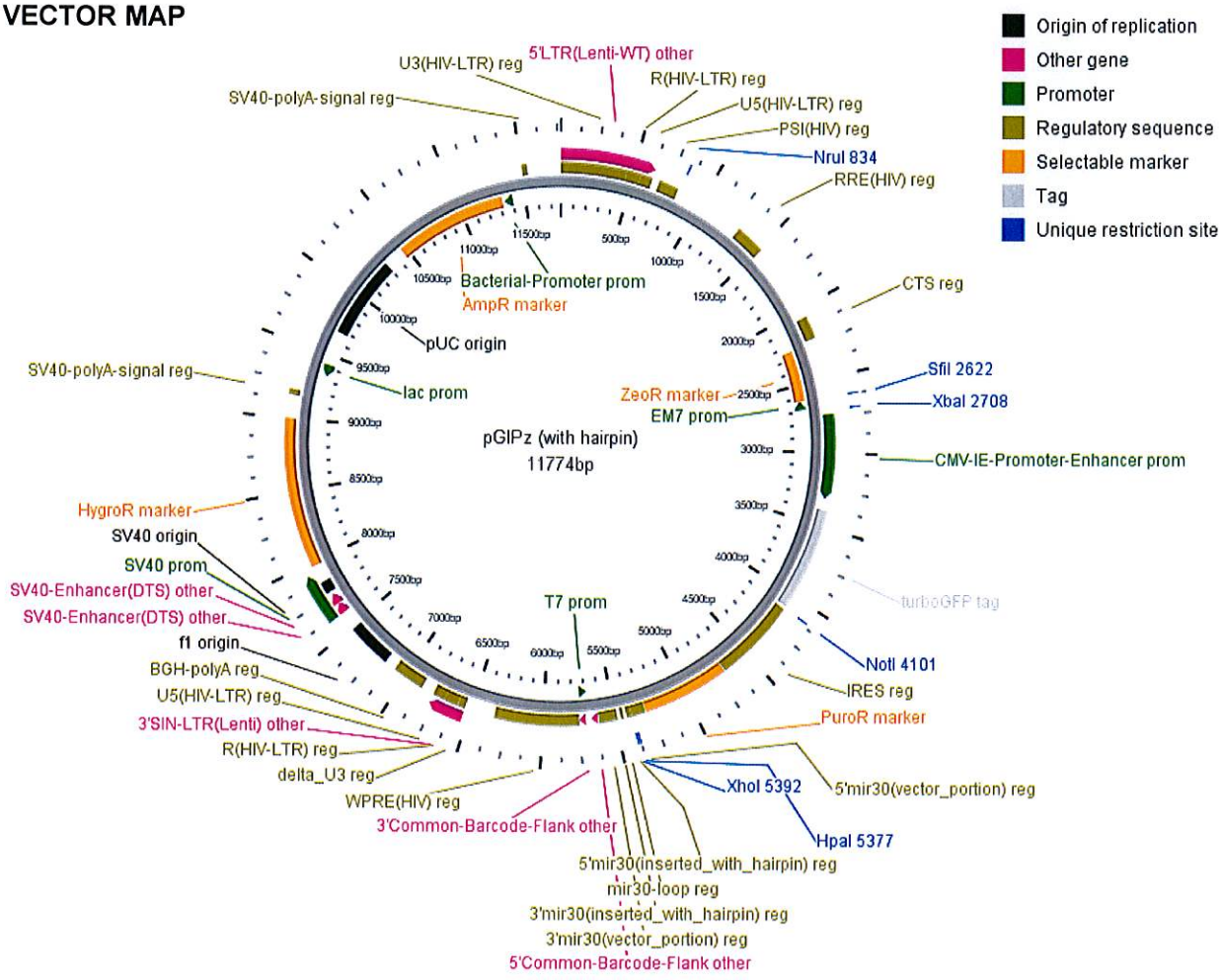

Figure 3. Detailed vector map of pGIPZ™ lentiviral vector.

ANTIBIOTIC RESISTANCE

pGIPZ™ contains 3 antibiotic resistance markers (Table 2).

Table 2. Antibiotic resistances conveyed by pGIPZ

Antibiotic	Concentration	Utility
Ampicillin (carbenicillin)	100µg/ml	Bacterial selection marker (outside LTRs)
Zeocin	25µg/ml	Bacterial selection marker (inside LTRs)
Puromycin	variable	Mammalian selectable marker

QUALITY CONTROL

The GIPZ lentiviral shRNAmir library has passed through internal QC processes to ensure high quality and low recombination (Figures 4 and 5).

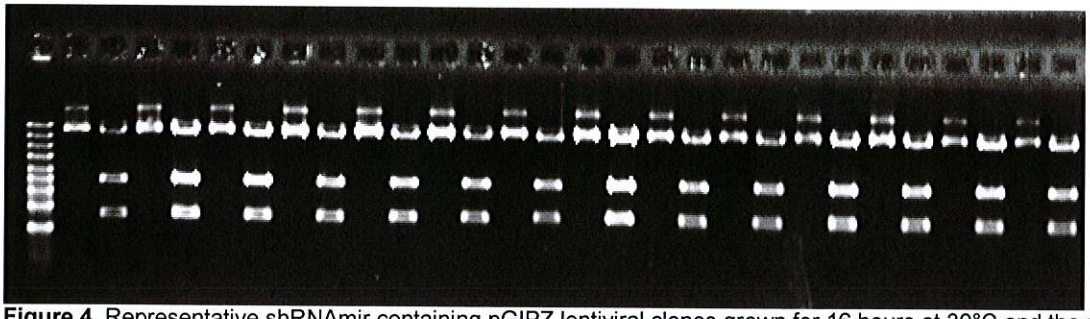

Figure 4. Representative shRNAmir containing pGIPZ lentiviral clones grown for 16 hours at 30°C and the plasmid isolated and normalized to a standard concentration. Clones were then digested with *Sac*II and run out on a gel. The expected band sizes are **1259bp, 2502bp, 7927bp.** No recombinant products are visible. 10kb molecular weight ladder (10kb, 7kb, 5kb, 4kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb)

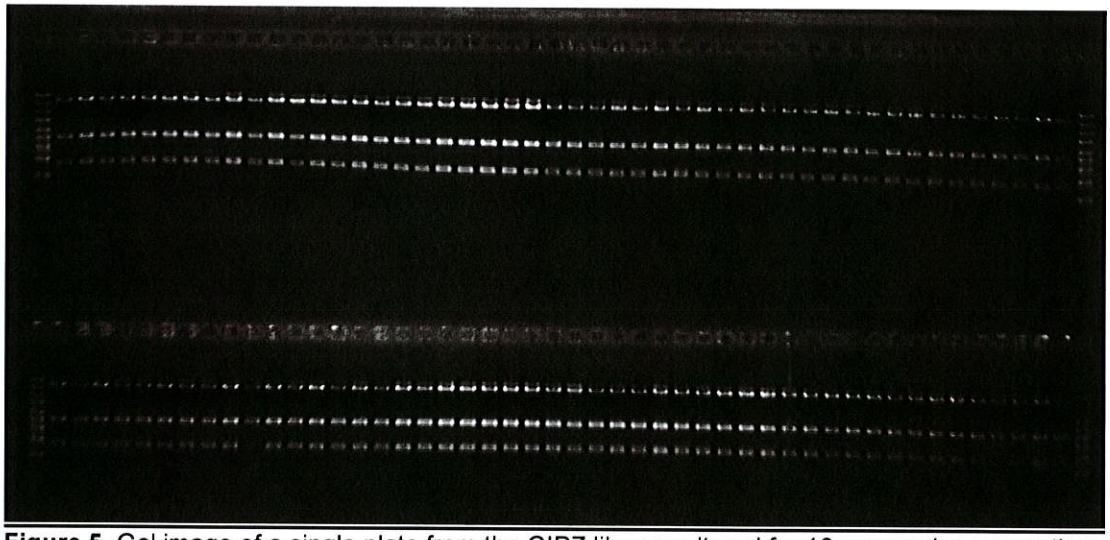

Figure 5. Gel image of a single plate from the GIPZ library cultured for 10 successive generations in an attempt to determine the tendency of the pGIPZ vector to recombine. Each generation was thawed, replicated and incubated overnight for 16 hours at 30°C then frozen, thawed and replicated. This process was repeated for 10 growth cycles. After the 10th growth cycle, plasmid was isolated and normalized to a standard concentration. Clones were then digested with *Sac*II and run on a gel. Expected band sizes **1259bp, 2502bp, 7927bp.** 10kb molecular weight ladder (10kb, 7kb, 5kb, 4kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb).The pGIPZ vector appears stable without showing any recombination.

PROTOCOL I - REPLICATION

Table 3. Materials for plate replication

Item	Vendor	Catalog Number
LB-Lennox broth (low salt)	VWR	EM1.00547.0500
Peptone, granulated, 2kg - Difco	VWR	90000-368
Yeast Extract, 500g, granulated	VWR	EM1.03753.0500
NaCl	Sigma	S-3014
Glycerol	VWR	EM-2200 or 80030-956
Carbenicillin or ampicillin	Novagen	69101-3
Zeocin	Invivogen	ant-zn-5p
Puromycin	Cellgro	61-385-RA
96 well microplates	Nunc	260860
Aluminum seals	Nunc	276014
Disposable replicators	Genetix	X5054
Disposable replicators	Scinomix	SCI-5010-OS

For archive replication, grow all pGIPZ™ clones at 30°C in LB-Lennox (low salt) media plus 25µg/ml zeocin and 100µg/ml carbenicillin in order to provide maximum stability of the clones. Prepare media with 8% glycerol* and the appropriate antibiotics.

Replication of plates

Prepare target plates by dispensing ~160µl of LB-Lennox (low salt) media supplemented with 8% glycerol* and appropriate antibiotic (25µg/ml zeocin and 100µg/ml 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 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 30°C incubator without shaking for 18-19 hours.

Freeze at -80°C for long term storage. Avoid long periods of storage at room temperature or higher in order to control background recombination products.

Note: Due to the tendency of all viral vectors to recombine, we recommend keeping the incubation times as short as possible and avoid subculturing.

**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.*

PROTOCOL II - PLASMID PREPARATION

Culture conditions for individual plasmid preparations

For plasmid preparation, grow all pGIPZ™ clones at 37°C in 2X-LB broth (low salt) media plus ***100µg/ml carbenicillin only***.

2X-LB broth (low salt) media preparation

LB-Broth-Lennox	20g/l
Peptone	10g/l
Yeast Extract	5g/l

Appropriate antibiotic(s) at recommended concentration(s)

Most plasmid mini-prep kits recommend a culture volume of 1-10ml for good yield. For shRNAmir constructs, 5ml of culture can be used for one plasmid mini-prep generally producing 5-10µg 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. Take a 10µl inoculum from the glycerol stock into 3-5ml of 2X-LB (low salt) with 100µg/ml carbenicillin. Return the glycerol stock(s) to -80°C.

   Note: If a larger culture volume is desired, incubate the 3-5ml culture for 8 hours at 37C with shaking and use as a starter inoculum. Dilute the starter culture 1:500-1:1000 into the larger volume.

4. Incubate at 37°C for 18-19 hours with vigorous shaking.
5. Pellet the 3-5ml culture and begin preparation of plasmid DNA.
6. Run 3-5µl of the plasmid DNA on a 1% agarose gel. pGIPZ with shRNAmir is 11774bp.

   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 for each plasmid preparation.

Culture conditions for 96 well bio-block plasmid preparation

Inoculate a 96 well bio-block containing 1ml per well of 2X-LB (low salt) media with 100µg/ml carbenicillin with 1µl of the culture. Incubate at 37°C with shaking (~170-200rpm). We have observed that incubation times between 18-19 hours produce good plasmid yield. For plasmid preparation, follow the protocols recommended by the plasmid isolation kit manufacturer.

Note: Open Biosystems uses the above 96 well bio-block plasmid preparation protocol in conjunction with a Qiagen Turbo kit (catalog no. 27191). We use 2 bio-blocks combined, do not perform the optional wash and elute the DNA in water.

PROTOCOL III - CLONING

Moving shRNAmir constructs from pSM2 to pGIPZ

1. Order the pSM2 vector already expressing the shRNAmir of interest from Open Biosystems.

2. Order the following PCR primers:
   pSM2 forward - 5' aagccctttgtacaccctaagcct 3'
   pSM2 reverse - 5' actggtgaaactcacccagggatt 3'

3. Order a KOD Hotstart Polymerase kit from Novagen (catalog no. 71086-5 for 20U)

4. Resuspend the PCR primers at a stock concentration of 50pmol/µl in sterile DEPC water. Dilute the stock 1:10 for a working concentration of 5pmol/µl in sterile DEPC water.

5. Set up the following PCR reaction at room temperature (Table 4). Add the components in the order listed. The following is for one 50ul reaction. To do more reactions simply multiply the master mix components by the desired number of reactions plus 10%. We recommend doing 4 reactions to ensure enough fragment will be available for cloning.

Table 4. PCR reaction

Component	**volume in µl**
H20 (DEPC)	25
5M Betaine	5
10X PCR buffer for KOD Hotstart Polymerase	5
dNTP's (2mM each)	5
MgSO4 25mM	2
pSM2 Forward Primer (5pmol/µl)	3
pSM2 Reverse Primer (5pmol/µl)	3
KOD Hotstart Polymerase (1U)	1
Total volume	49
Template (1ul of glycerol stock from your pSM2 clone of interest)	1

6. Input the following program into your thermocycler (Table 5):

Table 5. PCR program

PCR Program HS KOD		
	Temperature	**Time**
Hot Start Enzyme Activation	94˚C	2min
Melt	94˚C	15sec
Anneal	58˚C	30sec
Extension	72˚C	25sec
Cycles	30˚C	
Expected Product Size	1735bp	

7. Put the four PCR reactions through a Wizard SV Gel and PCR Clean-up System column according to the kit directions (Promega catalog no. A9281 for 50 preps), with the exception of eluting with 110µl of the provided nuclease free water. All four reactions can be run on a single SV Gel and PCR Clean-up column.

8. Set up the following restriction digest using the Clean-up column eluent (Table 6) and Incubate at 37˚C for 3 hours.

Table 6. Restriction digest

Component	volume in µl
PCR elutent	100
10X Restriction Enzyme Buffer	20
MluI	2
XhoI	2
Sterile Water	76
Total volume	200

9. Run the entire digest on a 1.2%-1.5% agarose gel. Three bands should be seen (789bp, 683bp, and 345bp) (Figures 6 and 7). Three bands will appear only if both *Mlu*I and *Xho*I have cut. Therefore the digest is diagnostic of the enzyme cuts in the following fashion (Table 7).

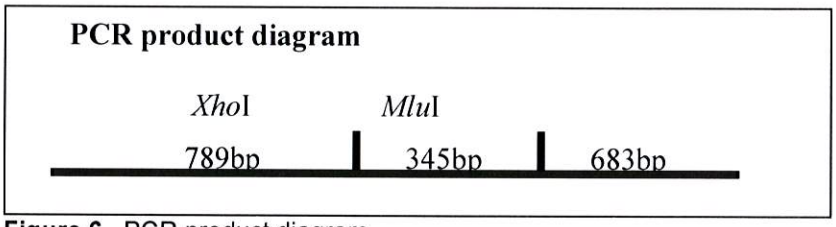

Figure 6. PCR product diagram

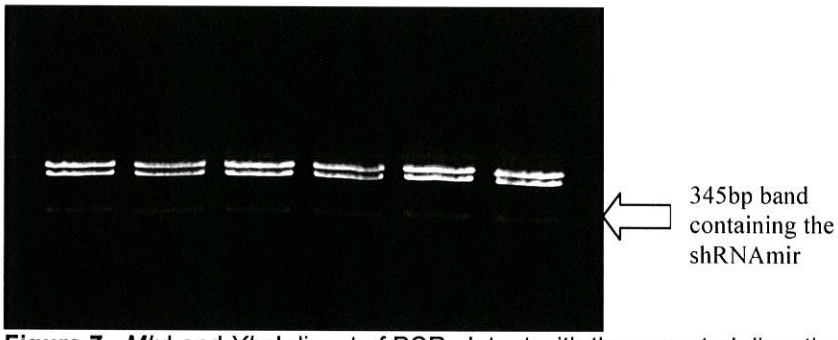

Figure 7. *Mlu*I and *Xho*I digest of PCR elutent with the expected digestion pattern (789bp, 683bp, 345bp)

Table 7. Possible digestion patterns

Band sizes seen	Conclusion
1735bp only	Neither *Mlu*I **or** *Xho*I cut
789bp, 683bp, and 345bp	Both *Mlu*I **and** *Xho*I cut
1028bp and 789bp	Only *Xho*I cut. *Mlu*I did not cut.
1134bp and 683bp	Only *Mlu*I cut. *Xho*I did not cut.

10. Excise the 345bp band containing the shRNAmir of interest and purify on a Wizard SV Gel and PCR Clean-up System column according to the kit directions (Promega catalog no. A9281 for 50 preps). Elute in 50µl nuclease free water.

11. Quantitate the insert fragment.

12. Prepare the pGIPZ empty vector for ligation to the shRNAmir insert. Set up the following restriction digest (Table 8).

Table 8. Restriction digest

Component	volume in µl
pGIPZ empty vector (250ng/µl)	12
10X Restriction Enzyme Buffer	10
MluI	2
XhoI	2
Sterile Water	74
Total volume	100

13. Mix the solution by pipetting and then gently spin the reaction for approximately 10 seconds to collect all the solution in the bottom of the tube. This will aid in decreasing contamination of uncut vector in your vector prep to follow. Incubate at 37˚C for 3 hours.

14. Run the entire digest on a 0.8% agarose gel. Make sure to run the gel through no less than 3cm length of agarose. This will also aid in decreasing contamination of uncut vector in your vector prep.

15. Gel isolate the 11429bp band using a Wizard SV Gel and PCR Clean-up System column according to the kit directions (Promega catalog no. A9281 for 50 preps). Elute in 50µl nuclease free water. You will likely not see a band representing the excised portion of the vector as it is too small (~259bp).

16. Quantitate the amount of cut vector per µl you have isolated.

17. Set up the following ligation reactions (Table 9):

Table 9. Ligation reactions

Component	No Insert control	shRNAmir ligation
shRNAmir insert cut with *MluI* and *XhoI* (total 7.4ng)	XXXXXXXXX	____ µl
pGIPZ vector cut with *MluI* and *XhoI* (total 250ng)	____ µl	____ µl
DEPC water	____ µl	____ µl
10X ligase buffer	2 µl	2 µl
Ligase	0.5 µl	0.5 µl
Total volume	20 µl	20 µl

Note: This setup yields a molar ratio of 1 vector to 1 insert.

18. Ligate for 3 hours at room temperature. Dilute the ligation mix by adding 160µl DEPC water.

19. Transform 5µl of the diluted ligation mix into PrimePlus™ competent *E.coli* (Open Biosystems catalog no. MBC4246). Follow the transformation protocol for the competent cells. Plate the transformed cells onto agar plates containing 100µg/ml carbenicillin and 25µg/ml zeocin. Be sure to transform the same volume of ligation mix and plate the same volume of cells for both the control and the experimental sample. Plating 100µl, 50µl, and 10µl aliquots is recommended.

20. Incubate plates at 30˚C overnight. Count colonies and determine the ratio of colonies on the control plate versus the experimental plates. Determine the number of colonies to screen.

21. Order the following PCR primers to screen your clones for insertion of the shRNAmir sequence of interest.
    X76 Forward - 5' acgtcgaggtgcccgaagga -3'
    M100 Reverse - 5' aagcagcgtatccacatagcgt -3'

22. Set up the following PCR reaction (Table 10). For template simply pick a colony with a toothpick, swirl in a small broth culture containing ampicillin and zeocin to maintain a stock and then swirl the same toothpick into your PCR well containing the appropriate amount of master mix. For a no insert control simply use 1ng of empty pGIPZ™ vector. The amounts in the table below are for a single 50µl reaction.

Table 10. PCR reaction

Component	**volume in µl**
H20 (DEPC)	26
5M Betaine	5
10X PCR buffer for KOD Hotstart Polymerase	5
dNTP's (2mM each)	5
$MgSO_4$ 25mM	2
X76 Forward Primer (5pmol/µl)	3
M100 Reverse Primer (5pmol/µl)	3
KOD Hotstart Polymerase (1U)	1
Total volume	49
Template colony picked with toothpick	---

23. Run the following PCR program (Table 11). Note the annealing temperature has changed.

Table 11. PCR program

PCR Program HS KOD#2		
	Temperature	**Time**
Hot Start Enzyme Activation	94°C	2min
Melt	94°C	15sec
Anneal	**56°C**	30sec
Extension	72°C	25sec
Cycles	30	

24. Expected band sizes are as follows:
    shRNA inserted = 603bp (clone with barcode); 543bp (clone without barcode)
    No shRNA inserted = 516bp

25. Sequence verify clones. The pGIPZ sequencing primer is as follows:
    5'- GCATTAAAGCAGCGTATC -3'
    Note: The binding site lies from base 5820-5842 and runs in the reverse complement direction. The melting temperature of this 18mer=52.7°C.

PROTOCOL IV - RESTRICTION DIGEST

The following is a sample protocol for restriction enzyme digestion using *Kpn*I, *Sac*II, *Sal*I, *Xho*I and/or *Not*I for diagnostic quality control of GIPZ lentiviral vectors.

1. Using filtered pipette tips and sterile conditions add the following components (Table 12), in the order stated, to a sterile PCR thin-wall tube.

Table 12. PCR Components

Component	Amount
Sterile, nuclease-free water	Xµl
Restriction enzyme 10X buffer	1µl
BSA (10X, 10mg/ml) if required	1µl
DNA sample 80-240ng, in water or TE buffer	Xµl
Restriction enzyme 20U	0.25µl
Final volume	10µl

2. Mix gently by pipetting.
3. Incubate in a thermalcycler at 37°C for 2 hours to digest
4. Load the gel with 10µl of each of the digested samples (*Kpn*I, *Sac*II, *Sal*I, *Xho*I and/or *Not*I) on a 1% agarose gel. Run uncut sample alongside the digested samples. (Figure 8)

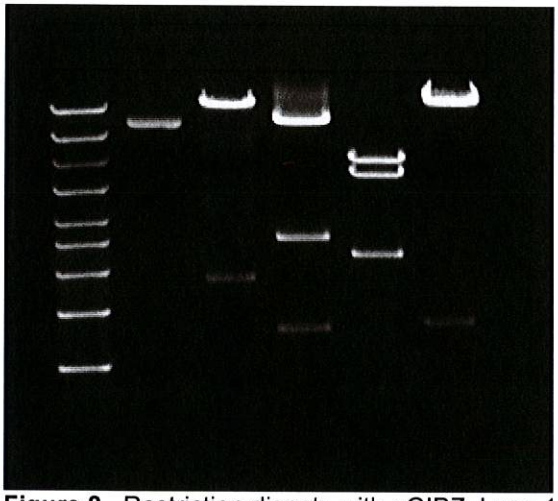

Figure 8. Restriction digests with pGIPZ. Lane 1- 10kb molecular weight ladder (10kb, 7kb, 5kb, 4kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb). Lane 2 - Uncut pGIPZ vector. Lane 3 - *Kpn*I digested pGIPZ produces 2 bands at 1750bp and 9860bp. Lane 4- *Sac*II digest produces 3 bands at 1178bp, 2502bp and 7930bp. Lane 5 -*Sal*I produces 3 bands at 2188bp, 4298bp and 5124bp. Lane 6 - *Xho*I, *Not*I double digest produces 2 bands at 1210bp and 10400bp.

PROTOCOL V - PUROMYCIN SELECTION

Puromycin Kill Curve and Puromycin Selection

In order to generate stable cell lines, it is important to determine the minimum amount of puromycin required to kill non-transfected/transduced cells. This can be done by generating a puromycin kill curve.

Puromycin Kill Curve

1. On day 0 plate 5 x 10^4 cells per well in a 24 well plate in enough wells to carry out your puromycin dilutions. Incubate overnight.
2. Prepare media specifically for your cells containing a range of antibiotic, for example: 0 - 15µg/ml puromycin.
3. The next day (day 1) replace the growth media with the media containing the dilutions of the antibiotic into the appropriate wells.
4. Incubate at 37°C.
5. Approximately every 2-3 days replace with freshly prepared selective media.
6. Monitor the cells daily and observe the percentage of surviving cells. Optimum effectiveness should be reached in 3-6 days under puromycin selection.
7. The minimum antibiotic concentration to use is the lowest concentration that kills 100% of the cells in 3-6 days from the start of antibiotic selection.

PROTOCOL VI - 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 13). 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 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 Arrest-In mixtures as they will be added to the cells.

Table 13. 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 (cm2)	Total serum free media volume per well (ml)	Plasmid DNA (µg)*	Arrest-In (µg)**
60 mm	20	2	4	21
35 mm	8	1	2	10
6 well	9.4	1	2	10
12 well	3.8	0.5	1	5
24 well	1.9	0.25	0.5	2.5
96 well	0.3	0.1	0.1 - 0.2	0.5 - 1

*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.

a. For each well to be transfected, dilute 500ng shRNA plasmid DNA into 50µl (total volume) of serum free medium in a microfuge tube.
b. For each well to be transfected, dilute 2.5µg (2.5µl) of Arrest-In into 50µl (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 by mass which is recommended for optimal transfection into HEK293T cells. Your total volume will be 100µl at this stage.

3. Aspirate the growth medium from the cells. Add an additional 150µl of serum free medium to each of the tubes containing transfection complexes (100µl). Add the total volume of the tube (250µl) to the cells and incubate for 5-6 hours in a CO_2 incubator at 37°C.

Your total volume will be 250µl at this stage (150µl serum-free medium + 100µl DNA:Arrest-In mixture).

4. Following the 5-6 hour incubation, add an equal volume of growth medium (250µl) 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 hands 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 24-72 hours of incubation, examine the cells microscopically for the TurboGFP expression.

Note: When visualizing TurboGFP expression, if less than 90% of all cells are green, it is recommended in these cases to utilize puromycin selection in order to reduce background expression of your gene of interest from untransfected cells (see Figure 11).

The working concentration of puromycin varies between cell lines. We recommend you determine the optimal concentration of puromycin required to kill your host cell line prior to selection for shRNAmir transfectants (see puromycin kill curve protocol, page 20). Typically, the working concentration ranges from 1-10µg/ml. You should use the lowest concentration that kills 100% of the cells in 1-4 days from the start of puromycin selection.

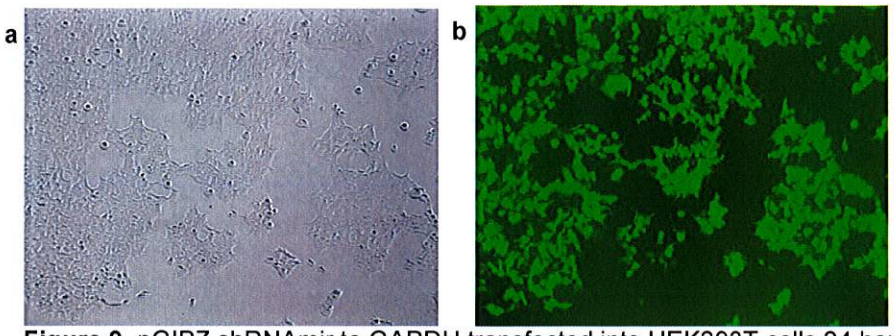

Figure 9. pGIPZ shRNAmir to GAPDH transfected into HEK293T cells 24 hours post transfection. a) Phase b) TurboGFP fluorescence. Note the high percentage of cells successfully transfected.

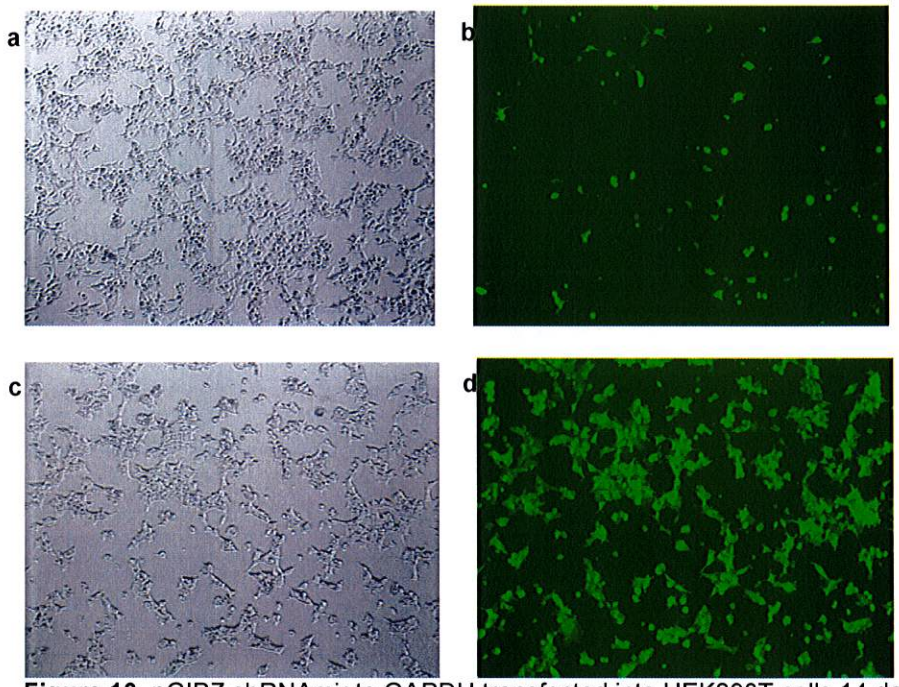

Figure 10. pGIPZ shRNAmir to GAPDH transfected into HEK293T cells 14 days post transfection. a) Phase. No puromycin selection. b) TurboGFP fluorescence. No puromycin selection. c) Phase. Puromycin selected. d) TurboGFP fluorescence. Puromycin selected. Note the decrease in the number of cells expressing TurboGFP when puromycin selection is not applied. When puromycin selection is maintained TurboGFP expression remains in all cells at high levels.

a. If adding puromycin, use the appropriate concentration as determined based on the above kill curve. Incubate.

b. Approximately every 2-3 days replace with freshly prepared selective media.

c. Monitor the cells daily and observe the percentage of surviving cells. At some time point almost all of the cells surviving selection will be harboring the shRNAmir. Optimum effectiveness should be reached in 3-6 days with puromycin.

6. If selecting for stably transfected cells (optional), change the medium on the cells to that containing puromycin for selection. It is important to wait at least 24 hours before beginning selection.

7. Once cells not expressing TurboGFP are virtually eliminated and/or you have selected for stably transfected cells (optional), you can proceed to 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 shRNAmir or other negative controls.

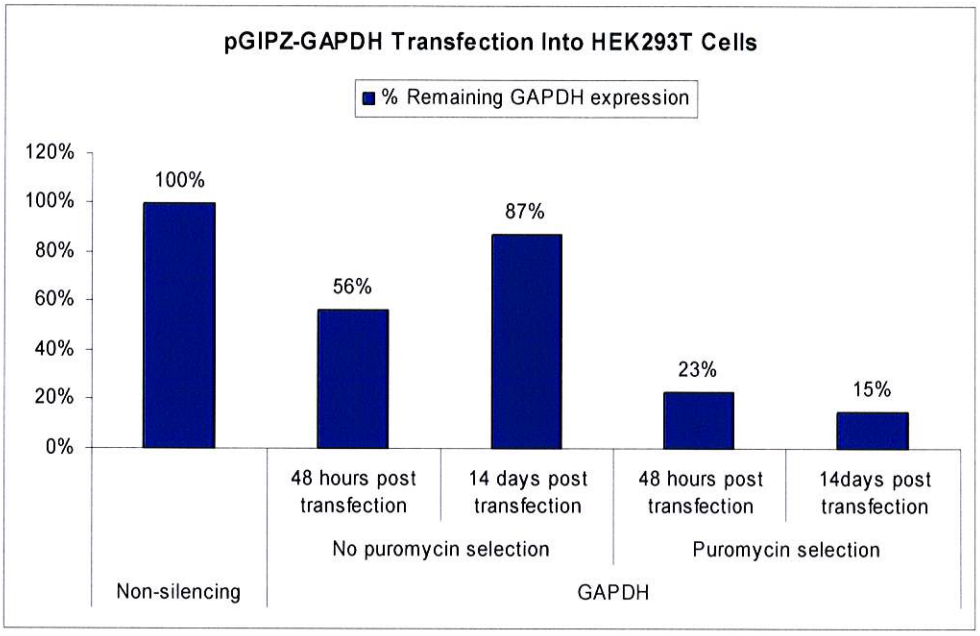

Figure 11. mRNA to GAPDH as measured by quantitative/real-time RT-PCR of GAPDH post transfection into HEK293T cells (48 hours and 14 days post transfection/puromycin selection). While transfection efficiency is high (Figure 9) the number of cells without the shRNA present is still high enough to mask the knockdown readout. These cells are eliminated via puromycin selection and the knockdown readout is increased.

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 knockdown. 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.

Factors affecting transfection efficiency are not limited to but include purity of plasmid DNA, health of transduced cells, and inconsistencies in number of cells plated.

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 13 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.

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 250µl serum free medium or to a total volume of 250µl of medium with serum (no antibiotics).

PROTOCOL VII - PACKAGING LENTIVIRUS

The pGIPZ vector is *tat* dependant, so you must use a packaging system that expresses the *tat* gene. For packaging our lentiviral shRNAmir constructs, we recommend the Trans-Lentiviral™ shRNA Packaging System (TLP4614, TLP4615). The Trans-Lentiviral shRNA Packaging System allows creation of a replication-incompetent (Shimada, et al. 1995), HIV-1-based lentivirus which can be used to deliver and express your gene or shRNAmir of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral shRNA Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes (Kappes and Wu 2001). For protocols and information on packaging pGIPZ™ with our Trans-Lentiviral shRNA Packaging System, please see the product manual available at the following link:
http://www.openbiosystems.com/collateral/rnai/pi/Trans-Lentiviral_GIPZ_Packaging_System.pdf

PROTOCOL VIII - TITERING

Viral Titering
Follow the procedure below to determine the titer of your lentiviral stock using the mammalian cell line of choice. This protocol uses the TLA-HEK293T™ cell line that is available as part of our Trans-Lentiviral shRNA Packaging System. You can use a standard HEK293T cell line as an alternative.

> *Note: If you have generated a lentiviral stock of the expression control (e.g. pGIPZ Non-Silencing), we recommend titering this stock as well.*

1. The day before transduction, seed a 24 well tissue culture plate with TLA-HEK293T cells at 5 x 10^4 cells per well in DMEM (10% FBS, 1% pen-strep).

   > *The following day, the well should be no more than 40-50% confluent. TLA-HEK293T (Open Biosystems catalog no. HCL4517).*

2. Make dilutions of the viral stock in a round bottom 96 well plate using serum-free media. Utilize the plate as shown in Figure 12 using one row for each virus stock to be tested. Use the procedure below (starting at step 4) for dilution of the viral stocks. The goal is to produce a series of 5-fold dilutions to reach a final dilution of 390625-fold.

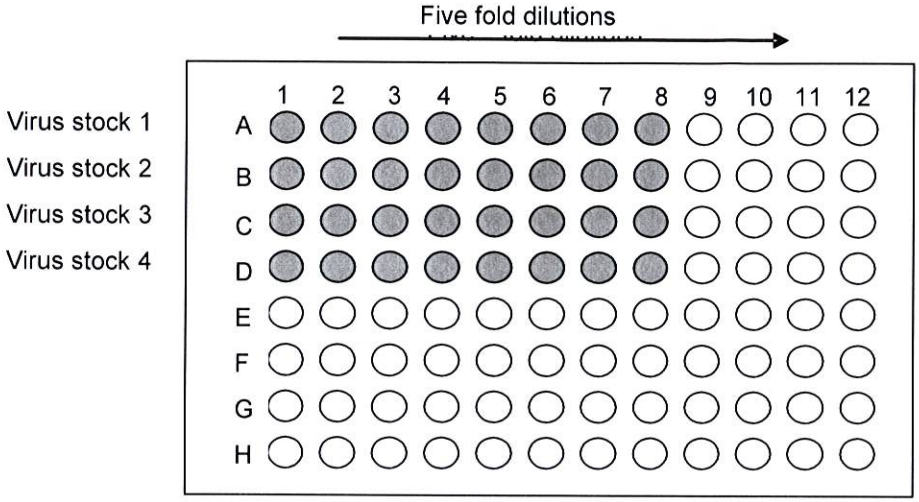

Figure 12. Five-fold serial dilutions of virus stock.

3. To each well add 80µl of serum-free media.

4. Add 20µl of thawed virus stock to each corresponding well in column 1 (5 fold dilution).

   Pipette contents of well up and down 10-15 times. Discard pipette tip.

5. With new pipette tips, transfer 20µl from each well of column 1 to the corresponding well in column 2.

   Pipette 10-15 times and discard pipette tips.

6. With new pipette tips, transfer 20µl from each well of column 2 to the corresponding well in column 3.

   Pipette 10-15 times and discard pipette tip.

7. Repeat transfers of 20µl from columns 3 through 8, pipetting up and down 10-15 times and changing pipette tips between each dilution.

   It is strongly recommended that you use a high quality multichannel pipettor when performing multiple dilutions. Pre-incubate the dilutions of the virus stock for 5 minutes at room temperature.

8. Label 24 well plate as shown in Figure 13 using one row for each virus stock to be tested.

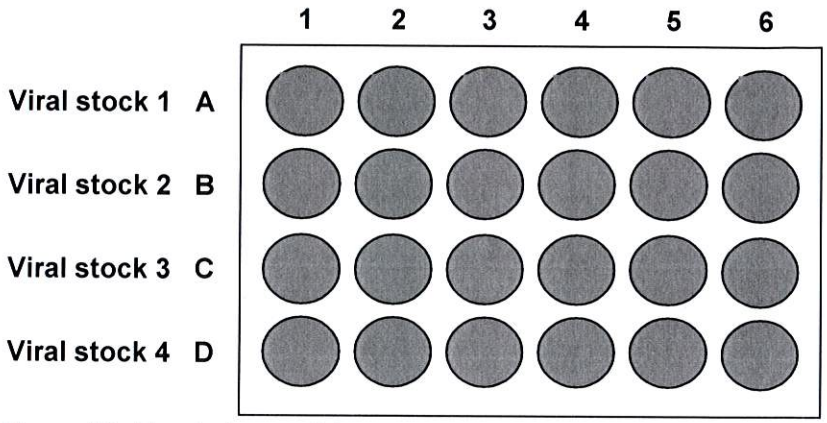

Figure 13. Twenty four well tissue culture plate, seeded with TLA-HEK293T™ cells, used to titer the virus.

9. Remove culture media from the cells in the 24 well plate.

10. Add 225µl of serum-free media to each well.

11. Transduce cells by adding 25µl of diluted virus from the original 96 well plate (Figure 12) to a well on the 24 well destination plate (Figure 13) containing the cells.

    For example, transfer 25µl from well A2 of the 96 well plate into well A1 in the 24 well plate (Table 14).

Table 14. Example of set up for dilutions

Well (Row A, B, C, or D)		Volume diluted virus used	Dilution Factor
Originating (96 well plate)	Destination (24 well plate)		
A1		25µl	5 *
A2	A1	25µl	25
A3	A2	25µl	125
A4	A3	25µl	625
A5	A4	25µl	3125
A6	A5	25µl	15625
A7	A6	25µl	78125
A8		25µl	390625 *

**Please note that when expecting very high or very low titers, it would be advisable to include either well 8 or well 1 respectively.*

12. Incubate transduced cultures at 37°C for 4 hours.

13. Remove transduction mix from cultures and add 1ml of DMEM (10% FBS, 1% pen-strep).

14. Culture cells for 48 hours.

15. Count the TurboGFP expressing cells or colonies of cells (Figure 14).

Count each multi-cell colony as 1 transduced cell, as the cells will be dividing over the 48 hour culture period. Figure 14 illustrates this principle of counting.

16. Transducing units per ml (TU/ml) can be determined using the following formula:
# of TurboGFP positive colonies counted x dilution factor x 40 = #TU/ml

Example: 55 TurboGFP positive colonies counted in well A3.
55 (TurboGFP positive colonies) x 625 (dilution factor) x 40 = 1.38×10^6 TU/ml

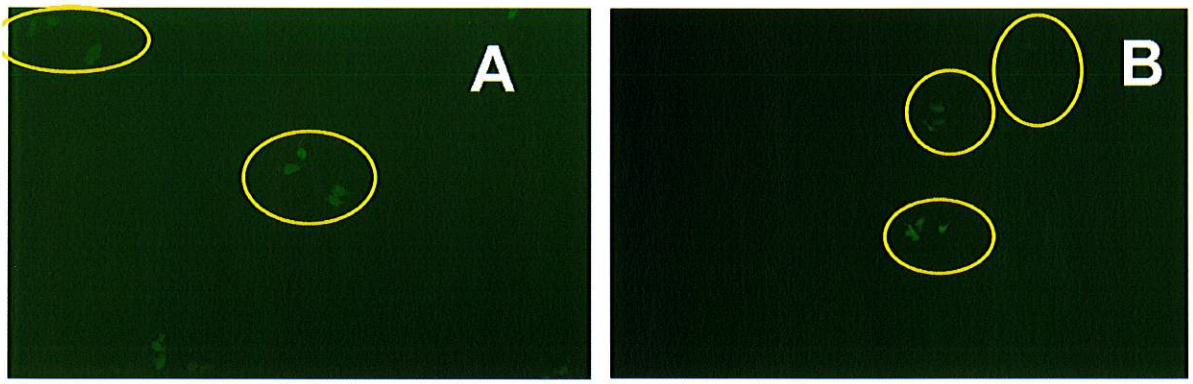

Figure 14. Examples of individual colonies

Once you have generated a lentiviral stock with a suitable titer, you are ready to transduce the lentiviral vector into the mammalian cell line of choice and assay for expression of your recombinant protein.

Multiplicity of Infection (MOI)
To obtain optimal expression of your gene of interest, you will need to transduce the lentiviral vector into your mammalian cell line of choice using a suitable MOI. MOI is defined as the number of transducing units per cell. Although this is cell line dependent, this generally correlates with the number of integration events per cell and as a result, level of expression.

Determining the Optimal MOI
A number of factors can influence determination of an optimal MOI including the nature of your mammalian cell (actively- versus non-dividing), its transduction efficiency, your application of interest, and the nature of your gene of interest. If you are transducing your lentiviral construct into the mammalian cell line of choice for the first time, after you have titered it, we recommend using a range of MOIs (e.g. 0, 0.5, 1, 2, 5, 10, 20) to determine the MOI required to obtain optimal expression for your particular application. It should be noted that to achieve single copy knockdown, an MOI of 0.3 is generally used, as less than 4% of your cells will have more than one insert.

PROTOCOL IX - TRANSDUCTION

Transduction of Target Cells

The protocol below is optimized for transduction of the lentiviral particles into HEK293T, OVCAR8 or MCF7 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 (see Table 13).

It is preferable that transduction be carried out in medium that is serum free and antibiotic free. A reduction in transduction efficiency occurs in the presence of serum, however it is possible to carry out successful transductions with serum present; you will have to optimize the protocol according to your needs.

1. On day 0 plate 5 x 10^4 cells per well in a 24 well plate. Incubate overnight.

   You will be using full medium (i.e. with serum) at this stage.

2. The next day (day 1), remove the medium and add the virus to the MOI you wish to use. Set up all desired experiments and controls in a similar fashion.

   Bring the total volume of liquid up so that it just covers the cells efficiently with serum-free media (See Table 15 for guidelines). If you are using concentrated virus you are likely to use very little virus volume and a lot of serum-free media; if you are using unconcentrated virus you will find you need much more virus volume.

Table 15. Suggested volumes of media per surface area per well of adherent cells.

Tissue Culture Dish	Surface area per well (cm^2)	Suggested total serum free medium volume per well (ml)
100mm	56	5
60mm	20	2
35mm	8	1
6 well	9.4	1
12 well	3.8	0.5
24 well	1.9	0.25
96 well	0.3	0.1

3. Approximately 4-6 hours post-transduction, add an additional 1ml of full media (serum plus pen-strep if you are using it) to your cells and incubate overnight.

   We have experienced low toxicity with transduction in the cell lines tested, therefore removal of virus is not required for many cell lines. In our hands higher transduction efficiencies have been achieved if the virus is not removed after 6 hours. However, if toxicity is a problem, aspirate the mixture after 3-6 hours and replace with fresh growth medium. Additionally, fresh growth medium should be replenished as required for continued cell growth.

4. At 48 hours post-transduction examine the cells microscopically for the presence of reporter expression as this will be your first indication as to the efficiency of your transduction.

Note: When visualizing TurboGFP expression, if less than 90% of all cells are green, it is recommended in these cases to utilize puromycin selection in order to reduce background expression of your gene of interest from untransduced cells.

d. If adding puromycin, use the appropriate concentration as determined based on the above kill curve. Incubate.

e. Approximately every 2-3 days replace with freshly prepared selective media.

f. Monitor the cells daily and observe the percentage of surviving cells. At some time point almost all of the cells surviving selection will be harboring the shRNAmir. Optimum effectiveness should be reached in 3-6 days with puromycin.

Please note that the higher the MOI you have chosen the more copies of the shRNAmir and puromycin resistance gene you will have per cell. When selecting on puromycin, it is worth remembering that at higher MOIs, cells containing multiple copies of the resistance gene can withstand higher puromycin concentrations than those at lower MOIs. Adjust the concentration of puromycin to a level that will select for the population of transduced cells you wish to select for, without going below the minimum antibiotic concentration you have established in your kill curve.

5. Once your transduction efficiency is at an acceptable level (with or without puromycin selection), you can proceed to assay cells for reduction in gene or reporter activity by quantitative/real-time PCR (QPCR), western blot or other appropriate functional assay; compare to untreated, reporter alone, non-silencing shRNAmir 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. QPCR generally gives the best indication of expression knockdown. 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.

PROTOCOL X - QPCR

QPCR Experimental Recommendations:

One of the biggest challenges of any QPCR experiment is to obtain reproducible reliable data. Due to the sensitivity of this multi-step technique care must be taken to ensure results obtained are accurate and trustworthy.

1. Experimental samples should be run in no less than duplicate. It should be noted that with duplicate experiments it will not be possible to assign error bars to indicate consistency from experimental sample to experimental sample. Using triplicate samples or higher will enable error bars to be assigned indicating the level of experimental variation.
2. QPCR should be done in no less than triplicate. Again, it should be noted that with duplicate reactions it will not be possible to assign error bars to indicate the consistency in your QPCR reactions. Using triplicate samples or higher will enable error bars to be assigned indicating the level of variation between QPCR reactions.

3. We have found that normalizing the RNA concentration prior to cDNA synthesis will increase consistency downstream.
4. Make sure the message you are using as your internal control for QPCR is expressed at a level higher than your target genes message.
5. Use only high-quality calibrated pipettes, in conjunction with well fitting barrier tips.
6. When pipetting, take the time to visually inspect the fluid in the tip(s) for accuracy and lack of bubbles, especially when using a multi-channel pipette.
7. Be sure to spin your QPCR plate prior to loading in the machine in order to collect the sample at the bottom of the well as well as eliminate any bubbles that may have developed.
8. With regard to knockdown experiments using shRNA, it is vitally important that you greatly reduce if not eliminate entirely those cells which are not transduced or transfected from the population (i.e. those cells that are not expressing the fluorescent marker). This can be done in several ways: increase the efficiency of your transfection, use a higher mulitplicity of infection (MOI) for your transduction, or utilize the puromycin selection marker and drug select against those cells that do not contain the shRNA.
9. Always utilize the non-silencing control as a reference for target gene expression, as opposed to an untreated sample. The non-silencing treated samples will most accurately reproduce the conditions in your experimental samples. The non-silencing best controls for changes in QPCR internal control gene expression.
10. You may also use an untreated sample to indicate substantial changes in target gene expression as seen in the non-silencing control due to generic consequences of viral infection/transfection reagents etc. However, it should be noted that small changes in expression levels between an untreated sample and the non-silencing control are to be expected.
11. Ct values greater than 35 should be avoided as they tend to be more variable. Samples with such high Ct values should be repeated at higher cDNA concentrations and with a lower expressing QPCR internal control (such as TBP).
12. Ct values less than 11 for the QPCR internal control should be avoided as it is difficult to determine a proper background subtraction using these values. If this occurs, use Ct values from both your internal control as well as your experimental target to determine an optimum cDNA concentration.
13. It may be necessary to change internal controls if conditions in steps 11 and 12 cannot be simultaneously met.

CONTROLS AND VALIDATION

RNAi*ntro* shRNAmir starter kits

The use of vector-based RNAi for gene silencing is a powerful and versatile tool. Successful gene silencing *in vitro* is dependent on several variables including 1) The target cell line being studied 2) Transfection and transduction efficiency 3) Abundance of the mRNA or protein of interest in the target cell line 4) Half life of the protein 5) Robust experimental protocols. For all these reasons it is very important to run controlled experiments where the transfection and transduction efficiencies are as high as possible and measurable.

Controls are a critical part of a gene silencing experiment. They enable accurate representation of knockdown data and provide confidence in the specificity of the response. Changes in the mRNA or protein levels in cells treated with negative or non-silencing controls reflect non-specific responses in cells and can be used as a baseline against which specific knockdown can be measured. Positive controls are useful to demonstrate that your experimental system is functional and your shRNA construct is successfully activating the RNAi pathway.

Controls

The pGIPZ™ EG5 and GAPDH lentiviral shRNAmir vectors have been validated as positive controls for RNAi experiments performed using the pGIPZ shRNAmir-containing lentiviral vectors. These shRNAmir have been tested in transduction based experiments and have shown efficient knockdown at both mRNA and protein levels. The EG5 control has been validated to knockdown human EG5 by means of QPCR and *in situ* hybridization of cells in tissue culture. The GAPDH control has been validated to knockdown human and mouse GAPDH by QPCR. The pGIPZ non-silencing lentiviral shRNAmir vector has been validated as negative control for RNAi experiments performed using the pGIPZ shRNAmir-containing lentiviral vectors.

Transduction based validation studies

HEK293T cells were trypsinized from a healthy, growing culture, seeded into 24 well plates at 5-8 x 10^4 cells per well and allowed to adhere for 24 hours in DMEM with 10% FCS.
DMEM containing serum was replaced with 200µl serum free media and lentiviral particles containing GAPDH or EG5 shRNAmir, non-silencing or non-transduced controls were added to the appropriate wells at three different multiplicity of infections (MOI) and incubated for 6 hours. DMEM containing serum was then added and the transduced cells were further incubated for a total of 48 hours

RNA Extraction and Validation

At 48 hours post-transduction, transduced cells were lysed and total RNA was extracted using the Qiagen RNAeasy Kit (catalog no. 74104). The RNA was converted to cDNA using the ABI-High Capacity cDNA RT Kit (catalog no. 4368813), using 500ng total RNA in a 100µl reaction. A 1/100 dilution of the cDNA was used in QPCR. Each gene was validated in triplicate, standardized to a 18s endogenous control and compared to non-silencing or non-transduced experimental controls. Knockdown was calculated as the percentage remaining gene expression normalized to the relevant non-silencing control (Figures 15, 16 and 17). The non-silencing control was shown to not knockdown endogenous genes (Figure 15).

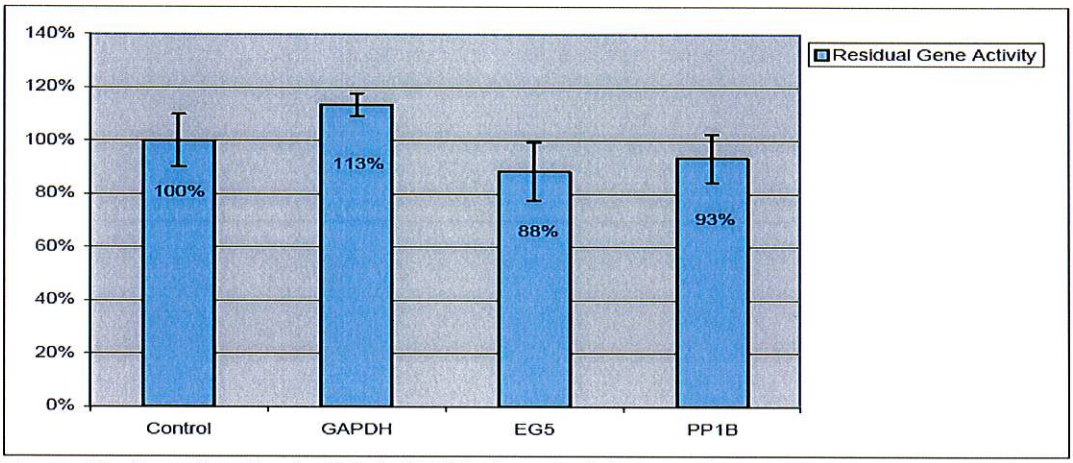

Figure 15. Non-silencing lentiviral shRNAmir control does not knockdown endogenous genes. The above data represents the baseline amount of GAPDH, EG5 or PP1B mRNA set at 100% in the control. The relative amounts of each of these mRNAs are then represented after treatment with non-silencing shRNAmir. Thus the non-silencing shRNAmir has no significant effect on endogenous gene expression.

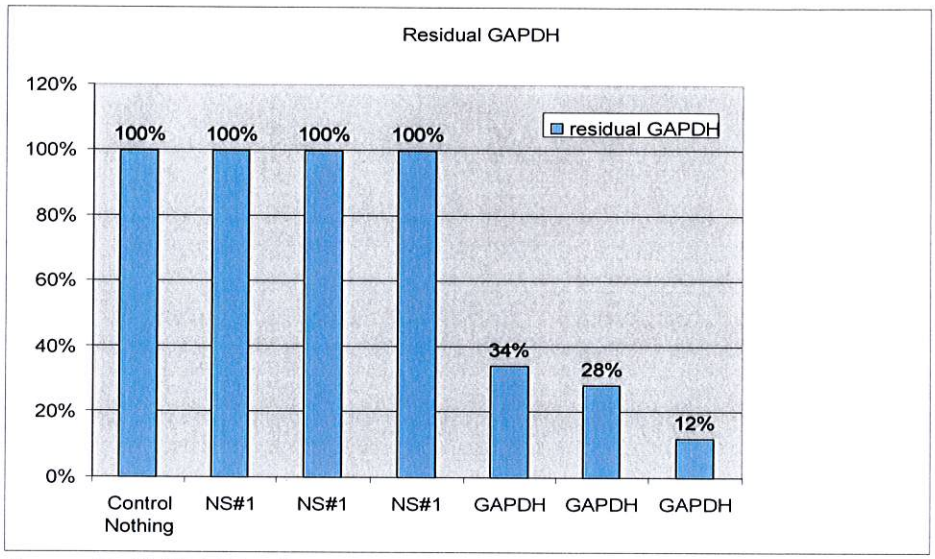

Figure 16. HEK293T cells were transduced with lentiviral particles expressing GAPDH or non-silencing shRNAmir at variable MOIs ranging from 9-48. The graph depicts the residual levels of GAPDH relative to its non-silencing control.

Knockdown of the EG5 (KIF11) gene allowed evaluation of phenotypic evidence of RNAi as well as its molecular manifestation (Figures 17 and 18).

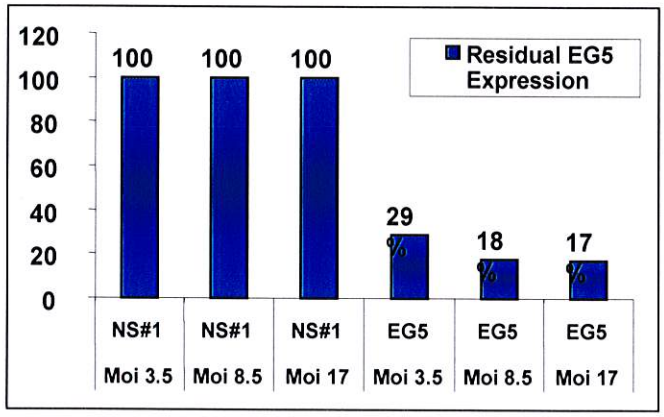

Figure 17. HEK293T cells were transduced with lentiviral particles expressing EG5 or non-silencing shRNAmir at MOIs of 3.5, 8.5 and 17. The graph depicts the residual levels of EG5 relative to its non-silencing control.

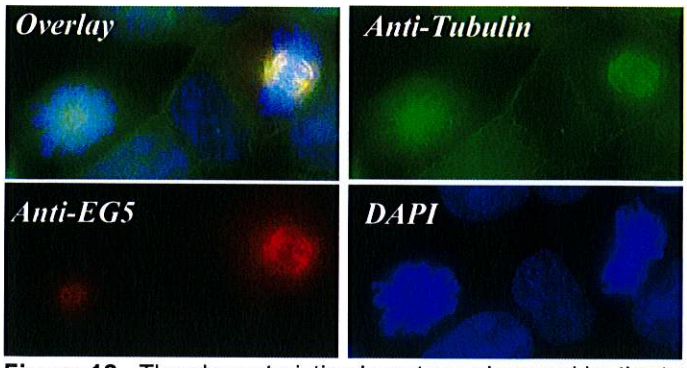

Figure 18. The characteristic phenotype observed by the targeting of the EG5 (KIF11) gene results in the formation of half spindles, mitotic arrest and monoastral microtubular arrays (green, see the cell on the left). By contrast, normal cells show bipolar spindles and microtubule networks in mitosis and in interphase (see the cell on the right). The comparative expression of EG5 (red) between the cell on the left and the right shows the extensive knockdown of EG5 in the cell displaying the phenotype (left). The cells were visualized at 100x magnification using a Leica DMIRB fluorescence microscope. HEK293T cells were stained for tubulin (anti-tubulin, green), DNA (DAPI, blue) and EG5 (anti-EG5, red).

RELATED REAGENTS

Table 16. Related Reagents

Reagent	Vendor	Catalog number
GAPDH verified positive control*	Open Biosystems	RHS4371
EG5 verified positive control*	Open Biosystems	RHS4480
Non-silencing verified negative control*	Open Biosystems	RHS4346
Arrest-In™ transfection reagent 0.5ml-10ml*	Open Biosystems	ATR1740-1743
pGIPZ™ empty vector	Open Biosystems	RHS4349
Trans-Lentiviral GIPZ Packaging System	Open Biosystems	TLP4614
Trans-Lentiviral GIPZ Packaging System (contains cell line)	Open Biosystems	TLP4615

**these items also available in the lentiviral RNAintro shRNAmir starter kit (catalog no. RHS4287)*

FAQS

For answers to questions that are not addressed here, please email technical support at **info@openbiosystems.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.

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 pGIPZ™ product page:
http://www.openbiosystems.com/RNAi/shRNAmirLibraries/GIPZLentiviralshRNAmir/

Where can I find the sequence of an individual shRNAmir construct?

If you are looking for the sequence an individual shRNAmir construct, you can use the gene search. Just enter the catalog number or clone ID of your hairpin into the gene search on the Open Biosystems website, hit submit and then click on the query result. If you then click on the oligo ID (the V2 number) and then click on the word "sequence" in the details grid, the hairpin sequence is listed with the target, mir-30 context and loop sequences annotated. If you are

looking for the sequence of several shRNAmir constructs, you can access this information in the data file of the collection. This data file can be downloaded from the lentiviral pGIPZ product page:
http://www.openbiosystems.com/RNAi/shRNAmirLibraries/GIPZLentiviralshRNAmir/

Which antibiotic should I use?
You should grow all pGIPZ constructs in both 25µg/ml zeocin and 100µg/ml carbenicillin for archive replication. You should grow the constructs in media containing **only 100µg/ml carbenicillin** for plasmid preparation.

What packaging cell line should I use for making lentivirus?
The pGIPZ vector is *tat* dependant, so you must use a packaging system that expresses the *tat* gene. For packaging our lentiviral shRNAmir constructs, we recommend the Trans-Lentiviral™ shRNA Packaging System (TLP4614, TLP4615). The Trans-Lentiviral shRNA Packaging System allows creation of a replication-incompetent (Shimada, et al. 1995), HIV-1-based lentivirus which can be used to deliver and express your gene or shRNAmir of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral shRNA Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes (Kappes and Wu 2001). For protocols and information on packaging pGIPZ™ with our Trans-Lentiviral shRNA Packaging System, please see the product manual available at the following link:
http://www.openbiosystems.com/collateral/rnai/pi/Trans-Lentiviral_GIPZ_Packaging_System.pdf

Can I use any 2nd generation packaging system with the GIPZ vector?
The pGIPZ vector is *tat* dependant, so you must use a packaging system that expresses the *tat* gene.

What does the number 40 refer to in the formula for the calculation of titer?
The titer units are given in transducing units (TU) per ml, so the number 40 is used to convert the 25µl used in the titration ("volume of diluted virus used", Table 3) to one milliliter.

What is the sequencing primer for GIPZ?
The pGIPZ sequencing primer is 5'- GCATTAAAGCAGCGTATC -3'
Notes: The binding site lies from base 5820-5842 and runs in the reverse complement direction. The melting temperature of this 18mer=52.7°C.

How can I make a stable cell line?
In order to generate stable cell lines, it is important to determine the minimum amount of puromycin required to kill non-transfected/transduced cells. This can be done by generating a puromycin kill curve. After you have determined the appropriate concentration of puromycin to use, you can transfect or transduce your cells with the shRNA construct and culture with puromycin in order to select for those cells that have a stable integrant. Cells not containing a stable integrant will not be selected for.

Where do you purchase puromycin?
We purchase puromycin from Cellgro™ (catalog no. 61-385-RA).

How many transfections are available in each volume size of Arrest-In™?

The number of transfections that can be performed depends on the size of the culture dish used and the volume size of Arrest-In purchased. Refer to Table 17 below for the approximate number of transfections.

Table 17. Number of transfections depending on culture dish size and volume of Arrest-In™ purchased.

Tissue Culture Dish	Surface area per well (cm^2)	Arrest-In (1mg/ml) (µg)*	0.5ml qty (rxns)**	1.0ml qty (rxns)**	5.0ml qty (rxns)**	10ml qty (rxns)**
60 mm	20	21	47-50	100	500	1000
35 mm	8	10	100	200	1000	2000
6 well	9.4	10	100	200	1000	2000
12 well	3.8	5	200	400	2000	4000
24 well	1.9	2.5	400	800	4000	8000
96 well	0.3	0.5-1	1000	2000	10000	20000

**Recommended starting amounts of Arrest-In reagent as defined in Table 1.

**Approximate number of transfections based on recommended starting amount of Arrest-In. Individual results may vary depending on amounts of Arrest-In used.

TROUBLESHOOTING

For help with transfection or transduction of your lentiviral 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. our GAPDH or EG5 validated positive controls and the validated non-silencing negative control)?
5. What were the results of the controlled experiments?
6. How was knockdown measured (i.e. quantitative 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 and purity of your DNA using 260/280nm 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. 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 transduction into your cell line is unsuccessful, you may need to consider the following list of factors influencing successful transduction:

1. Transduction efficiency is integrally related to the quality and the quantity of the virus you have produced. Factors to bear in mind when transducing include MOI (related to accurate titer), the presence of serum in the media, the use of polybrene in the media, length of expose to virus, and viral toxicity to your particular cells.
2. High quality transfer vector DNA and the appropriate and efficient viral packaging are required to make high quality virus able to transduce cells effectively. See also suggestions 3-6 for factors influencing successful transfection (above).
3. All cell lines are not equally permissible to transduction by lentivirus. You may consider testing additional cell lines to find one more suitable for your experiments.

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

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Retroviral Gene Transfer and Expression User Manual

PT3132-1 (PR631543)
Published 13 March 2006

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Note: The viral supernatants produced by these retroviral systems could, depending on your DNA insert, contain potentially hazardous recombinant virus. Due caution must be exercised in the production and handling of recombinant retrovirus. **The user is strongly advised not to create retroviruses capable of expressing known oncogenes in amphotropic or polytropic host range viruses.**

Please refer to the appropriate regional and institutional guidelines on handling retroviruses. Please contact your on-site safety officer for specific requirements in your facility. In the United States, NIH guidelines require that retroviral production and transduction be performed in a Biosafety Level 2 facility. For more information, see appropriate HHS publications. Section IV in this User Manual contains a brief description of Biosafety Level 2 as well as other general information and precautions. **http://bmbl.od.nih.gov** and **www.niehs.nih.gov/odhsb/biosafe/nih/rdna-apr98.pdf**

I. Introduction

Retroviral gene transfer is a technique for efficiently introducing stable, heritable genetic material into the genome of any dividing cell type (Ausubel et al., 1995; Coffin et al., 1996). This User Manual supports many Clontech packaging cell lines, retroviral vectors, and retroviral expression systems.

Retroviral Gene Transfer Technology

Current retroviral gene transfer technology is based on the coordinated design of packaging cell lines and retroviral expression vectors. The development of packaging lines—cell lines that package recombinant retroviral RNAs into infectious, replication-incompetent particles—created a new level of safety and control (Figure 1; Mann et al., 1983; Miller & Buttimore, 1986). To develop a packaging cell line, the viral gag, pol, and env genes—necessary for particle formation and replication—are stably integrated into the genome of the packaging cell line. The separate introduction and integration of the structural genes minimizes the chances of producing replication-competent virus due to recombination events during cell proliferation (Morgenstern & Land, 1990; Miller & Chen, 1996). Retroviral expression vectors provide the packaging signal Ψ^+, transcription and processing elements, and a target gene. Inserts of up to 6.5 kb can be efficiently packaged. Transfection of the retroviral vector into a packaging cell line produces high-titer, replication-incompetent virus.

The viral env gene, expressed by the packaging cell line, encodes the envelope protein, which determines the range of infectivity (tropism) of the packaged virus. Viral envelopes are classified according to the receptors used to enter host cells. For example, ecotropic virus can recognize a receptor found on only mouse and rat cells. Amphotropic virus recognizes a receptor found on a broad range of mammalian cell types. Dualtropic virus recognizes two different receptors found on a broad range of mammalian cell types.

A pantropic packaging cell line provided a major advancement in retroviral gene transfer, as this cell line produces virus that can infect both mammalian and non-mammalian cells (Burns et al., 1993). Using this cell line, virions are pseudo-typed with the envelope glycoprotein from the vesicular stomatitis virus (VSV-G). Unlike other viral envelope proteins, VSV-G mediates viral entry through lipid binding and plasma membrane fusion (Emi et al., 1991). Stable expression of the VSV-G envelope protein is toxic; thus, the packaging cell line only contains the viral gag and pol genes. Virus is produced by transiently cotransfecting a retroviral expression vector and pVSV-G into a pantropic packaging cell line.

Once a packaging cell line is transfected with a retroviral expression vector that contains a packaging signal, the viral genomic transcript containing the target gene and selectable marker are packaged into infectious virus within 48–72 hrs. Alternatively, you can use antibiotic selection to select cells that stably express the integrated vector. Stable virus-producing cells can be frozen and used in later experiments. Virus produced by both transient and stable transfections can infect target cells and transmit target genes; however, it cannot replicate within target cells because the viral structural genes are absent.

I. Introduction *continued*

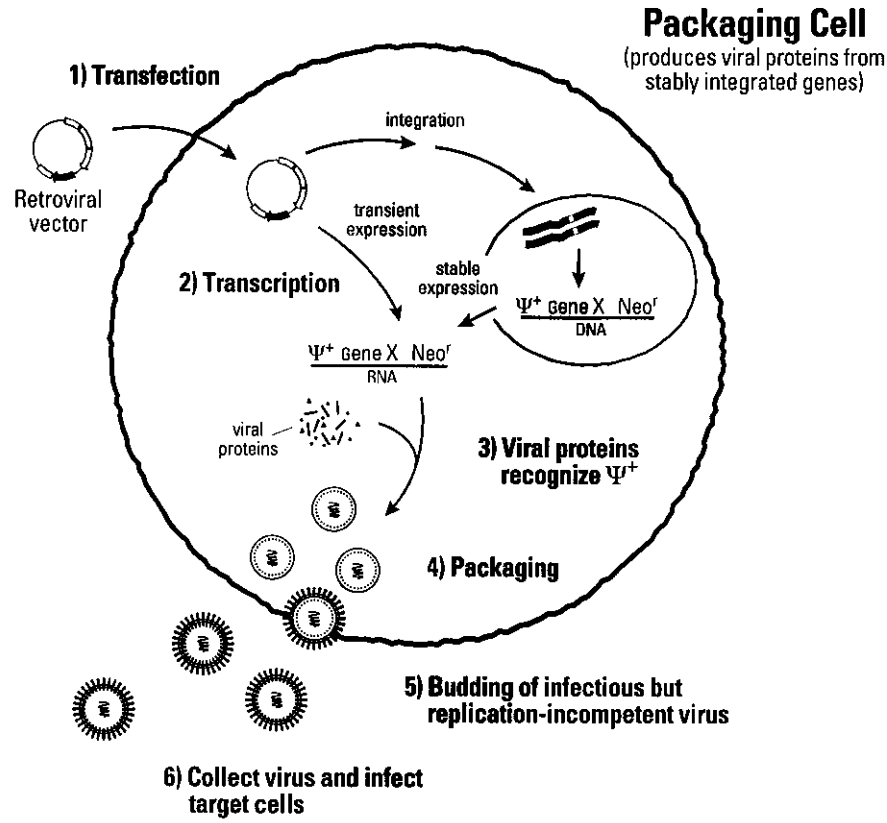

Figure 1. Virus production in packaging cell lines. The *gag, pol* and *env* genes required for viral production are integrated into the packaging cells genome. The vector provides the viral packaging signal, commonly denoted Ψ^+, a target gene, and drug-resistance marker.

The Retro-X™Universal Packaging System (Cat. No. 631530) is a transient packaging system that allows you to select the envelope according to the tropism needed for your experiments. It includes the GP2-293 cell line, which has the viral gag and pol genes incorporated in its genome. The remaining portion of the packaging function, the viral env gene, must be cotransfected with the retroviral expression vector bearing the gene of interest. The kit includes vectors that encode ecotropic, amphotropic, dualtropic (10A1) and pantropic (VSV-G) envelope proteins. This allows you to cater the tropism or host range of the packaged virus to your needs by determining which envelope protein is used.

I. Introduction *continued*

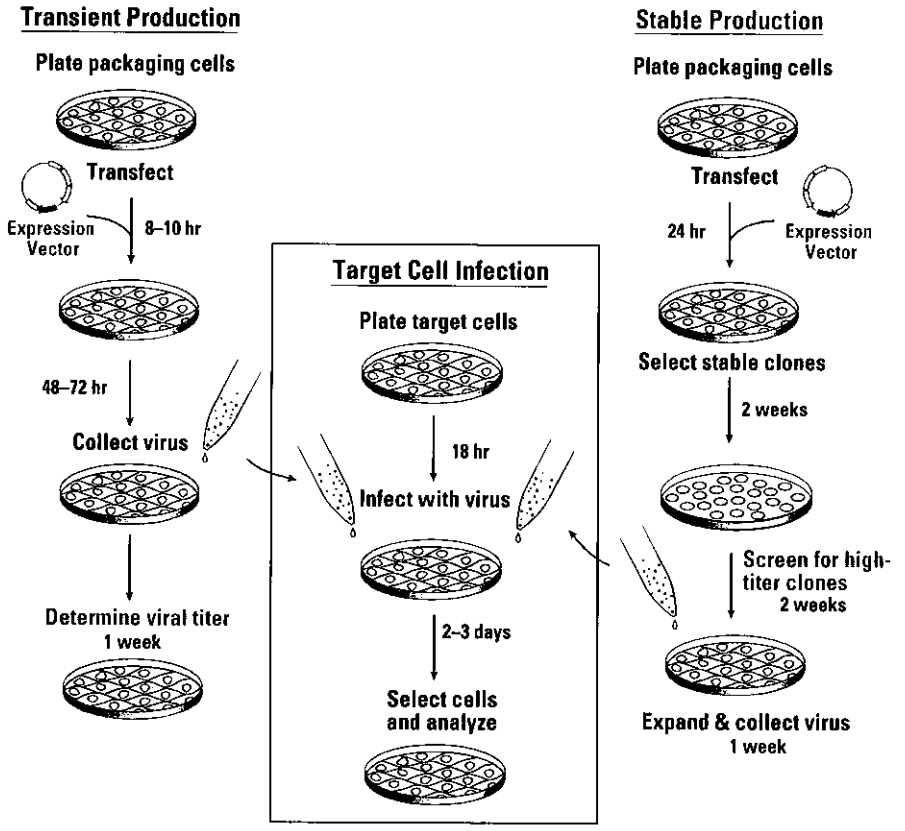

Figure 2. Overview of transient and stable virus production. To produce high-titer virus transiently, transfect a retroviral expression vector into an HEK 293-based packaging cell line. After 48–72 hours, collect virus and determine the viral titer or infect a target cell line. Alternatively, you can use antibiotic selection to develop clones that stably produce high-titer retrovirus.

In addition, Clontech offers a variety of stable packaging cell lines. Table II provides a detailed overview of each cell line.

- **RetroPack™ PT67 Cell Line**

  The RetroPack PT67 Cell Line (Cat. No. 631510) is derived from a mouse fibroblast (NIH 3T3) cell line designed for stably producing high-titer retrovirus. RetroPack PT67 cells package virus with a dualtropic (or polytropic) envelope, 10A1, that recognizes receptors on mouse, rat, human, hamster, mink, cat, dog, and monkey cells. Virus produced by these cells can enter target cells via two surface molecules, the amphotropic retrovirus receptor, RAM1 (Pit2), and the GALV (Pit1) receptor. Two viral receptors means that if one receptor is not abundantly expressed by a given species or cell type, the alternate

I. Introduction *continued*

TABLE I: HOST RANGE OF PACKAGING CELL LINES EXPRESSING DIFFERENT ENVELOPES

Target Cells[b]	Envelopes[a]			
	Dualtropic	Amphotropic	Ecotropic	Pantropic[c]
Mouse	+	+	+	+
Rat	+	+	+	+
Hamster	+	+/–	–	+
Mink	+	+	–	+
Cat	+	+	–	+
Dog	+	+	–	+
Monkey	+	+	–	+
Human	+	+	–	+
Avian	-	-	-	+
Fish	-	-	-	+
Insect	-	-	-	+

[a]See Table II: Packaging Cell Lines for a description of the envelope proteins.

[b] This listing of the most common target cells is not intended to be exclusive. Other cells which are not listed may also be infected.

[c] Virus packaged with the pantropic envelope also infects molusk, amphibian, ameoba and nematode cells.

receptor may still allow viral entry. Thus, virus packaged by RetroPack PT67 has a broad mammalian host range (Table I; Miller & Miller, 1994; Miller, 1996). These cells are best suited for stable virus production.

- **EcoPack2™-293 Cell Line**

  The EcoPack2-293 Packaging Cell Line (Cat. No. 631507) is a human embryonic kidney, HEK 293-derived cell line designed for rapid, transient production of high-titer, ecotropic retrovirus (Figure 2). EcoPack2-293 cells can also be used to produce retrovirus stably. Bleomycin- and hygromycin-resistance genes were used to separately introduce the viral gag-pol and env genes. Virus produced by EcoPack2-293 cells possess an ecotropic envelope (gap70), and thus can infect both mouse and rat cells (Table I). Retroviral sequence within the cell genome has been minimized, reducing the likelihood that replication-competent virus will be produced through recombination. EcoPack2-293 cells are more adherent and produce higher viral titers in comparison to our original EcoPack™-293 cells.

- **AmphoPack™-293 Cell Line**

  The AmphoPack-293 Packaging Cell Line (Cat. No. 631505) is a human embryonic kidney, HEK 293-derived cell line designed for rapid, transient production of high-titer, amphotropic retrovirus (Figure 2). AmphoPack-293

I. Introduction *continued*

TABLE II: PACKAGING CELL LINES

	Retropack™ PT67	EcoPack2™ -293	AmphoPack™ -293	GP2-293
Cell type	NIH 3T3	HEK 293	HEK 293	HEK 293
Tropism	Dualtropic	Ecotropic	Amphotropic	Pantropic[a] Env-specific[b]
Target cells	Broad mammalian	Murine, rat	Broad mammalian	Mammalian, or non-mammalian
Envelope	10A1	gap70	4070A	VSV-G[a] Env-specific[b]
Receptors	GALV (Pit1), RAM1 (Pit2)	mCAT-1	RAM1	phosphatidylserine, phosphatidyl-inositol, & G_{M3} ganglioside[a]
Markers[c] ***gag-pol*** ***env***	TK DHFR	Bleo Hyg	Bleo Puro	DHFR –

cells can also be used to produce high-titer retrovirus stably. Bleomycin- and puromycin-resistance genes were used to separately introduce the viral gag-pol and env genes. Therefore, the popular neomycin and hygromycin selection markers can be used to develop clones that stably produce high-titer virus. Virus produced by AmphoPack-293 cells express an amphotropic envelope (4070A), and thus can infect a broad range of mammalian cell types (Table I).

- **GP2-293 Packaging Cell Line**

  The Pantropic Retroviral Expression System (Cat. No. 631512) features GP2-293, a HEK 293-based packaging cell line that stably expresses the viral gag and pol genes. To produce infectious virus, cotransfect GP2-293 with a retroviral expression vector and pVSV-G, a plasmid that expresses VSV-G from the CMV promoter (Yee et al., 1994). The VSV-G envelope must be cotransfected with the vector due to toxicity caused by the fusogenic properties of the VSV-G protein. A positive control cell line, GP2-293 Luc, allows verification that pVSV-G is functioning properly and that target cells can be infected. This system takes advantage of the envelopes ability to infect non-mammalian cells (Table I).

  The Retro-X™ Universal Expression System (Cat. No. 631530) also features the GP2-293 cell line. To produce infectious virus, cotransfect GP2-293 with a retroviral expression vector and the vector that encodes the envelope of your choice: pAmpho, pEco, p10A1, or pVSV-G. A positive control vector, pQCLIN, allows verification that the envelope vector is functioning properly

I. Introduction *continued*

and that target cells can be infected. The major advantages of the Universal Packaging System are as follows:

- The system can be used with any MMLV-based vector.
- The tropism can be changed to accomodate the desired cell type.
- High titers are generated (10^6–10^7).
- Infectious virus can be obtained in 48 hours, and the packaging system is therefore ideal for testing multiple constructs.
- Excess Gag and Pol proteins are not generated, which may be detrimental (Yap et al., 2000).

Retroviral Expression Vectors

Clontech offers a wide range of retroviral expression vectors that can all be used with our various packaging cell lines. For more detailed descriptions of our vectors and sequence information, visit our **www.clontech.com** and navigate to the vector information page. All vectors contain the extended retroviral packaging signal, Ψ^+, which promotes high-titer virus production. With the exception of the expression vectors in the MSCV Retroviral Expression System (Cat. No. 634401), all vectors are derived from Moloney murine leukemia virus (MMLV). Each vector contains a different antibiotic resistance marker—neomycin, hygromycin, or puromycin—allowing you to choose the cloning vector appropriate for the desired selection method.

The MSCV Vectors contain a specifically designed long terminal repeat (LTR) from the murine stem cell PCMV virus. PCMV stands for PCC4-cell-passaged myeloproliferative sarcoma virus (Hilberg et al., 1987, Hawley et al., 1994). This LTR differs from the MMLV LTR by several point mutations and a deletion. These changes enhance transcriptional activation and decrease transcriptional suppression in embryonic stem and embryonal carcinoma cells. As a result, the LTR drives high-level constitutive expression of a target gene in stem cells and other mammalian cell lines (Hawley et al., 1994).

The Retro-X™ Q Vectors are self-inactivating bicistronic expression vectors designed to express a target gene along with an antibiotic selection marker without the risk of promoter interference from the 5' LTR. In the case of pQCXIX two target genes may be expressed. Upon transfection into a packaging cell line, Q Vectors can transiently express, or integrate and stably express a viral genomic transcript containing the CMV immediate early promoter, gene-of-interest, IRES and antibiotic selection marker. Also included in the viral genomic transcript are the necessary viral RNA processing elements including the LTRs, packaging signal (ψ^+), and tRNA primer binding site. The self-inactivating feature of the vectors is provided by a deletion in the 3' LTR enhancer region (U3). During reverse transcription of the retroviral transcript in the infected cell, the inactivated 3' LTR is copied and replaces the 5' LTR, resulting in inactivation of the 5' LTR promoter (CMV). The gene of interest and antibiotic resistance gene are expressed from

I. Introduction *continued*

an internal CMV promoter and co-translated, via the internal ribosome entry site (IRES), as a bicistronic message in mammalian cells (Jackson et al., 1990; Jang et al., 1988).

Creator™ Compatibility for Diverse Gene Expression Studies

Our retroviral systems are fully compatible with the Creator™ Gene Cloning and Expression System. This system uses Cre-loxP recombination to transfer a gene of interest directly from a single donor vector into numerous acceptor expression vectors without the need for subcloning. This strategy provides easy access to retroviral expression as well as fluorescent protein tagging, yeast two-hybrid studies, tetracycline-regulated gene expression, bacterial expression, and more. Creator™ Acceptor Vectors, such as pLP-LNCX and pLP-RevTRE, serve as entry points into our standard retroviral expression and tetracycline-regulated retroviral expression systems. Additionally, RevTet-Off™ and RevTet-On™ expression systems are available that are compatible with Creator™ technology. See Section XII for ordering information. Further details on the Creator System, including the Creator™ DNA Cloning Kits User Manual (PT3460-1), are available at **www.clontech.com**.

Retroviral Delivery of RNAi Constructs

Our pSIREN-Retro-Q Vector is designed for gene silencing experiments based on the RNA interference phenomenon (July 2003 Clontechniques). Expression of silencing RNAs (shRNA) can decrease expression of a target gene in vivo (Xia et al., 2002). For more information about gene silencing technology, and use of the pSIREN vectors, please refer to the Knockout RNAi Systems User Manual (PT3739-1). Viral delivery of shRNAs has the following advantages:

1. Retroviral expression systems are capable of highly efficient gene delivery.
   - Viral vectors take advantage of viral mechanisms that allow efficient delivery of their nucleic acids to susceptible cell targets. Since recombinant viruses can infect nearly 100% of a cell population, the selection process used to enrich the population for a construct can be eliminated. If less than 100% of cells carry the construct, expression of specified gene may still be detected.
   - This efficiency is difficult to achieve in primary cells with transfection.
   - Retroviruses integrate into the host cell's genome promoting permanent and stable gene transfer as well as persistent expression of the shRNA cassette.
   - Viral infection provides consistent, reproducible transfer of the sequence of interest. However, transfection efficiency can be low and inconsistent.
2. Copy number can be controlled with retroviral vectors. The shRNA dosage is important in maintaining gene silencing (Barton & Medzhitov, 2002).
3. Retroviral vector-based shRNAs produced within a retroviral packaging cell do not affect the titer or production of virus particles (Brummelkamp et al.,

I. Introduction *continued*

2002).

4. Because shRNA molecules anneal to specific sequences, developing vectors that target specific cell types is unnecessary because only those cells that express the targeted sequence will be affected by the vector.
5. Retroviral shRNA expression is more economical than chemical synthesis of small RNA, which is expensive for labs to do on a continuous basis.
6. Retroviral-vector based shRNA expression provides the option for stable expression.

Adeno-X™ Adenoviral Gene Expression

For experiments requiring transient gene expression in non-dividing or difficult-to-transfect cells, we recommend our Adeno-X™ Expression Systems (see Related Products). These adenovirus-based systems enable high-level protein expression in a wide variety of cell types (dividing or non-dividing) without the need for plaque purification (January 2000 & April 2003 Clontechniques).

II. List of Components

Store cell lines in liquid nitrogen (–196°C). Store all plasmids and primers at –20°C.

Retro-X™ System (Cat. No. 631508)

- 1 ml RetroPack PT67 Cell Line (2 x 10^6 cells/ml)
- 40 µl pLNCX2 Retroviral Vector (0.5 µg/µl)
- 40 µl pLXSN Retroviral Vector (0.5 µg/µl)
- 40 µl pLAPSN Retroviral Vector (0.5 µg/µl)
- 100 µl pLNCX Seq/PCR Primer (20 µM)
- 100 µl pLXSN Seq/PCR Primer (20 µM)

Retro-X™ Q Vector Set (Cat. No. 631516)

- 20 µg pQCXIN Retroviral Vector (500 ng/µl)
- 20 µg pQCXIH Retroviral Vector (500 ng/µl)
- 20 µg pQCXIP Retroviral Vector (500 ng/µl)
- 20 µg pQCLIN Retroviral Vector (500 ng/µl)
- 100 µl 5' pQC Seq/PCR Primer (20 µM)
- 100 µl 3' pQC Seq/PCR Primer (20 µM)

LRCX Retroviral Vector Set (Cat. No. 631511)

- 20 µg pLNCX2 Retroviral Vector (0.5 µg/µl)
- 20 µg pLHCX Retroviral Vector (0.5 µg/µl)
- 20 µg pLPCX Retroviral Vector (0.5 µg/µl)
- 100 µl 5' pLNCX Seq/PCR Primer (20 µM)
- 100 µl 3' pLNCX Seq/PCR Primer (20 µM)

MSCV Retroviral Expression System (Cat. No. 634401)

- 1 ml RetroPack PT67 Cell Line (2 x 10^6 cells/ml)
- 20 µg pMSCVneo Retroviral Vector (0.5 µg/µl)
- 20 µg pMSCVhyg Retroviral Vector (0.5 µg/µl)
- 20 µg pMSCVpuro Retroviral Vector (0.5 µg/µl)
- 100 µl 5' pMSCV Primer (20 µM)
- 100 µl 3' pMSCV Primer (20 µM)

Pantropic Retroviral Expression System (Cat. No. 631512)

- 1 ml GP2-293 Packaging Cell Line (2 x 10^6 cells/ml)
- 1 ml GP-293 Luc Packaging Cell Line (2 x 10^6 cells/ml)
- 20 µg pLNHX Vector (0.5 µg/µl)
- 20 µg pLXRN Vector (0.5 µg/µl)
- 20 µg pLLRN Control Vector (0.5 µg/µl)

II. List of Components *continued*

- 20 µg pVSV-G Vector (0.5 µg/µl)

Retro-X™ Universal Packaging System (Cat. No. 631530)

- 1 ml GP2-293 Packaging Cell Line (2 x 10^6 cells/ml)
- 20 µg p10A1 Vector (0.5 µg/µl)
- 20 µg pAmpho Vector (0.5 µg/µl)
- 20 µg pEco Vector (0.5 µg/µl)
- 20 µg pVSV-G Vector (0.5 µg/µl)
- 20 µg pQCLIN Control Vector (0.5 µg/µl)

RetroPack™ PT67 Cell Line (Cat. No. 631510)

- 1 ml RetroPack PT67 Cell Line (2 x 10^6 cells/ml)

EcoPack2™-293 Cell Line (Cat. No.631507)

- 1 ml EcoPack2-293 Cell Line (2 x 10^6 cells/ml)

AmphoPack™-293 Cell Line (Cat. No. 631505)

- 1 ml AmphoPack-293 Cell Line (2 x 10^6 cells/ml)

III. Additional Materials Required

- **Dulbecco's Modified Eagle's Medium** (high glucose with sodium pyruvate & glutamine; Sigma Cat. No. D5796)
- **Fetal bovine serum** (FBS). **Note:** serum need not be heat inactivated.
- **200 mM L-Glutamine** (Sigma Cat. No. G7513)
- Solution of 10,000 units/ml **Penicillin G sodium** and 10,000 µg/ml **Streptomycin sulfate** (Sigma Cat. No. P0781)
- **Complete Medium**
  Dulbecco's Modified Eagle's Medium DMEM [or Minimum Essential Medium, α Modification (α-MEM)] supplemented with 100 units/ml penicillin G sodium, 100 µg/ml streptomycin, 4 mM L-glutamine, 1 mM sodium pyruvate and 10% fetal bovine serum (FBS).
- **G418** (Cat. No. 631307)
  Note: Make a 10 mg/ml active stock solution by dissolving 1 g of powder in approximately 70 ml of complete medium without supplements. Filter sterilize and store at 4°C. G418 can also be purchased as a premade solution.
- **Hygromycin** (Cat. No. 631309)
- **Puromycin** (Cat. No. 631305)
- **Aminopterin** (Calbiochem Cat. No. 454125)
- **Hypoxanthine** (Calbiochem Cat. No. 4010)
- **Thymidine** (Calbiochem Cat. No. 6060)
- **Zeocin** (Invitrogen Cat. No. R250-01)
- **Polybrene** (Hexadimethrine Bromide; Sigma Cat. No. H9268)
- **Trypsin-EDTA** (Trypsin; Sigma Cat. No. T3924)
- **TNE** (50 mM Tris-HCl [pH 7.8], 130 mM NaCl, 1 mM EDTA)
- **Dulbecco's phosphate buffered saline** (DPBS; VWR Cat. No. 82020-066)
- **Cell Freezing Medium** (Sigma Cat. No. C6164) or **DMSO** (Sigma Cat. No. D2650)
- **Tissue culture plates and flasks**
- **BD Biocoat Collagen Type I 12-well plates** (BD Biosciences Cat. Nos. 354500 & 356500)
- **Cloning cylinders** (PGC Scientific Cat. No. 62-6150-40, -45)
- **NIH-3T3 cells** (ATCC Cat. No. CRL-1658)
- **CalPhos™ Mammalian Transfection Kit** (Cat. No. 631312)
- **CLONfectin™ Transfection Reagent** (Cat. No. 631301)
- **Chloroquine** (Sigma Cat. No. C6628)

IV. Safety & Handling of Retroviruses

The protocols in this User Manual require producing, handling, and storing infectious retrovirus. A thorough understanding of safe laboratory practices and potential retroviral hazards is essential.

MMLV does not naturally infect human cells; however, viruses packaged from the MMLV-based vectors described here are capable of infecting human cells if packaged in a cell line with the proper tropism. This statement is also true for PCMV-based vectors. The viral supernatants produced by these retroviral systems could, depending on your retroviral insert, contain potentially hazardous recombinant virus.

For these reasons, exercise due caution when producing and handling recombinant retrovirus. **The user is strongly advised not to create retroviruses capable of expressing known oncogenes in amphotropic, dualtropic or pantropic packaging cell lines.**

Appropriate NIH, regional, and institutional guidelines apply, as well as specific guidelines for other countries. Please contact your on-site safety officer for specific requirements in your facility. In the United States, NIH guidelines require that retroviral production and transduction be performed in a Biosafety Level 2 (BL2) facility. A brief description of BL2 is given below. It is neither detailed nor complete. More information about BL2 guidelines is available at **http://bmbl.od.nih.gov/contents.htm** and more information about the risk group assessment for our viral systems is available at **http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html**. If possible, observe and learn the practices described below from someone who has experience working with retroviruses. For more information, see the following reference:

- Biosafety in Microbiological and Biomedical Laboratories, Fourth Edition (May 1999) HHS Pub. No. (CDC) 93-8395. U.S. Department of Health and Human Services, PHS, CDC, NIH.

Practices

- Perform work in a limited access area
- Post biohazard warning signs
- Minimize production of aerosols
- Decontaminate potentially infectious wastes before disposal
- Take precautions with sharps

Safety equipment

- Use a laminar flow hood with a HEPA filter
- Wear protective laboratory coat, face protection, and double gloves

Facilities

- Autoclave for decontamination of solid and liquid waste
- Use unrecirculated exhaust air
- Stock chemical disinfectants for spills

V. Plasmid Manipulations

A. Propagating Plasmids

1. To ensure that you have a renewable source of DNA, transform each plasmid into a suitable E. coli host strain (e.g., DH5α).
2. Purify plasmids with a NucleoBond® or NucleoSpin® Plasmid Kit. Alternatively, isolate plasmids by banding on a CsCl gradient (Sambrook & Russell, 2001).

B. Generating Expression Vectors

Use standard molecular biology techniques to transfer your target gene into an expression vector (Sambrook & Russell, 2001).

1. Purify your gene fragment by any standard method. The cDNA or gene fragment must contain an ATG initiation codon. Adding a Kozak consensus ribosome binding site may improve expression levels in mammalian systems (Kozak, 1987). Please note that all sequences placed into a retroviral vector must be compatible with the retroviral life cycle and allow complete transcription of the full-length viral genome. Sequences such as poly-A signals must **not** be included (Coffin et al., 1996).

   You can generate the fragment using compatible restriction sites that are on either side of the gene and in the cloning vector. If no such sites are present, use PCR to incorporate suitable restriction sites into your gene fragment. PCR fragments can be conveniently cloned into any vector using our In-Fusion™ PCR Cloning Kits (See XII. Related Products).
2. Digest the vector with the appropriate restriction enzyme(s), treat with phosphatase, and purify.
3. Ligate the digested vector and the target gene fragment.
4. Transform ligation mixture into E. coli.
5. Identify the desired recombinant plasmid by restriction analysis, and confirm orientation and junctions by sequencing.

VI. Culturing Packaging Cell Lines

A. General Considerations

The protocols in this section are intended for use with packaging cell lines from Clontech.

- The RetroPack PT67 cell line has a very short doubling time (<16 hr). Split the culture before it becomes confluent.
- The doubling time for EcoPack2-293, AmphoPack-293, and GP2-293 cell lines is 24–36 hr.
- If you experience low packaging cell line viability, grow the cells for a longer period of time to allow for cell recovery and expansion.
- All our packaging cell lines should be grown at 37°C in a humidified chamber with 5–10% CO_2. See the Product Analysis Certificate for details particular to each cell line.

B. Starting Cultures from Frozen Stocks

The protocols in this User Manual provide only general guidelines for mammalian cell culture techniques. Perform all steps involving cell culture using sterile technique in a suitable hood. For those requiring more information on mammalian cell culture, we recommend the following general references:

- Culture of Animal Cells, Fourth Edition, ed. by R. I. Freshney (2000, Wiley-Liss)
- Current Protocols in Molecular Biology, ed. by F. M. Ausubel et al. (1995, Wiley & Sons)

Note: Frozen cells should be cultured immediately upon receipt or as soon thereafter as possible. Increased loss of viability may occur after shipping if culturing is delayed.

1. Transfer the vial of frozen cells from liquid N_2 to a 37°C water bath until just thawed. To prevent osmotic shock and to maximize cell survival, perform the following:
   a. Rinse the outside of the tube with 70% ethanol.
   b. Add 1 ml complete medium (prewarmed to 37°C) to tube. Transfer mixture to a 15-ml tube.
   c. Add 5 ml complete medium and mix gently. Repeat. The final volume should be 12 ml.
   d. Centrifuge at 250 x g for 10 min.
   e. Remove supernatant.
2. Gently resuspend cells in 10 ml complete medium: DMEM [or Minimum Essential Medium, α Modification (α-MEM)] supplemented with 100 units/ml penicillin G sodium, 100 µg/ml streptomycin, 4 mM L-glutamine, 1 mM sodium pyruvate and 10% fetal bovine serum.
3. Incubate cells at 37°C with 5% CO_2.

VI. Culturing Packaging Cell Lines *continued*

C. Maintaining Packaging Cell Lines

Generally, cells should be plated at 10^6 per 100-mm plate and split every 2–3 days when they reach 70–80% confluency.

Note: Plate HEK 293-based packaging cell lines on collagen-coated plates initially to promote adherence after thawing. These cells may be cultured on non-coated plates/flasks after recovery; however, if adherence is poor, we recommend collagen-coated vessels for all culturing purposes including viral packaging.

Split the cells as follows:

1. Remove medium, and wash cells once with room-temperature PBS.
   Note: If cells are over-confluent, omit the wash since the cells may detach from the plate.
2. Treat with 2 ml of trypsin-EDTA solution for 0.5–1 min. Depending on the cell line, you may need to treat the cells longer.
3. Add 3 ml of media + serum to inhibit trypsinization.
4. Resuspend cells gently by pipetting.
5. Add a predetermined portion of cells to a 100-mm plate in 10 ml of complete medium. Rock the plate to distribute the cells evenly.
   Note: Split RetroPack PT67 cells at a ratio of up to 1:20, and split HEK 293-based cells at a ratio of 1:10.
6. If cell viability is low, grow cells for a longer period of time, maintain higher cell densities, and verify culture conditions.

D. Freezing Packaging Cell Lines

Once a stable cell culture is established, we recommend that several aliquots of cells be frozen for future use. Prepare frozen aliquots of the packaging cells to ensure a renewable source as follows:

1. Expand the cell line into the desired number of flasks or plates.
2. When the desired number of flasks/plates reaches ~80% confluency, wash the cells once with PBS or HBSS, trypsinize using standard tissue-culture procedures (Freshney, 2000), add 2–4 volumes of complete medium to neutralize trypsin, and harvest cells.
3. Count the cells using a hemocytometer (Freshney, 2000), and collect by centrifugation (250 x g for 10 min at room temperature).
4. Resuspend in 4°C cell freezing medium containing 10% DMSO at 1–2 x 10^6 cells/ml.
5. Dispense 1-ml aliquots into labeled freezing vials and place in a cell freezing container (reduces temperature ~1°C/min) at –80°C overnight. Alternatively, place the vials on ice or at –20°C for 1–2 hr, transfer to an insulated container such as a foam ice chest, and place in a –80°C freezer for several hours to overnight.
6. Transfer vials to liquid nitrogen.
7. Two or more weeks later. To confirm viability of frozen stocks, start a fresh culture of each frozen cell type, as described in Section B above.

VII. Virus Production

This section provides detailed procedures for virus production, target cell infection, and stable clone selection. For more detailed information or related protocols, see Coffin & Varmus (1996) or Ausubel et al. (1995). Figure 3 provides an overview of methods for producing high-titer virus using RetroPack PT67, EcoPack2-293, Amphopack-293, and GP2-293 cells.

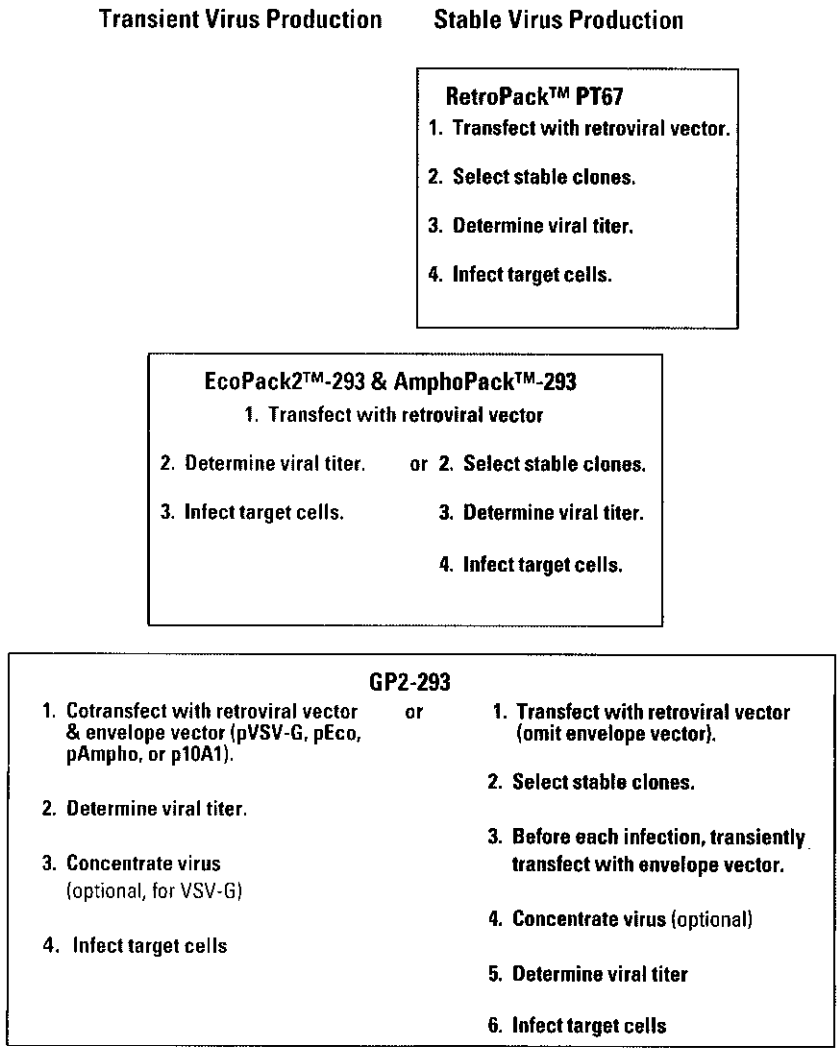

Figure 3. Overview of producing infectious retrovirus.

VII. Virus Production *continued*

A. Transfecting Retroviral Vectors

Transfect by any standard method. We routinely use 60-mm plates for culturing packaging cell lines; See Additional Materials Required. Typically, transfections are done in smaller volumes than culturing.

For maximal transfection efficiencies, we recommend the CalPhos™ Mammalian Transfection Kit (Cat. No. 631312). For maximal transfection efficiency in liposome-mediated transfections, we recommend Clonfectin™ Transfection Reagent (Cat. No. 631301). To optimize your transfection protocol, you can transfect the host cell line with a non-inducible reporter expression vector, such as our pLAPSN included in our Retro-X™ System (Cat. No. 631508), or Living Colors™ Vectors, and assay for reporter gene activity.

After choosing a method of transfection, optimize cell density (usually 60–80% confluency or 1–2 x 10^6 cells/60 mm plate), the amount and purity of the DNA, media conditions, and transfection time. If a transfection method is already established in your laboratory, proceed with those conditions. Keep optimized parameters constant to obtain reproducible results.

1. Clone your target gene into a retroviral expression vector, or use the provided control vector for control experiments.

   Note: Use only high quality plasmid DNA. We recommend using a NucleoBond or NucleoSpin Plasmid Kit.

2. 12–24 hr before transfection, plate packaging cells on a 60-mm plate at 60–80% confluency (1–2 x 10^6 cells/60-mm plate).

   Note: Adding 25 µM chloroquine just prior to transfection may increase transfection efficiency 2–3 fold. Prepare a 25 mM stock of chloroquine in distilled water and filter sterilize. 1–2 hr before transfection, replace medium with medium containing chloroquine (Pear et al., 1993).

3. Transfect each 60-mm plate with the following amount of plasmid DNA:

   For RetroPack™ PT67, EcoPack™ 2-293 & AmphoPack™-293: ~5–10 µg of plasmid DNA

   For GP2-293: ~5 µg of expression vector and ~5 µg envelope vector

   Notes:

   - When using GP2-293 cells, envelope vector must be cotransfected.
   - When using a $CaPO_4$-based transfection method, **the final volume of transfection mixture should not exceed 0.5 ml for a 60-mm plate or 1 ml for a 100-mm plate.** More than 1 ml of $CaPO_4$ precipitants can be toxic to cells. Add the transfection solution to the medium and evenly distribute the solution on the cells. If toxicity is observed, perform transfection with 0.5 ml of the transfection mix.
   - 6–8 hr after transfection, you may perform glycerol shock treatment to increase the uptake of DNA (Freshney, 2000).

4. For RetroPack PT67 cells, aspirate culture medium 10–24 hr after transfection. Wash RetroPack PT67 cells twice with PBS, and add

VII. Virus Production *continued*

3 ml of complete medium. Proceed to Section VII.B.

5. For HEK 293-based cell lines, aspirate culture medium 8–10 hr after transfection, and add 3 ml of complete medium.
6. Incubate the culture for an additional 48–72 hr to allow viral titer to increase. The viral titer reaches a maximum ~48 hr after transfection and is generally at least 30% of the maximum beyond 72 hr after transfection.

Alternative Method: Infecting packaging cells (Ping-Pong)

This method can be used to deliver the viral construct to the packaging cell line, an objective that can be accomplished by transfection, electroporation, or even infection (**Note:** Retro-X Q vectors can only be delivered by transfection). This method can also eliminate the need for selecting individual clones when making stable packaging cell lines (Parente & Wolfe, 1996). Alternatively, infect the packaging cells with virus obtained from another packaging cell line. Table II details the appropriate packaging cell lines to use for infection. A protocol for infection follows in Section VIII.

This approach produces high-titer virus for several reasons:

- More cells acquire the construct, and copy number is higher and more consistent (1–2 copies per cell per single round of infection depending upon titer of virus stock).
- Virus-producing clones derived from transduced cells are more stable than those derived from transfected cells (Parente & Wolfe, 1996).
- Allows the host-range of a vector to be changed.

Important Notes:

- This method requires previously transfected, virus-producing packaging cells.
- **You cannot infect cells that are already expressing the same or similar (eg. Ampho and 10A1) envelope protein. For example, virus produced from RetroPack™ PT67 cells cannot efficiently infect AmphoPack™-293 cells and vice versa.**
- Virus packaged in GP2-293 cells can be used to infect any other cell line depending on the envelope (pVSV-G, pEco, pAmpho or p10A1) that was cotransfected with the expression vector.
- Virus produced by EcoPack2-293 cells can only infect mouse and rat cells, such as RetroPack PT67 cells.

VII. Virus Production *continued*

B. Selecting Stable Virus-Producing Cell Lines

1. Prior to using antibiotics to establish stable cell lines, you must titrate antibiotic stocks to determine the optimal concentration for selection (see Appendix B). This procedure is commonly called a kill curve.
2. Plate transfected packaging cells in selection medium 24–36 hr after transfection.
3. Culture cells for one week with the appropriate antibiotic.
4. Isolate large, healthy colonies and transfer them to individual plates or wells.

   Note: We generally isolate clones using cloning cylinders or cloning disks. The selected cell populations usually produce titers of ~ 10^5 cfu/ ml. If you require higher titer clones, pick individual clones for propagation. Determine viral titer as described in Section VIII. You must screen 20–50 clones to isolate a clone of acceptably high titer. Once clones are isolated, withdraw antibiotic from the medium.

C. Concentrating Virus (VSV-G enveloped virions only, Burns et al, 1994)

1. Remove cell debris and aggregated virus by low speed centrifugation for 5 min at 4°C.
2. Pellet the virus at 50,000 x g for 90 min at 4°C. Remove the supernatant.
3. Resuspend the virus to 0.5–1% of the original volume in TNE (See Additional Materials required), and incubate overnight at 4°C.

   Note: If desired, perform a second round of ultracentrifugation (Steps 1–2).
4. Determine the viral titers of pre- and post-concentrated viral supernatants.
5. Infect target cells (Section IX.B.3).

D. Producing Virus from Stable Packaging Cell Clone (PT67)

1. Remove clone from liquid nitrogen and follow thaw procedures outlined in Section VI.B.
2. Culture the clone, until cell culture reaches the desired culture volume.
3. Retaining one plate for the continuation of the culture, plate the remaining cells at 60–80% confluency in the desired number of culture vessels.
4. Viral supernatants can then be harvested in 24 hr intervals until cells are no longer viable. Discard all cells once the virus has been harvested.

E. Storage of Viral Stocks

VII. Virus Production *continued*

1. Once viral supernatant has been collected, briefly centrifuge sample to remove cellular debris at 500 x g for 10 min. Pool all similar stocks at this time.
2. Aliquot cleared supernatant into single-use tubes to avoid multiple freeze-thaw cycles.
3. Store tubes at –70°C. No cryoprotectant is required.

   Note: Avoid multiple freeze-thaw cycles, since titers can drop as much as 2–4 fold with each cycle (Higashikawa & Chang, 2001; Kwon et al., 2003).

VIII. Determining Viral Titer

A. General Considerations

Determining the viral titer is necessary for three reasons:

- Confirmation that viral stocks are viable.
- Determination of the proper transduction conditions for your particular cell type by adjusting the MOI for the desired transduction efficiency. (i.e., control of copy number)

  MOI = No. of virus particles per target cell
- Determination of the maximum number of target cells that can be infected for a given virus volume.

B. Procedure for Determining Viral Titer

1. Plate NIH 3T3 cells one day prior to beginning this procedure. Plate cells in 6-well plates at a density of 0.5–1 x 10^5 cells per well. Add 2 ml of medium per well.
2. Prepare 20 ml of complete medium and add 60 µl of 4 mg/ml polybrene.

   Note: Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane.
3. Collect virus-containing medium from packaging cells.
4. Filter medium through a 0.45-µm cellulose acetate or polysulfonic (low protein binding) filter. **Do not use a nitrocellulose filter** because nitrocellulose binds proteins in the retroviral membrane and destroys the virus. This is the viral stock.
5. Prepare six 10-fold serial dilutions as follows:
   a. Add 1.35 ml of medium (Step 2) to each of six 1.5-ml microcentrifuge tubes.
   b. Add 150 µl of virus-containing medium (Step 4) to the first tube. Mix.
   c. Transfer 150 µl of viral stock dilution from tube 1 to tube 2. Continue serial dilutions byt transferring 150 µl of eac hsuccessive dilution to the next prepared tube.
6. Infect NIH 3T3 cells by adding 1 ml of the diluted virus medium (Step 5) to the wells. Final polybrene concentration will be 4 µg/ml in ~3 ml.
7. If you used pLAPSN from the Retro-X™ System for virus production, stain cells after 48 hr by assaying for alkaline phosphatase expression. Use any standard alkaline phosphatase assay (Ausubel et al., 1995).

   For other vectors, subject cells to antibiotic selection 24 hr after infection for one week.

   See Appendix B for Kill Curve information.
8. The viral titer corresponds to the number of colonies present at the

VIII. Determining Viral Titer *continued*

highest dilution that contains colonies, multiplied by the dilution factor. For example, the presence of four colonies in the 10^6 dilution would represent a viral titer of 4×10^6.

4 colony forming units (cfu) x 10^6 = 4×10^6 cfu/ml

For virus produced from RetroPack PT67, EcoPack2-293, AmphoPack-293, and GP2-293 cells, a good viral titer is >10^6 cfu/ml.

C. Alternative Methods

We recommend that you determine viral titer by infecting NIH 3T3 cells with serially diluted viral supernatants produced with a control vector such as pLAPSN, part of our Retro-X™ System (Cat. No. 631508). Infect both NIH 3T3 cells and your target cells. See Section IX for instructions on infecting NIH 3T3 cells. Infecting your target cell line will give you a rough, but rapid, estimation of infection success. You can use your cells of choice to determine viral titer (e.g., HeLa or Mink cells), but NIH 3T3 cells are widely accepted as the standard target cell for titering retrovirus because of the efficiency at which these cells become infected. The same virus preparation can give different "apparent" titers on different cells lines due to differential receptor expression and cell cycle rates. For more information on determining viral titer, please refer to Ausubel, et al. (1995).

The method described in this manual is a standard gene transducing unit assay that measures the **functional** titer of a particular virus stock—the virus ability to infect is assayed. Another method is a drug-resistance colony assay, in which antibiotic selection of the infected cells gives rise to a countable number of colonies after roughly 10–14 days. Some variations of this method describe: a transduction, followed by a shorter selection period (3 days; Byun et al., 1996), recently-infected target cells (Tafuro et al., 1996; Miyao et al., 1995), and in situ PCR (PRINS; Claudio et al., 2001) with similar results. Other markers include LacZ, EGFP (Cashion et al., 1999, Muldoon et al., 1997), and luciferase.

Although it relates directly to the infectious viral particles, functional titer does not provide a consistent measurement of virion concentration because it depends upon the transduction efficiency of the cell line being used to determine titer. Therefore, direct quantitation for determining virus particle concentration may be more desirable. Also, physical quantitation lends itself for more high-throughput applications, such as screening of stable virus-producing clones for high-titer variants. Direct quantitation of virus concentration in supernatant does not rely on antibiotic selection and therefore all viruses, regardless of sequence can be quantitated. Methods for the direct quantitation of virus particles include slot blots (Nelson et al., 1998; Murdoch, et al., 1997; Onodera, et al., 1997) and PCR applied to viral supernatants (Quinn & Trevor, 1997; Morgan et al., 1990). Reverse transcriptase activity has also been used (Goff et al., 1981). Some have used protein production (encoded by the retroviral transgene) from pack-

IX. Infecting Target Cells

aging cells as a method for screening high titer clones, but this method is flawed because protein production does not correlate with the number of infectious virions (Tasaki et al., 1997).

A. General Considerations

The following protocols are general recommendations for infecting adherent cells, such as NIH 3T3 or HeLa. Use them as a starting point for determining optimal conditions for your experiments. If these protocols do not work for your cell type, please refer to Appendix C for alternative infection methods.

Important:

Multiple rounds of infection can improve your results by increasing the number of infected cells as well as increasing the copy number per cell.

Virus produced with the VSV-G envelope can be concentrated by ultracentrifugation to titers of up to 10^9 cfu/ml (See Section VII.C).

B. Infecting Target Cells

1. Plate the target cells 12–18 hr before infection, at a cell density of 1–2 x 10^5 per 60-mm plate.

   If you will be using infected cells for a biological assay, ensure that the control cells are treated with an insert-free virus under identical conditions.

   Note: The viral pre-integration complex enters the nuclei of actively dividing cells only.

2. For infection, collect medium from packaging cells and filter medium through a 0.45-µm cellulose acetate or polysulfonic (low protein binding) filter. **Do not use a nitrocellulose filter** because it binds proteins in the retroviral membrane and destroys the virus.

   Optional: For VSV-G enveloped virus, you can concentrate virus as described in Section VII.C.

3. Add virus to target cells. Until you have determined the viral titer, use as much virus-containing medium as possible for the infection. Store remaining viral supernatant at –80°C.

   Notes:

   - Titer will decrease ~2–4-fold per freeze-thaw cycle.
   - The optimal final concentration of polybrene may need to empirically determined but generally falls within a range of 2–12 µg/ml.
   - Excessive exposure to polybrene (>24 hr) can be toxic to cells.

   Alternatively, perform infections sequentially, ~12 hr apart. Doing so increases the efficiency of infection, but also increases copy number. Cellular receptors can be occupied by soluble envelope and/or nonfunctional virions. Therefore, to ensure that cellular receptors will be unoccupied by viral envelope, allow cells to rest for a minimum of 12 hr

IX. Infecting Target Cells *continued*

between each infection.

4. Add polybrene to a final concentration of 4–8 μg/ml.
5. Replace medium with fresh medium after 24 hr of incubation.
6. To determine the efficiency of infection, subject a small subpopulation of cells to antibiotic treatment. The infected cells should be used for experiments or for selection as soon as possible, but not earlier than 24 hr after the last infection.

   The growth of some target cells is strongly affected by media conditioned by the packaging cells. You can take certain precautions to avoid an adverse effect induced by the packaging cell-derived supernatants:

   - Dilute virus-containing media at least 2-fold with fresh medium.
   - Expose target cells to the virus for 4–6 hr and then replace with fresh medium.
   - For cells that prove more difficult to infect, please see references located in Appendix C.

X. Troubleshooting Guide

A. Cloning

DNA does not cut as expected	Incomplete digest: repeat digest with more enzyme, a different enzyme lot, or for a longer period of time.
Low yield of plasmid	Retroviral constructs use a low copy pBR322 ori. Grow more liquid culture and purify using low-copy purification procedures.
Plasmid is difficult to grow or clone.	Plasmid may rearrange due to presence of LTR's. Switch to alternate E. coli strain for unstable DNA sequences.

B. Packaging Cells

Poor viability upon thawing	Improper thawing procedures: follow thawing proce-dures in Section VI.B.
	Improper culture medium: all packaging cell lines will grow in DMEM + 10% FBS
	Improper tissue culture plasticware: use collagen I-coated plates to aid adherence during initial seeding.
Slow growth	Improper culture medium: all packaging cell lines will grow in DMEM + 10% FBS.
	Improper incubator conditions: grow cells at 37°C in humidified incubator with 5–10% CO_2.
Cells do not attach to plate	Improper culture medium: all packaging cell lines will grow in DMEM + 10% FBS.
	Improper tissue culture plasticware: use collagen I-coated plates to aid adherence during initial seeding.
Cells appear morphologically different	Subclone parental cell line.
Cannot select for packaging	Incorrect amount of antibiotic: we do not recommend reselecting for packaging function.

X. Troubleshooting Guide *continued*

C. Virus Production

Poor Transfection efficiency	Cells are overly confluent: plate fewer cells (60–80% confluency, 1–2 x 10^6 cells/60mm). Transfection is toxic to cells: Optimize DNA and transfection reagent amounts and exposure time.
	Assaying for positive cells too early: wait 48 hr after transfection for maximal gene expression to determine efficiency.
Low titer (<10^5 cfu/ml)	Poor transfection efficiency: optimize transfection. Concentrate virus if using VSV-G.
	Truncated viral RNA: check sequence for presence of poly(A) between LTRs.
	Virus harvested too early: harvest virus 48–72 hr after transfection.
	Vector too large (The limit of packaging function is 8.3 kb from LTR to LTR). Concentrate virus for large vectors or reduce size of inserts.
	Low virus production from cell population (PT67): Pick and screen for stable, higher-titer clones.
	No polybrene added during titration: add polybrene (4–8 µg/ml) to viral supernatant.
	Virus exposed to multiple freeze-thaw cycles: each cycle drops the titer approximately 2–4 fold. Limit the number of freeze-thaws.
	Sub-optimal selection procedure during titration: perform an antibiotic kill curve on titration targets prior to titration.

X. Troubleshooting Guide *continued*

D. Infection of Target Cells

Poor infection efficiency	Low titer: see above section.
	Infection protocol not optimized: see Appendix C for references for optimizing transduction protocols.
	Target cells not dividing: plate cells at lower confluency, activate with mitogen, or use another method to induce cell division.
	Optimize culture conditions for targets prior to infection.
Target cell viability poor during infection	Packaging cell line-conditioned media may be affecting cell growth: dilute viral medium or shorten exposure time to viral supernatant.
	Excessive exposure to polybrene: optimize amount of polybrene (titrate) or shorten exposure time to viral supernatant.
	Low infection efficiency (See Section D, above).
Low expression level	Possible promoter inactivation: split cells, activate with mitogen, treat cells with 5-azacytidine.
	Choose a tissue-specific promoter.
	Poor cell viability: check growth parameters.

XI. References

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Retroviral Expression	
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• RevTet™-On System	631021
• pRevTet-On Vector	631007
• pRevTet-Off Vector	631003
• pRevTet-Off-IN Vector	631001
• pRevTRE Vector	631002
• RNAi-Ready pSIREN-Retro-Q Vector	631526
Creator™ Systems	
• RevTet™-Off Retroviral Gene Expression System with Creator™ technology	631023
• RevTet™-On Retroviral Gene Expression System with Creator™ technology	631024
• pLP-LNCX Acceptor Vector	631504
• pLP-RevTRE	631015
• pDNR Cloning Kit	631615
Adeno-X™ Adenoviral Expression Systems	
• Adeno-X™ Expression System 1	631513
• Adeno-X™ System 1Viral DNA (linear)	631026
• Adeno-X™ Accessory Kit	631027
• Adeno-X™ Tet-Off Expression System 1	631022
• Adeno-X™ Tet-On Expression System 1	631050
• Adeno-X™ Rapid Titer Kit	631028
• Adeno-X™ System 1 PCR Primer Set 2	631030
• Adeno-X-DsRed2 Adenovirus	632417
• Adeno-X-LacZ Adenovirus	631029

XII. Related Products *continued*

Products	Cat. No.
Adeno-X™ Adenoviral Expression Systems cont.	
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• Adeno-X™ Expression System 2	631524
• Adeno-X™ Virus Purification Kit	631532 631533
• Adeno-X™ Virus Purification Mega Kit	631534
• Adeno-X™ Tet-On Expression System 2	631057
• Adeno-X™ Tet-Off Expression System 2	631058
Transfection and Selection	
• Tet System Approved FBS (US Sourced)	631101
• Tet System Approved FBS (USDA Approved)	631106
• CalPhos™ Mammalian Transfection Kit	631312
• CLONfectin™ Transfection Reagent	631301
• G418	631307
• Hygromycin B	631309
• Puromycin	631305
Miscellaneous	
• NucleoSpin® Virus Kits	many
• NucleoBond® Kits	many
• In-Fusion™ PCR Cloning Kit	631774 631775
• In-Fusion™ Dry-Down PCR Cloning Kit	639602 639604 639605
• Knockout Adenoviral RNAi System 1	631528
• Knockout Adenoviral RNAi System 2	631529

Appendix A: Culture Plate Conversions

TABLE III: CULTURE PLATE CONVERSION			
Size of Plate	**Growth Area (cm^2)**	**Relataive area***	**Recommended Volume**
96 well	0.32	0.04 X	200 µl
24 well	1.88	0.25 X	500 µl
12 well	3.83	0.5 X	1.0 ml
6 well	9.4	1.2 X	2.0 ml
35 mm	8.0	1.0 X	2.0 ml
60 mm	**21**	**2.6 X**	**5.0 ml**
10 cm	55	7 X	10.0 ml
Flasks	25 75	3 X 9 X	5.0 ml 12.0 ml

* Relative area is expressed as a factor of the growth area of a 35-mm culture plate.

Appendix B: Titration of Antibiotic Stocks (Kill Curves)

Prior to using G418, hygromycin or puromycin to establish stable packaging cell lines, it is important to titrate your selection agent stocks to determine the optimal concentration for selection with the chosen cell line. This is also important because of lot-to-lot variation in the potency of these drugs. Therefore, you should titrate each new lot of antibiotic to determine the optimal concentration. We recommend that you perform two experiments for each drug: (1) a titration to determine the optimal drug concentration, and (2) an experiment to determine the optimal plating density.

1. Titrate at fixed cell density.
   a. Plate 2 x 10^5 cells in each of six 10-cm tissue culture dishes containing 10 ml of the appropriate complete medium plus varying amounts (0, 50, 100, 200, 400, 800 µg/ml) of hygromycin or G418. For puromycin, add the drug at 0, 1, 2.5, 5, 7.5, and 10 µg/ml.
   b. Incubate the cells for 10–14 days, replacing the selective medium every four days (or more often if necessary).
   c. Examine the dishes for viable cells every two days.

   For selecting stable transformants, use the lowest concentration that begins to give massive cell death in ~5 days and kills all the cells within two weeks.
2. Determine optimal plating density.

   Once you have determined the optimal drug concentration, determine the optimal plating density by plating cells at several different densities in the presence of a constant amount of drug. If cells are plated at too high a density, they will reach confluency before the selection takes effect. Optimal plating density is dependent on population doubling time and cell surface area. For example, cells that double rapidly have a lower optimal plating density than cells that double slowly.
   a. Plate cells at several different densities in each of six 10-cm tissue culture dishes containing 10 ml of the appropriate selective medium. Suggested densities (cells/10-cm dish): 5 x 10^6, 1 x 10^6, 5 x 10^5, 2 x 10^5, 1 x 10^5, and 5 x 10^4.
   b. Incubate the cells for 5–14 days, replacing the selective medium every four days.
   c. Examine the dishes for viable cells every two days.

   For selecting stable transfectants, use a plating density that allows the cells to reach ~80% confluency before massive cell death begins (at about day 5). This is the cell density at which cells should be plated for selection of stable transfectants.

Appendix C: Additional Viral Infection Methods

These references are provided for fine-tuning your transduction protocols to achieve the desired infection frequency in target cells. This list is not intended to be comprehensive. These protocols will work for a wide range of cell types; however you must determine which works best for your targets. While each technique can provide modest increases in efficiency, they may be combined to create an additive effect. For ease of analysis, we recommend our retroviral vectors that express our Living Colors™ fluorescent proteins (See Related Products) for detection and quantitation of gene transfer efficiency during testing.

A. Transduction of cells at 32°C: Decrease in temperature increases viral half-life during transduction

Bunnell, B. A., Muul, L. M., Donahue, R. E., Blaese, R. M., Morgan, R. A. (1995) High-efficiency retroviral-mediated gene transfer into human and nonhuman primate peripheral blood lymphocytes. Proc. Natl. Acad. Sci. USA **92**(17):7739–7743.

Zhou, P., Lee, J., Moore, P., Brasky, K. M. (2001) High-efficiency gene transfer into rhesus macaque primary T lymphocytes by combining 32 degrees C centrifugation and CH-296-coated plates: effect of gene transfer protocol on T cell homing receptor expression. Hum. Gene Ther. **12**(15):1843–1855.

Kotani, H., Newton, P. B. 3rd, Zhang, S., Chiang, Y. L., Otto, E., Weaver, L., Blaese, R. M., Anderson, W. F. & McGarrity, G. J. (1994) Improved methods of retroviral vector transduction and production for gene therapy. Hum. Gene Ther. **5**(1):19–28.

Higashikawa, F. & Chang, L. (2001) Kinetic analyses of stability of simple and complex retroviral vectors. Virology **280**(1):124–131.

B. Centrifugation during transduction (Spinoculation): believed to counteract diffusion of virus when binding target cells

Bunnell, B. A., Muul, L. M., Donahue, R. E., Blaese, R. M. & Morgan, R. A. (1995) High-efficiency retroviral-mediated gene transfer into human and nonhuman primate peripheral blood lymphocytes. Proc. Natl. Acad. Sci. USA **92**(17):7739–7743.

Ohkubo, T., Barcena, A., Smith, C. A., Harrison, M. R. & Muench, M. O. (2001) High-efficiency retroviral transduction of fetal liver CD38-CD34++ cells: implications for in utero and ex utero gene therapy. Fetal Diagn. Ther. **16**(5):299–307.

Movassagh, M., Boyer, O., Burland, M. C., Leclercq, V., Klatzmann, D. & Lemoine F. M. (2000) Retrovirus-mediated gene transfer into T cells: 95% transduction efficiency without further in vitro selection. Hum. Gene Ther. **11**(8):1189–1200.

Bahnson, A. B., Dunigan, J. T., Baysal, B. E., Mohney, T., Atchison, R. W., Nimgaonkar, M. T., Ball, E. D. & Barranger, J. A. (1995) Centrifugal enhancement of retroviral mediated gene transfer. J. Virol. Methods **54**:131–143.

C. Precipitation to increase titer (concentration)

Pham, L., Ye, H., Cosset, F. L., Russell, S. J. & Peng, K. W. (2001) Concentration of viral vectors by co-precipitation with calcium phosphate. J. Gene Med. **3**(2):188–194.

Darling, D., Hughes, C., Galea-Lauri, J., Gaken, J., Trayner, I. D., Kuiper, M. & Farzaneh, F. (2000) Low-speed centrifugation of retroviral vectors absorbed to a particulate substrate: a highly effective means of enhancing retroviral titre. Gene Ther. **7**(11):914–923.

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Appendix C: Additional Viral Infection Methods *continued*

particles provide a choice of three affinity-based capture and magnetic concentration strategies for retroviral vectors. Mol. Ther. **3**(4):623–630.

D. Precipitation (during transduction): facilitates greater contact between the target cells and virions

Le Doux, J. M., Landazuri, N., Yarmush, M. L. & Morgan, J. R. (2001) Complexation of retrovirus with cationic and anionic polymers increases the efficiency of gene transfer. Hum. Gene Ther. **12**(13):1611–1621.

Morling, F. J. & Russell, S. J. (1995) Enhanced transduction efficiency of retroviral vectors coprecipitated with calcium phosphate. Gene Ther. **2**(7):504–508.

Hennemann, B., Chuo, J. Y., Schley, P. D., Lambie, K., Humphries, R. K. & Eaves, C. J. (2000) **High-efficiency** retroviral transduction of mammalian cells on positively charged surfaces. Hum. Gene Ther. **11**(1):43–51.

E. Increase transduction rate by phosphate depletion: results in up regulation of GLVR-1 and GLVR-2 (RAM1) receptors (for amphotropic or 10A1 pseudotyped virus)

Bunnell, B. A., Muul, L. M., Donahue, R. E., Blaese, R. M. & Morgan, R. A. (1995) High-efficiency retroviral-mediated gene transfer into human and nonhuman primate peripheral blood lymphocytes. Proc. Natl. Acad. Sci. USA **92**(17):7739–7743.

Zhou, P., Lee, J., Moore P, Brasky K. M. (2001) High-efficiency gene transfer into rhesus macaque primary T lymphocytes by combining 32 degrees C centrifugation and CH-296-coated plates: effect of gene transfer protocol on T cell homing receptor expression. Hum. Gene Ther. **12**(15):1843–1855.

F. Flow through transduction: concentrating cells and virus together in small culture systems

Pan, D., Shankar, R., Stroncek, D. F. & Whitley, C. B. (1999) Combined ultrafiltration-transduction in a **hollow-fiber** bioreactor facilitates retrovirus-mediated gene transfer into peripheral blood lymphocytes from patients with mucopolysaccharidosis type II. Hum. Gene Ther. **10**(17):2799–2810.

Chuck, A. S. & Palsson, B. O. (1996) Consistent and high rates of gene transfer can be obtained using **flow-through** transduction over a wide range of retroviral titers. Hum. Gene Ther. **7**(6):743–750.

G. Addition of fibronectin: adhesion domains within fibronectin allow binding to both target cells and virions to facilitate co-localization

Zhou, P., Lee, J., Moore, P. & Brasky, K. M. (2001) High-efficiency gene transfer into rhesus macaque primary T lymphocytes by combining 32 degrees C centrifugation and CH-296-coated plates: effect of gene transfer protocol on T cell homing receptor expression. Hum. Gene Ther. **12**(15):1843–1855.

Moritz, T., Dutt, P., Xiao, X., Carstanjen, D., Vik, T., Hanenberg, H. & Williams D. A. (1996) Fibronectin improves transduction of reconstituting hematopoietic stem cells by retroviral vectors: evidence of direct viral binding to chymotryptic carboxy-terminal fragments. Blood **88**(3):855–862.

Hanenberg, H., Xiao, X. L., Dilloo, D., Hashino, K., Kato, I. & Williams, D. A. (1996) Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. Nat. Med. **2**(8):876–882.

Appendix C: Additional Viral Infection Methods *continued*

Bajaj, B., Lei, P. & Andreadis, S. T. (2001) High efficiencies of gene transfer with immobilized recombinant retrovirus: kinetics and optimization. Biotechnol. Prog. **17**(4):587–596.

H. Cocultivation of target cells and packaging cells: Allows targets to be continuously in contact with freshly-produced viral supernatant

Casal, M. L. & Wolfe, J. H. (1997) Amphotropic and ecotropic retroviral vector viruses transduce midgestational murine fetal liver cells in a dual-chambered cocultivation system. Gene Ther. 4(1):39–44.

Germeraad, W. T., Asami, N., Fujimoto, S., Mazda, O. & Katsura, Y. (1994) Efficient retrovirus-mediated gene transduction into murine hematopoietic stem cells and long-lasting expression using a transwell coculture system. Blood **84**(3):780–788.

I. Use of cationic liposomes: Enhance virus-to-cell fusion

Kaneko, Y. & Tsukamoto, A. (1996) Cationic liposomes enhance retrovirus-mediated multinucleated cell formation and retroviral transduction. Cancer Lett. **105**(1):39–44.

Porter, C. D., Lukacs, K. V., Box, G., Takeuchi, Y. & Collins, M. K. (1998) Cationic liposomes enhance the rate of transduction by a recombinant retroviral vector in vitro and in vivo. J. Virol. **72**(6):4832–4840.

J. Use of histone deacetylase inhibitors to increase titer: Relieves repression of viral expression by hyperacetylation of histones

Chen, W. Y., Bailey, E. C., McCune, S. L., Dong, J. Y. & Townes, T. M. (1997) Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase. Proc. Natl. Acad. Sci. USA **94**:5798–5803.

Tobias, C. A., Kim, D. & Fischer, I. (2000) Improved recombinant retroviral titers utilizing trichostatin A. Biotechniques **29**:884–890.

Notes

Notes

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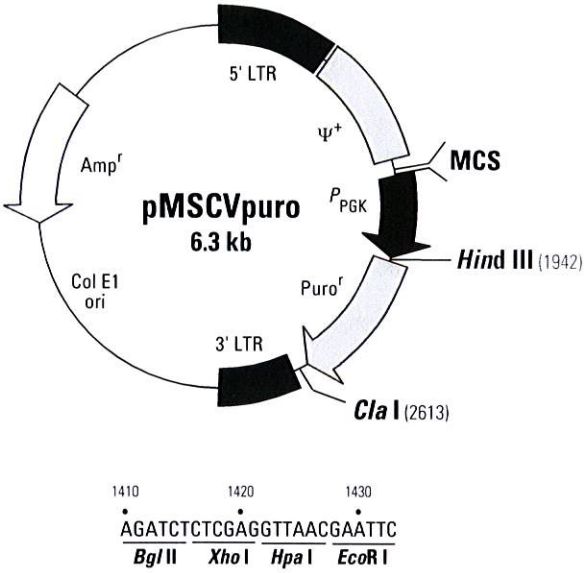
pMSCVpuro Vector Information PT3303-5

GenBank Accession #: Submission in progress. Sold as part of Cat. No. 634401



Restriction Map and Multiple Cloning Site (MCS) of pMSCVpuro. Unique restriction sites are in bold.

Description

The Murine Stem Cell Virus (MSCV) vectors were derived from the Murine Embryonic Stem Cell Virus (MESV) and the LN retroviral vectors (1,2). Upon transfection into a packaging cell line, pMSCVpuro transiently expresses, or integrates and stably expresses, a transcript containing the extended viral packaging signal Ψ^+, the puromycin resistance gene, and a gene of interest. The vectors achieve stable, high-level gene expression in hematopoietic and embryonic stem cells through a specifically designed 5' long terminal repeat (LTR). This LTR is from the murine stem cell PCMV virus, and it differs from the MoMuLV LTR used in other retroviral vectors by several point mutations and a deletion. These changes enhance transcriptional activation and prevent transcriptional suppression in embryonic stem and embryonal carcinoma cells. As a result, the LTR drives high-level constitutive expression of a target gene in stem cells or other mammalian cell lines (3). A gene can be cloned into the multiple cloning site immediately downstream of this LTR. The murine phosphoglycerate kinase (PKG) promoter (P_{PKG}) controls expression of the puromycin resistance gene (Puro^r) for antibiotic selection in eukaryotic cells. pMSCVpuro also contains the pUC origin of replication and *E. coli* Amp^r gene for propagation and antibiotic selection in bacteria.

Use

pMSCVpuro can be transfected into a packaging cell line such as the RetroPack™ PT67 Cell Line (Cat. No. 631510). Once in the cell, RNA from the vector is packaged into infectious, replication-incompetent retroviral particles. pMSCVpuro does not contain the *gag*, *pol*, and *env* structural genes necessary for particle formation and replication: these genes are stably integrated into the PT67 genome (4–7). Introduction of pMSCVpuro into a packaging cell line results in production of high-titer, replication-incompetent infectious virus particles. These particles can infect target cells and transmit the gene of interest to them. However, they cannot replicate because the target cells lack viral structural genes. The process of separately introducing and integrating the structural genes into the packaging cell line minimizes the chances of producing replication-competent virus due to recombination events during cell proliferation.

(PR37258; published 23 July 2003)

Location of features:

- 5' PCMV LTR: 1–515
- Ψ^+ (extended packaging signal): 516–1404
- Puromycin resistance gene (Puro^r): 1958–2557
- PKG promoter ($P_{CMV\ IE}$): 1429–1937
- Multiple Cloning Site: 1410–1433
- 3' PCMV LTR: 2687–3170
- Col E1 origin of replication:
  Site of replication initiation: 3741
- Ampicillin resistance gene (β-lactamase): 5361–4504

Sequencing primer locations:

- pMSCV Primers:
  5' primer (1333–1355): 5'-CCCTTGAACCTCCTCGTTCGACC-3'
  3' primer (1660–1682): 5'-GAGACGTGCTACTTCCATTTGTC-3'

Propagation in *E. coli:*

- Suitable host strains: DH5α, HB101, and other general purpose strains.
- Selectable marker: plasmid confers resistance to ampicillin (50 µg/ml) to *E. coli* hosts.
- *E. coli* replication origin: Col E1
- Copy number: low

Note: The viral supernatants produced by this retroviral vector could, depending on your cloned insert, contain potentially hazardous recombinant virus. Due caution must be exercised in the production and handling of recombinant retrovirus. Appropriate NIH, regional, and institutional guidelines apply.

References:

1. Grez, M., *et al.* (1990) *Proc. Natl. Acad. Sci. USA* **87**:9202–9206.
2. Miller, A. D. & Rosman, G. J. (1989) *BioTechniques* **7**:980–990.
3. Hawley, T.S. *et al.* (1994) *Gene Ther.* **1**:136–138.
4. Mann, R., *et al.* (1983) *Cell* **33**:153–159.
5. Miller, A. D. & Buttimore, C. (1986) *Mol. Cell. Biol.* **6**:2895–2902.
6. Morgenstern, J. P. & Land, H. (1990) *Nucleic Acids Res.* **18**:3587–3590.
7. Miller, A. D. & Chen, F. (1996) *J. Virol.* **70**:5564–5571.

Note: The attached sequence file has been compiled from information in the sequence databases, published literature, and other sources, together with partial sequences obtained by Clontech. This vector has not been completely sequenced.

Conforms to ANSI Z400.1 Standard (United States)

Material Safety Data Sheet

Agilent Technologies

Stratagene pBC KS- Phagemid, Catalog #212218

1. Product and company identification

Product name	: Stratagene pBC KS- Phagemid, Catalog #212218	
Material uses	: Analytical reagent.	
	pBC KS- Phagemid	0.02 ml
	XL1-Blue MRF' Strain	0.5 ml
Supplier/Manufacturer	: Agilent Technologies, Inc. 1834 State Highway 71 West Cedar Creek, TX 78612	
Part No. (Chemical Kit)	: 212218	
Part No.	: pBC KS- Phagemid	212218-51
	XL1-Blue MRF' Strain	200201-81
Validation date	: 01/31/2011	
In case of emergency	: 1-800-894-1304	

Material Safety Data Sheets

2. Hazards identification

Physical state	: pBC KS- Phagemid	Liquid.
	XL1-Blue MRF' Strain	Liquid.
Odor	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	Not available.
OSHA/HCS status	: pBC KS- Phagemid	While this material is not considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200), this MSDS contains valuable information critical to the safe handling and proper use of the product. This MSDS should be retained and available for employees and other users of this product.
	XL1-Blue MRF' Strain	This material is considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200).

Emergency overview

Signal word	: pBC KS- Phagemid	No signal word.
	XL1-Blue MRF' Strain	CAUTION!
Hazard statements	: pBC KS- Phagemid	NOT EXPECTED TO PRODUCE SIGNIFICANT ADVERSE HEALTH EFFECTS WHEN THE RECOMMENDED INSTRUCTIONS FOR USE ARE FOLLOWED.
	XL1-Blue MRF' Strain	MAY CAUSE RESPIRATORY TRACT, EYE AND SKIN IRRITATION. CONTAINS MATERIAL THAT MAY CAUSE TARGET ORGAN DAMAGE, BASED ON ANIMAL DATA.
Precautions	: pBC KS- Phagemid	No known significant effects or critical hazards. Avoid prolonged contact with eyes, skin and clothing.
	XL1-Blue MRF' Strain	Avoid breathing vapor or mist. Avoid contact with eyes, skin and clothing. Use only with adequate ventilation. Keep container tightly closed and sealed until ready for use. Wash thoroughly after handling.
Routes of entry	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	Not available.

Potential acute health effects

2. Hazards identification

Inhalation	: pBC KS- Phagemid XL1-Blue MRF' Strain	No known significant effects or critical hazards. Slightly irritating to the respiratory system.
Ingestion	: pBC KS- Phagemid XL1-Blue MRF' Strain	No known significant effects or critical hazards. No known significant effects or critical hazards.
Skin	: pBC KS- Phagemid XL1-Blue MRF' Strain	No known significant effects or critical hazards. Slightly irritating to the skin.
Eyes	: pBC KS- Phagemid XL1-Blue MRF' Strain	No known significant effects or critical hazards. Moderately irritating to eyes.

Potential chronic health effects

Chronic effects	: pBC KS- Phagemid XL1-Blue MRF' Strain	No known significant effects or critical hazards. Contains material that may cause target organ damage, based on animal data.
Carcinogenicity	: pBC KS- Phagemid XL1-Blue MRF' Strain	No known significant effects or critical hazards. No known significant effects or critical hazards.
Mutagenicity	: pBC KS- Phagemid XL1-Blue MRF' Strain	No known significant effects or critical hazards. No known significant effects or critical hazards.
Teratogenicity	: pBC KS- Phagemid XL1-Blue MRF' Strain	No known significant effects or critical hazards. No known significant effects or critical hazards.
Developmental effects	: pBC KS- Phagemid XL1-Blue MRF' Strain	No known significant effects or critical hazards. No known significant effects or critical hazards.
Fertility effects	: pBC KS- Phagemid XL1-Blue MRF' Strain	No known significant effects or critical hazards. No known significant effects or critical hazards.
Target organs	: pBC KS- Phagemid XL1-Blue MRF' Strain	Not available. Contains material which may cause damage to the following organs: kidneys, upper respiratory tract, skin, eye, lens or cornea.

Over-exposure signs/symptoms

Inhalation	: pBC KS- Phagemid XL1-Blue MRF' Strain	No specific data. Adverse symptoms may include the following: respiratory tract irritation coughing
Ingestion	: pBC KS- Phagemid XL1-Blue MRF' Strain	No specific data. No specific data.
Skin	: pBC KS- Phagemid XL1-Blue MRF' Strain	No specific data. Adverse symptoms may include the following: irritation redness
Eyes	: pBC KS- Phagemid XL1-Blue MRF' Strain	No specific data. Adverse symptoms may include the following: irritation watering redness
Medical conditions aggravated by over-exposure	: pBC KS- Phagemid XL1-Blue MRF' Strain	None known. Pre-existing disorders involving any target organs mentioned in this MSDS as being at risk may be aggravated by over-exposure to this product.

See toxicological information (Section 11)

3. Composition/information on ingredients

Name	CAS number	%
XL1-Blue MRF' Strain		
Glycerol	56-81-5	10 - 30
Sodium chloride	7647-14-5	0.1 - 1

There are no additional ingredients present which, within the current knowledge of the supplier and in the concentrations applicable, are classified as hazardous to health or the environment and hence require reporting in this section.

4. First aid measures

Eye contact

: pBC KS- Phagemid — Check for and remove any contact lenses. Immediately flush eyes with plenty of water for at least 15 minutes, occasionally lifting the upper and lower eyelids. Get medical attention if symptoms occur.

XL1-Blue MRF' Strain — Check for and remove any contact lenses. Immediately flush eyes with plenty of water for at least 15 minutes, occasionally lifting the upper and lower eyelids. Get medical attention immediately.

Skin contact

: pBC KS- Phagemid — In case of contact, immediately flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Wash clothing before reuse. Clean shoes thoroughly before reuse. Get medical attention if symptoms occur.

XL1-Blue MRF' Strain — In case of contact, immediately flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Wash clothing before reuse. Clean shoes thoroughly before reuse. Get medical attention immediately.

Inhalation

: pBC KS- Phagemid — Move exposed person to fresh air. If not breathing, if breathing is irregular or if respiratory arrest occurs, provide artificial respiration or oxygen by trained personnel. Loosen tight clothing such as a collar, tie, belt or waistband. Get medical attention if symptoms occur.

XL1-Blue MRF' Strain — Move exposed person to fresh air. If not breathing, if breathing is irregular or if respiratory arrest occurs, provide artificial respiration or oxygen by trained personnel. Loosen tight clothing such as a collar, tie, belt or waistband. Get medical attention immediately.

Ingestion

: pBC KS- Phagemid — Wash out mouth with water. Do not induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. Get medical attention if symptoms occur.

XL1-Blue MRF' Strain — Wash out mouth with water. Do not induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. Get medical attention immediately.

4. First aid measures

Protection of first-aiders	: pBC KS- Phagemid	No action shall be taken involving any personal risk or without suitable training.
	XL1-Blue MRF' Strain	No action shall be taken involving any personal risk or without suitable training. It may be dangerous to the person providing aid to give mouth-to-mouth resuscitation.
Notes to physician	: pBC KS- Phagemid	No specific treatment. Treat symptomatically. Contact poison treatment specialist immediately if large quantities have been ingested or inhaled.
	XL1-Blue MRF' Strain	No specific treatment. Treat symptomatically. Contact poison treatment specialist immediately if large quantities have been ingested or inhaled.

5. Fire-fighting measures

Flammability of the product	: pBC KS- Phagemid	In a fire or if heated, a pressure increase will occur and the container may burst.
	XL1-Blue MRF' Strain	In a fire or if heated, a pressure increase will occur and the container may burst.
Extinguishing media		
Suitable	: pBC KS- Phagemid	Use an extinguishing agent suitable for the surrounding fire.
	XL1-Blue MRF' Strain	Use an extinguishing agent suitable for the surrounding fire.
Not suitable	: pBC KS- Phagemid	None known.
	XL1-Blue MRF' Strain	None known.
Special exposure hazards	: pBC KS- Phagemid	No action shall be taken involving any personal risk or without suitable training.
	XL1-Blue MRF' Strain	No action shall be taken involving any personal risk or without suitable training.
Hazardous thermal decomposition products	: pBC KS- Phagemid	No specific data.
	XL1-Blue MRF' Strain	Decomposition products may include the following materials: carbon dioxide carbon monoxide halogenated compounds metal oxide/oxides
Special protective equipment for fire-fighters	: Fire-fighters should wear appropriate protective equipment and self-contained breathing apparatus (SCBA) with a full face-piece operated in positive pressure mode.	

6. Accidental release measures

Personal precautions	: pBC KS- Phagemid	No action shall be taken involving any personal risk or without suitable training. Evacuate surrounding areas. Keep unnecessary and unprotected personnel from entering. Do not touch or walk through spilled material. Put on appropriate personal protective equipment (see Section 8).
	XL1-Blue MRF' Strain	No action shall be taken involving any personal risk or without suitable training. Evacuate surrounding areas. Keep unnecessary and unprotected personnel from entering. Do not touch or walk through spilled material. Avoid breathing vapor or mist. Provide adequate ventilation. Wear appropriate respirator when ventilation is inadequate. Put on appropriate personal protective equipment (see Section 8).

6. Accidental release measures

Environmental precautions :	pBC KS- Phagemid	Avoid dispersal of spilled material and runoff and contact with soil, waterways, drains and sewers. Inform the relevant authorities if the product has caused environmental pollution (sewers, waterways, soil or air).
	XL1-Blue MRF' Strain	Avoid dispersal of spilled material and runoff and contact with soil, waterways, drains and sewers. Inform the relevant authorities if the product has caused environmental pollution (sewers, waterways, soil or air).
Methods for cleaning up :	pBC KS- Phagemid	Stop leak if without risk. Move containers from spill area. Dilute with water and mop up if water-soluble. Alternatively, or if water-insoluble, absorb with an inert dry material and place in an appropriate waste disposal container. Dispose of via a licensed waste disposal contractor.
	XL1-Blue MRF' Strain	Stop leak if without risk. Move containers from spill area. Dilute with water and mop up if water-soluble. Alternatively, or if water-insoluble, absorb with an inert dry material and place in an appropriate waste disposal container. Dispose of via a licensed waste disposal contractor.

7. Handling and storage

Handling :	pBC KS- Phagemid	Put on appropriate personal protective equipment (see Section 8). Eating, drinking and smoking should be prohibited in areas where this material is handled, stored and processed. Workers should wash hands and face before eating, drinking and smoking. Remove contaminated clothing and protective equipment before entering eating areas.
	XL1-Blue MRF' Strain	Potentially biohazardous material. Put on appropriate personal protective equipment (see Section 8). Eating, drinking and smoking should be prohibited in areas where this material is handled, stored and processed. Workers should wash hands and face before eating, drinking and smoking. Remove contaminated clothing and protective equipment before entering eating areas. Do not ingest. Avoid contact with eyes, skin and clothing. Avoid breathing vapor or mist. Use only with adequate ventilation. Wear appropriate respirator when ventilation is inadequate. Keep in the original container or an approved alternative made from a compatible material, kept tightly closed when not in use. Empty containers retain product residue and can be hazardous. Do not reuse container.
Storage :	pBC KS- Phagemid	Store in accordance with local regulations. Store in original container protected from direct sunlight in a dry, cool and well-ventilated area, away from incompatible materials (see section 10) and food and drink. Keep container tightly closed and sealed until ready for use. Containers that have been opened must be carefully resealed and kept upright to prevent leakage. Do not store in unlabeled containers. Use appropriate containment to avoid environmental contamination.
	XL1-Blue MRF' Strain	Store in accordance with local regulations. Store in

7. Handling and storage

original container protected from direct sunlight in a dry, cool and well-ventilated area, away from incompatible materials (see section 10) and food and drink. Keep container tightly closed and sealed until ready for use. Containers that have been opened must be carefully resealed and kept upright to prevent leakage. Do not store in unlabeled containers. Use appropriate containment to avoid environmental contamination.

8. Exposure controls/personal protection

Ingredient	Exposure limits
XL1-Blue MRF' Strain Glycerol	**ACGIH TLV (United States, 2/2010).** TWA: 10 mg/m³ 8 hour(s). Form: Inhalable fraction. See Appendix C, paragraph A. Inhalable Particulate Mass TLVs (IPM–TLVs) for those materials that are hazardous when deposited anywhere in the respiratory tract. **OSHA PEL (United States, 6/2010).** TWA: 5 mg/m³ 8 hour(s). Form: Respirable fraction TWA: 15 mg/m³ 8 hour(s). Form: Total dust **OSHA PEL 1989 (United States, 3/1989).** TWA: 5 mg/m³ 8 hour(s). Form: Respirable fraction TWA: 10 mg/m³ 8 hour(s). Form: Total dust

Recommended monitoring procedures : If this product contains ingredients with exposure limits, personal, workplace atmosphere or biological monitoring may be required to determine the effectiveness of the ventilation or other control measures and/or the necessity to use respiratory protective equipment.

Engineering measures : Use only with adequate ventilation. If user operations generate dust, fumes, gas, vapor or mist, use process enclosures, local exhaust ventilation or other engineering controls to keep worker exposure to airborne contaminants below any recommended or statutory limits.

Hygiene measures : Handle as biohazard material (Biosafety level 1).
Wash hands, forearms and face thoroughly after handling chemical products, before eating, smoking and using the lavatory and at the end of the working period. Appropriate techniques should be used to remove potentially contaminated clothing. Wash contaminated clothing before reusing. Ensure that eyewash stations and safety showers are close to the workstation location.

Personal protection

Respiratory : Use a properly fitted, air-purifying or air-fed respirator complying with an approved standard if a risk assessment indicates this is necessary. Respirator selection must be based on known or anticipated exposure levels, the hazards of the product and the safe working limits of the selected respirator.

Hands : Chemical-resistant, impervious gloves complying with an approved standard should be worn at all times when handling chemical products if a risk assessment indicates this is necessary.

Eyes : Safety eyewear complying with an approved standard should be used when a risk assessment indicates this is necessary to avoid exposure to liquid splashes, mists or dusts.

Skin : Personal protective equipment for the body should be selected based on the task being performed and the risks involved and should be approved by a specialist before handling this product.

Environmental exposure controls : Emissions from ventilation or work process equipment should be checked to ensure they comply with the requirements of environmental protection legislation. In some cases, fume scrubbers, filters or engineering modifications to the process equipment will be necessary to reduce emissions to acceptable levels.

8. Exposure controls/personal protection

Other protection : Not available.

9. Physical and chemical properties

Physical state	: pBC KS- Phagemid	Liquid.
	XL1-Blue MRF' Strain	Liquid.
Flash point	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	[Product does not sustain combustion.]
Auto-ignition temperature	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	Not available.
Flammable limits	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	Not available.
Color	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	Not available.
Odor	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	Not available.
pH	: pBC KS- Phagemid	7.5
	XL1-Blue MRF' Strain	7
Boiling/condensation point	: pBC KS- Phagemid	100°C (212°F)
	XL1-Blue MRF' Strain	Not available.
Melting/freezing point	: pBC KS- Phagemid	0°C (32°F)
	XL1-Blue MRF' Strain	Not available.
Specific gravity	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	Not available.
Vapor pressure	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	Not available.
Vapor density	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	Not available.
Volatility	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	Not available.
Odor threshold	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	Not available.
Evaporation rate	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	Not available.
Viscosity	: pBC KS- Phagemid	Not available.
	XL1-Blue MRF' Strain	Not available.
Solubility	: pBC KS- Phagemid	Easily soluble in the following materials: cold water and hot water.
	XL1-Blue MRF' Strain	Easily soluble in the following materials: cold water and hot water.

10. Stability and reactivity

Chemical stability	: pBC KS- Phagemid	The product is stable.
	XL1-Blue MRF' Strain	The product is stable.
Conditions to avoid	: pBC KS- Phagemid	No specific data.
	XL1-Blue MRF' Strain	No specific data.
Materials to avoid	: pBC KS- Phagemid	No specific data.
	XL1-Blue MRF' Strain	No specific data.
Hazardous decomposition products	: pBC KS- Phagemid	Under normal conditions of storage and use, hazardous decomposition products should not be produced.
	XL1-Blue MRF' Strain	Under normal conditions of storage and use, hazardous decomposition products should not be produced.

10. Stability and reactivity

Possibility of hazardous reactions : pBC KS- Phagemid — Under normal conditions of storage and use, hazardous reactions will not occur.

XL1-Blue MRF' Strain — Under normal conditions of storage and use, hazardous reactions will not occur.

11. Toxicological information

Acute toxicity

Product/ingredient name	Result	Species	Dose	Exposure
XL1-Blue MRF' Strain				
Glycerol	LD50 Oral	Rat	12600 mg/kg	-
Sodium chloride	LC50 Inhalation Dusts and mists	Rat	>42 g/m³	1 hours
	LD50 Oral	Rat	3000 mg/kg	-

Irritation/Corrosion

Product/ingredient name	Result	Species	Score	Exposure	Observation
XL1-Blue MRF' Strain					
Glycerol	Eyes - Mild irritant	Rabbit	-	-	-
	Skin - Mild irritant	Rabbit	-	-	-
Sodium chloride	Eyes - Moderate irritant	Rabbit	-	-	-
	Skin - Mild irritant	Rabbit	-	-	-

Sensitizer

Conclusion/Summary : Not available.

Chronic toxicity / Carcinogenicity / Mutagenicity / Teratogenicity / Reproductive toxicity

Not available.

Other adverse symptoms : pBC KS- Phagemid — Not available.

XL1-Blue MRF' Strain — Not available.

12. Ecological information

Ecotoxicity : No known significant effects or critical hazards.

Aquatic ecotoxicity

Product/ingredient name	Result	Species	Exposure
XL1-Blue MRF' Strain			
Glycerol	Acute LC50 54 to 57 ml/L Fresh water	Fish - Oncorhynchus mykiss - 0.9 g	96 hours
Sodium chloride	Acute EC50 402600 to 469200 ug/L Fresh water	Daphnia - Daphnia magna	48 hours
	Acute LC50 >5600 ppm Fresh water	Crustaceans - Asellus communis	48 hours
	Acute LC50 1000000 ug/L Fresh water	Fish - Morone saxatilis - LARVAE	96 hours

Conclusion/Summary : Not available.

Partition coefficient: n-octanol/water : pBC KS- Phagemid — Not available.

XL1-Blue MRF' Strain — Not available.

Other adverse effects : No known significant effects or critical hazards.

13. Disposal considerations

Waste disposal : The generation of waste should be avoided or minimized wherever possible. Significant quantities of waste product residues should not be disposed of via the foul sewer but processed in a suitable effluent treatment plant. Dispose of surplus and non-recyclable products via a licensed waste disposal contractor. Disposal of this product, solutions and any by-products should at all times comply with the requirements of environmental protection and waste disposal legislation and any regional local authority requirements. Waste packaging should be recycled. Incineration or landfill should only be considered when recycling is not feasible. This material and its container must be disposed of in a

13. Disposal considerations

safe way. Care should be taken when handling emptied containers that have not been cleaned or rinsed out. Empty containers or liners may retain some product residues. Avoid dispersal of spilled material and runoff and contact with soil, waterways, drains and sewers.

Disposal should be in accordance with applicable regional, national and local laws and regulations. Local regulations may be more stringent than regional or national requirements.

The information presented below only applies to the material as supplied. The identification based on characteristic(s) or listing may not apply if the material has been used or otherwise contaminated. It is the responsibility of the waste generator to determine the toxicity and physical properties of the material generated to determine the proper waste identification and disposal methods in compliance with applicable regulations.

Refer to Section 7: HANDLING AND STORAGE and Section 8: EXPOSURE CONTROLS/PERSONAL PROTECTION for additional handling information and protection of employees.

14. Transport information

Regulatory information

DOT / IMDG / IATA / : Not regulated.

15. Regulatory information

HCS Classification : pBC KS- Phagemid — Not regulated.
XL1-Blue MRF' Strain — Irritating material
Target organ effects

U.S. Federal regulations : **TSCA 8(a) IUR**: Partial exemption

United States inventory (TSCA 8b): Not determined.

SARA 302/304/311/312 extremely hazardous substances: No products were found.
SARA 302/304 emergency planning and notification: No products were found.
SARA 302/304/311/312 hazardous chemicals: Glycerol
SARA 311/312 MSDS distribution - chemical inventory - hazard identification: Glycerol: Immediate (acute) health hazard, Delayed (chronic) health hazard

Clean Water Act (CWA) 311: Edetic acid

Clean Air Act Section 112(b) Hazardous Air Pollutants (HAPs) : Not listed

Clean Air Act Section 602 Class I Substances : Not listed

Clean Air Act Section 602 Class II Substances : Not listed

DEA List I Chemicals (Precursor Chemicals) : Not listed

DEA List II Chemicals (Essential Chemicals) : Not listed

State regulations

Massachusetts : The following components are listed: GLYCERINE MIST

New York : None of the components are listed.

New Jersey : The following components are listed: GLYCERIN; 1,2,3-PROPANETRIOL

Pennsylvania : The following components are listed: 1,2,3-PROPANETRIOL

California Prop. 65

15. Regulatory information

WARNING: This product contains a chemical known to the State of California to cause birth defects or other reproductive harm.

Ingredient name	Cancer	Reproductive	No significant risk level	Maximum acceptable dosage level
XL1-Blue MRF' Strain Tetracycline	No.	Yes.	No.	No.

16. Other information

Label requirements : pBC KS- Phagemid — NOT EXPECTED TO PRODUCE SIGNIFICANT ADVERSE HEALTH EFFECTS WHEN THE RECOMMENDED INSTRUCTIONS FOR USE ARE FOLLOWED.

XL1-Blue MRF' Strain — MAY CAUSE RESPIRATORY TRACT, EYE AND SKIN IRRITATION. CONTAINS MATERIAL THAT MAY CAUSE TARGET ORGAN DAMAGE, BASED ON ANIMAL DATA.

Date of issue : 01/31/2011

Date of previous issue : No previous validation.

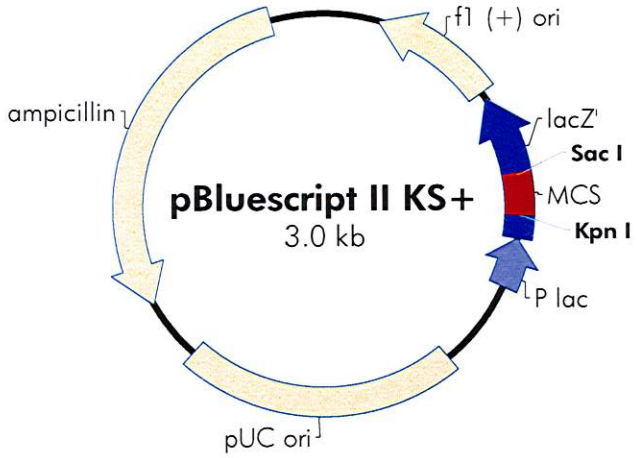
Version : 1

Indicates information that has changed from previously issued version.

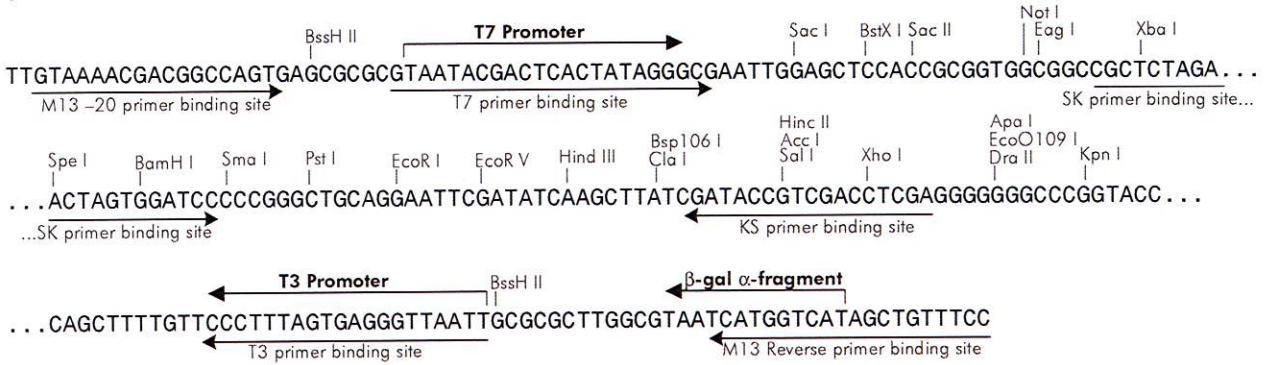
Notice to reader

Disclaimer: The information contained in this document is based on Agilent's state of knowledge at the time of preparation. No warranty as to its accurateness, completeness or suitability for a particular purpose is expressed or implied.

f1 (+) origin 135–441
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
***lac* promoter** 817–938
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833



pBluescript II KS (+/–) Multiple Cloning Site Region (sequence shown 598–826)



invitrogen™

Material Safety Data Sheet

Revision Date: 20-Oct-2008

1. IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND THE COMPANY/UNDERTAKING

Product code 350524
Product name pcDNA™ 3.1/ His/ lacZ

Company/Undertaking Identification

INVITROGEN CORPORATON
5791 VAN ALLEN WAY
PO BOX 6482
CARLSBAD, CA 92008
760-603-7200

INVITROGEN CORPORATION
5250 MAINWAY DRIVE
BURLINGTON, ONT
CANADA L7L 6A4
800-263-6236

GIBCO PRODUCTS
INVITROGEN CORPORATION
3175 STALEY ROAD P.O. BOX 68
GRAND ISLAND, NY 14072
716-774-6700

2. COMPOSITION/INFORMATION ON INGREDIENTS

Hazardous/Non-hazardous Components
The product contains no substances which at their given concentration, are considered to be hazardous to health

3. HAZARDS IDENTIFICATION

Emergency Overview
The product contains no substances which at their given concentration, are considered to be hazardous to health

Form
Liquid

Principle Routes of Exposure/ Potential Health effects

Eyes	No information available
Skin	No information available
Inhalation	No information available

Ingestion No information available

Specific effects

Carcinogenic effects	No information available
Mutagenic effects	No information available
Reproductive toxicity	No information available
Sensitization	No information available

Target Organ Effects No information available

HMIS

Health	0
Flammability	0
Reactivity	0

Skin contact	Wash off immediately with plenty of water
Eye contact	Rinse thoroughly with plenty of water, also under the eyelids.
Ingestion	Never give anything by mouth to an unconscious person
Inhalation	Move to fresh air
Notes to physician	Treat symptomatically.

Suitable extinguishing media	Dry chemical
Special protective equipment for firefighters	Wear self-contained breathing apparatus and protective suit

Personal precautions	Use personal protective equipment
Methods for cleaning up	Soak up with inert absorbent material.

Handling	No special handling advice required
Storage	Keep in properly labelled containers

Occupational exposure controls

Exposure limits

Engineering measures Ensure adequate ventilation, especially in confined areas

Personal protective equipment

Respiratory protection	In case of insufficient ventilation wear suitable respiratory equipment
Hand protection	Protective gloves
Eye protection	Safety glasses with side-shields
Skin and body protection	Lightweight protective clothing.
Hygiene measures	Handle in accordance with good industrial hygiene and safety practice

Environmental exposure controls	Prevent product from entering drains.

General Information

Form	Liquid

Important Health Safety and Environmental Information

Boiling point/range	**°C** No data available	**°F** No data available
Melting point/range	**°C** No data available	**°F** No data available
Flash point	**°C** No data available	**°F** No data available
Autoignition temperature	**°C** No data available	**°F** No data available
Oxidizing properties	No information available	
Water solubility	No data available	

Stability	Stable.
Materials to avoid	No information available
Hazardous decomposition products	No information available
Polymerization	Hazardous polymerisation does not occur.

Acute toxicity

Principle Routes of Exposure/ Potential Health effects

Eyes	No information available
Skin	No information available
Inhalation	No information available
Ingestion	No information available

Specific effects

Carcinogenic effects	No information available
Mutagenic effects	No information available
Reproductive toxicity	No information available
Sensitization	No information available

Target Organ Effects No information available

Ecotoxicity effects	No information available.
Mobility	No information available.
Biodegradation	Inherently biodegradable.
Bioaccumulation	Does not bioaccumulate.

Dispose of in accordance with local regulations

14. TRANSPORT INFORMATION

IATA

Proper shipping name	Not classified as dangerous in the meaning of transport regulations
Hazard Class	No information available
Subsidiary Class	No information available
Packing group	No information available
UN-No	No information available

15. REGULATORY INFORMATION

International Inventories

U.S. Federal Regulations

SARA 313
This product is not regulated by SARA.

Clean Air Act, Section 112 Hazardous Air Pollutants (HAPs) (see 40 CFR 61)
This product does not contains HAPs.

U.S. State Regulations

California Proposition 65
This product does not contain chemicals listed under Proposition 65

WHMIS hazard class:
Non-controlled

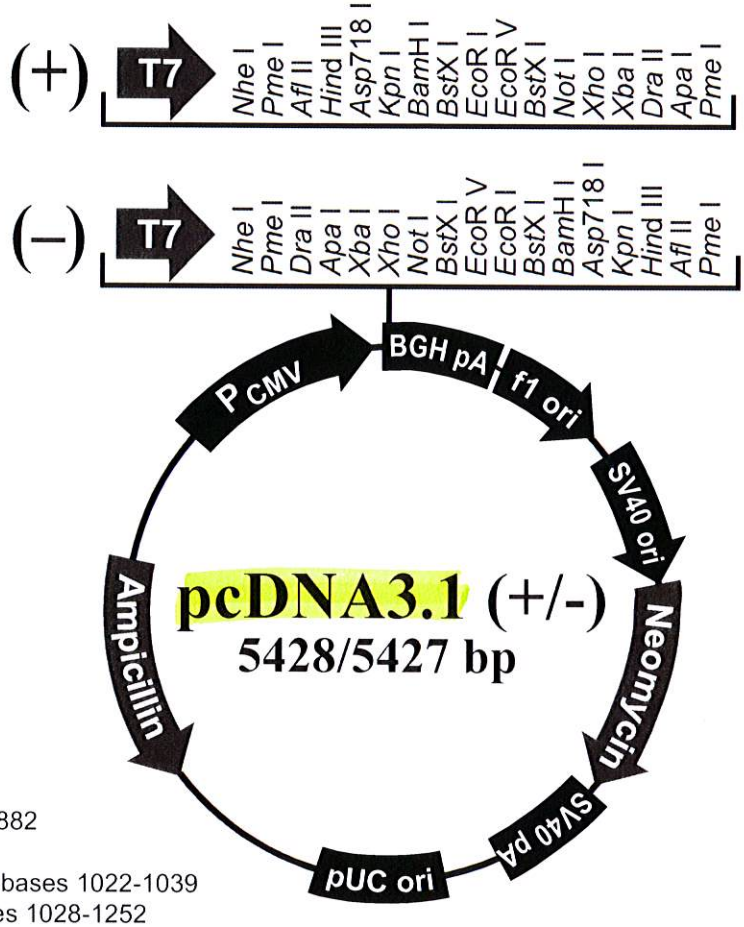
This product has been classified according to the hazard criteria of the CPR and the MSDS contains all of the information required by the CPR

16. OTHER INFORMATION

This material is sold for research and development purposes only. It is not for any human or animal therapeutic or clinical diagnostic use. It is not intended for food, drug, household, agricultural, or cosmetic use. An individual technically qualified to handle potentially hazardous chemicals must supervise the use of this material.

The above information was acquired by diligent search and/or investigation and the recommendations are based on prudent application of professional judgment. The information shall not be taken as being all inclusive and is to be used only as a guide. All materials and mixtures may be present unknown hazards and should be used with caution. Since Invitrogen Corporation cannot control the actual methods, volumes, or conditions of use, the Company shall not be held liable for any damages or losses resulting from the handling or from contact with the product as described herein. THE INFORMATION IN THIS MSDS DOES NOT CONSTITUTE A WARRANTY, EXPRESS OR IMPLIED, INCLUDING ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR ANY PARTCULAR PURPOSE.

End of Safety Data Sheet



Comments for pcDNA3.1 (+)
5428 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
pcDNA3.1/BGH reverse priming site: bases 1022-1039
BGH polyadenylation sequence: bases 1028-1252
f1 origin: bases 1298-1726
SV40 early promoter and origin: bases 1731-2074
Neomycin resistance gene (ORF): bases 2136-2930
SV40 early polyadenylation signal: bases 3104-3234
pUC origin: bases 3617-4287 (complementary strand)
Ampicillin resistance gene (*bla*): bases 4432-5428 (complementary strand)
ORF: bases 4432-5292 (complementary strand)
Ribosome binding site: bases 5300-5304 (complementary strand)
bla promoter (P3): bases 5327-5333 (complementary strand)

1. IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND THE COMPANY/UNDERTAKING

Product code 54357
Product name pUC 19 Control DNA

Company/Undertaking Identification

INVITROGEN CORPORATON
5791 VAN ALLEN WAY
PO BOX 6482
CARLSBAD, CA 92008
760-603-7200

INVITROGEN CORPORATION
5250 MAINWAY DRIVE
BURLINGTON, ONT
CANADA L7L 6A4
800-263-6236

GIBCO PRODUCTS
INVITROGEN CORPORATION
3175 STALEY ROAD P.O. BOX 68
GRAND ISLAND, NY 14072
716-774-6700

24 hour Emergency Response (Transport): 866-536-0631
301-431-8585
Outside of the U.S. ++1-301-431-8585

For research use only

2. COMPOSITION/INFORMATION ON INGREDIENTS

Hazardous/Non-hazardous Components
The product contains no substances which at their given concentration, are considered to be hazardous to health. We recommend handling all chemicals with caution.

3. HAZARDS IDENTIFICATION

Emergency Overview
The product contains no substances which at their given concentration, are considered to be hazardous to health

Form
Liquid

Principle Routes of Exposure/ Potential Health effects

Eyes	No information available
Skin	No information available
Inhalation	No information available
Ingestion	May be harmful if swallowed.

Specific effects

Carcinogenic effects	No information available
Mutagenic effects	No information available
Reproductive toxicity	No information available
Sensitization	No information available

Target Organ Effects No information available

HMIS

Health	0
Flammability	0
Reactivity	0

Skin contact	Wash off immediately with plenty of water. If symptoms persist, call a physician.
Eye contact	Rinse thoroughly with plenty of water, also under the eyelids. If symptoms persist, call a physician.
Ingestion	Never give anything by mouth to an unconscious person. If symptoms persist, call a physician.
Inhalation	Move to fresh air. If symptoms persist, call a physician.
Notes to physician	Treat symptomatically.

Suitable extinguishing media	Dry chemical
Special protective equipment for firefighters	Wear self-contained breathing apparatus and protective suit

Personal precautions	Use personal protective equipment
Methods for cleaning up	Soak up with inert absorbent material.

Handling	No special handling advice required
Storage	Keep in properly labelled containers

Occupational exposure controls

Exposure limits

Engineering measures	Ensure adequate ventilation, especially in confined areas

Personal protective equipment

Respiratory Protection	In case of insufficient ventilation wear suitable respiratory equipment
Hand protection	Protective gloves
Eye protection	Safety glasses with side-shields
Skin and body protection	Lightweight protective clothing.
Hygiene measures	Handle in accordance with good industrial hygiene and safety practice
Environmental exposure controls	Prevent product from entering drains.

General Information

Form	Liquid

Important Health Safety and Environmental Information

Boiling point/range	**°C** No data available	**°F** No data available
Melting point/range	**°C** No data available	**°F** No data available
Flash point	**°C** No data available	**°F** No data available
Autoignition temperature	**°C** No data available	**°F** No data available
Oxidizing properties	No information available	
Water solubility	Extremely soluble in water.	

Stability	Stable.
Materials to avoid	No information available
Hazardous decomposition products	No information available
Polymerization	Hazardous polymerisation does not occur.

Acute toxicity

Principle Routes of Exposure/ Potential Health effects

Eyes	No information available
Skin	No information available
Inhalation	No information available

Ingestion	May be harmful if swallowed.

Specific effects **(Long Term Effects)**

Carcinogenic effects	No information available
Mutagenic effects	No information available
Reproductive toxicity	No information available
Sensitization	No information available

Target Organ Effects No information available

Ecotoxicity effects	No information available.
Mobility	No information available.
Biodegradation	Inherently biodegradable.
Bioaccumulation	Does not bioaccumulate.

Dispose of in accordance with local regulations

IATA

Proper shipping name	Not classified as dangerous in the meaning of transport regulations
Hazard Class	No information available
Subsidiary Class	No information available
Packing group	No information available
UN-No	No information available

International Inventories

U.S. Federal Regulations

SARA 313
This product is not regulated by SARA.

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End of Safety Data Sheet

pUC19

GenBank Accession #: L09137

Feature	Coordinates	Source
lacZα	469-146	–
origin	1455-867	pMB1 (mutant)
bla (Ap^R)	2486-1626	*Tn3*

ori = origin of replication
Ap = ampicillin

There are no restriction sites for the following enzymes: AarI(x), AfeI, AflII, AgeI, AleI, ApaI, AscI, AsiSI, AvrII, BaeI, BbsI, BbvCI, BclI, BglII, BlpI, BmgBI, BmtI, Bpu10I, BsaAI, BsaBI, BseRI, BsgI, BsiWI, BsmFI, BsmI, BspDI, BspEI, BsrGI, BssHII, BstBI, BstEII, BstXI, BstZ17I, Bsu36I, BtgI, BtgZI, ClaI, CspCI, DraIII, EagI, EcoNI, EcoRV, FseI, FspAI(x), HpaI, I-CeuI, I-SceI, MfeI, MluI, MscI, NaeI, NcoI, NgoMIV, NheI, NotI, NruI, NsiI, PI-PspI, PI-SceI, PacI, PaeR7I, PflFI, PflMI, PmeI, PmlI, PpuMI, PshAI, PsiI, PspOMI, PspXI, RsrII, SacII, SanDI(x), SexAI, SfiI, SgrAI, SnaBI, SpeI, SrfI(x), StuI, StyI, SwaI, TliI, Tth111I, XcmI, XhoI

(x) = enzyme not available from NEB

pUC19 is a small, high-copy number *E. coli* plasmid cloning vector containing portions of pBR322 and M13mp19 (1). It contains the pMB1 origin of replication from pBR322, but it lacks the *rop* gene and carries a point mutation in the RNAII transcript (G 2975 in pBR322 to A 1308 in pUC19; 2). These changes together result in a temperature-dependent copy number of about 75 per cell at 37°C and >200 per cell at 42°C (2, 3). The multiple cloning site (MCS) is in frame with the *lacZα* gene, allowing screening for insertions using α-complementation.

pUC18 is identical to pUC19 except that the MCS region (nt 397-454) is inverted.

pNEB193 is also identical to pUC19 except for the addition of several restriction endonuclease sites to the MCS. Its total length is 2713 bp.

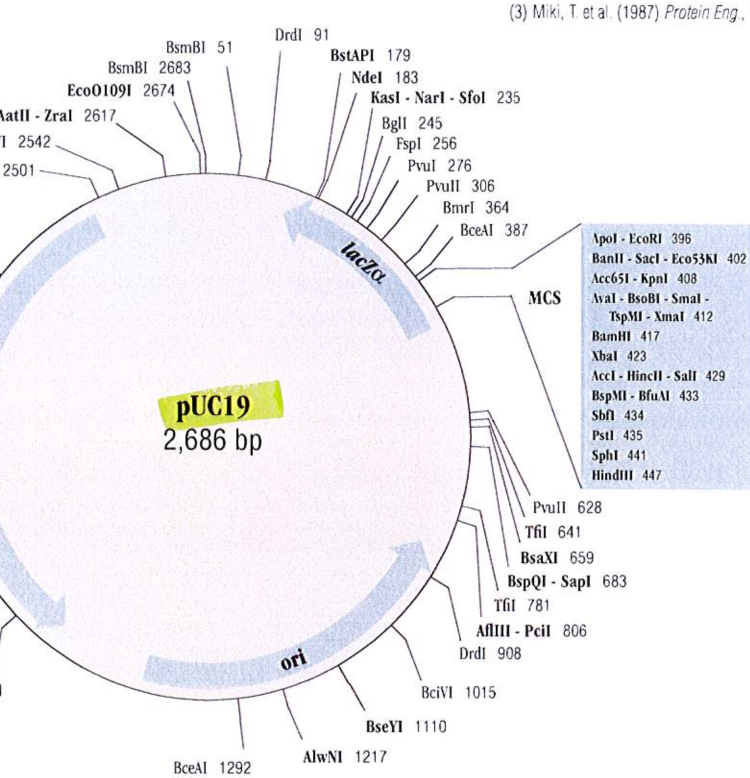
Enzymes with unique restriction sites are shown in **bold** type, and enzymes with two restriction sites are shown in regular type. Location of sites of all NEB restriction enzymes can be found on the NEB web site (choose Technical Reference > DNA Sequences and Maps). Restriction site coordinates refer to the position of the 5´-most base on the top strand in each recognition sequence.

Open reading frame (ORF) coordinates are in the form "translational start – translational stop"; numbers refer to positions on the top (clockwise) strand, regardless of the direction of transcription and include the start and stop codons.

Origin of replication coordinates include the region from the -35 promoter sequence of the RNAII transcript to the RNA/DNA switch point. *bla* (Ap^R) gene coordinates include the signal sequence.

References

(1) Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene*, 33, 103–119.
(2) Lin-Chao, S., Chen, W.-T. and Wong, T.-T. (1992) *Mol. Microbiol.*, 6, 3385–3393.
(3) Miki, T. et al. (1987) *Protein Eng.*, 1, 327–332.



pUC19 MCS

```
           SacI        SmaI         XbaI         SbfI
   EcoRI         KpnI        BamHI         SalI  PstI  SphI  HindIII
agtgAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGcgtaatcatggtcat
       .           .           .           .           .           .           .
      400         410         420         430         440         450         460
...S  N  S  S  P  V  R  P  D  E  L  T  S  R  C  A  H  L  S  P  T  I  M  T  M
```

← *lacZα* translational start

pNEB193 MCS

```
           SacI        SmaI    BssHII        PacI                             SbfI
   EcoRI         KpnI        AscI     BamHI            XbaI   SalI    PmeI     PstI  SphI  HindIII
agtgAATTCGAGCTCGGTACCCGGGGGCGCGCCGGATCCTTAATTAAGTCTAGAGTCGACTGTTTAAACCTGCAGGCATGCAAGCTTGGcgtaatcatggtcat
       .           .           .           .           .           .           .           .           .           .
      400         410         420         430         440         450         460         470         480         490
...S  N  S  S  P  V  R  P  R  A  P  D  K  I  L  D  L  T  S  Q  K  F  R  C  A  H  L  S  P  T  I  M  T  M
```

← *lacZα* translational start

Material Safety Data Sheet
acc. to ISO/DIS 11014

Printing date 03/08/2011 Reviewed on 03/08/2011

*
1 Identification of the substance/mixture and of the company/undertaking

Product identifier

Trade name: pGEM®-T Easy

Article number: A137
Application of the substance / the preparation Laboratory chemicals

Details of the supplier of the safety data sheet
Manufacturer/Supplier:

Promega Corporation
2800 Woods Hollow Road
Madison, WI 53711
U.S.A.
1-800-356-9526 or (608)-274-4330

Information department: MSDS author: Regulatory.Affairs@promega.com
Emergency telephone number:
For Chemical Emergency ONLY (spill, leak, fire, exposure or accident), call CHEMTREC at 1-800-424-9300
For call originating outside the United States dial 001-703-527-3887

2 Composition/information on ingredients

Chemical characterization: Mixtures
Description: Mixture of the substances listed below with nonhazardous additions.

Dangerous components: Void
Additional information: For the wording of the listed risk phrases refer to section 15.

*
3 Hazards identification

Classification of the substance or mixture

Classification according to Directive 67/548/EEC or Directive 1999/45/EC
Not applicable. Product has been classified as non-hazardous.
Information concerning particular hazards for human and environment:
The product does not have to be labelled due to the calculation procedure of international guidelines.
Classification system:
The classification was made according to the latest editions of international substances lists, and is expanded upon by company and technical literature data.

Label elements

Labelling according to EU guidelines:
Observe the general safety regulations when handling chemicals.
The product is not subject to identification regulations according to directives on hazardous materials.

(Contd. on page 2)

Material Safety Data Sheet
acc. to ISO/DIS 11014

Printing date 03/08/2011 Reviewed on 03/08/2011

Trade name: pGEM®-T Easy

(Contd. of page 1)

Classification system:
NFPA ratings (scale 0 - 4)
Health = 0
Fire = 0
Reactivity = 0
HMIS-ratings (scale 0 - 4)
Health = 0
Fire = 0
Reactivity = 0
OSHA Hazard Overview (Criteria according to 29CFR1910.1200): *Not applicable*
Target Organ(s): *Not applicable or unknown*

*
4 First aid measures

General information: *No special measures required.*
After inhalation: *Supply fresh air; consult doctor in case of complaints.*
After skin contact: *Generally the product does not irritate the skin.*
After eye contact: *Rinse opened eye for several minutes under running water.*
After swallowing: *If symptoms persist consult doctor.*

*
5 Firefighting measures

Suitable extinguishing agents:
CO2, extinguishing powder or water spray. Fight larger fires with water spray or alcohol resistant foam.
Special hazards arising from the substance or mixture *None known*
Protective equipment: *No special measures required.*

*
6 Accidental release measures

Personal precautions, protective equipment and emergency procedures *Not required.*
Environmental precautions: *No special measures required.*
Methods and material for containment and cleaning up:
Absorb with liquid-binding material (sand, diatomite, acid binders, universal binders, sawdust).
Reference to other sections
No dangerous substances are released.
See Section 7 for information on safe handling.
See Section 13 for disposal information.

*
7 Handling and storage

Handling:
Precautions for safe handling *No special measures required.*
Information about protection against explosions and fires: *The product is not flammable.*
Storage:
Requirements to be met by storerooms and receptacles: *No special requirements.*
Information about storage in one common storage facility: *Not required.*
Further information about storage conditions: *None.*
Specific end use(s) *No further relevant information available.*

(Contd. on page 3)

Material Safety Data Sheet
acc. to ISO/DIS 11014

Printing date 03/08/2011 Reviewed on 03/08/2011

Trade name: pGEM®-T Easy

(Contd. of page 2)

8 Exposure controls/personal protection

Components with limit values that require monitoring at the workplace:
The product does not contain any relevant quantities of materials with critical values that have to be monitored at the workplace.
Additional information: The lists that were valid during the creation were used as basis.
Personal protective equipment:
General protective and hygienic measures:
The usual precautionary measures for handling chemicals should be followed.
Breathing equipment: Not required.
Protection of hands:
Protective gloves
Selection of the glove material on consideration of the penetration times, rates of diffusion and the degradation
Material of gloves
The selection of the suitable gloves does not only depend on the material, but also on further marks of quality and varies from manufacturer to manufacturer. As the product is a preparation of several substances, the resistance of the glove material can not be calculated in advance and has therefore to be checked prior to the application.
Eye protection: Goggles recommended during refilling.

9 Physical and chemical properties

General Information	
Appearance:	
Form:	Fluid
Color:	Colorless
Odor:	Characteristic
Odour threshold:	Not determined.
pH-value at 20°C (68 °F):	7.4
Change in condition	
Melting point/Melting range:	0°C (32 °F)
Boiling point/Boiling range:	100°C (212 °F)
Flash point:	Not applicable.
Flammability (solid, gaseous):	Not applicable.
Ignition temperature:	
Decomposition temperature:	Not determined.
Auto igniting:	Product is not selfigniting.
Danger of explosion:	Product does not present an explosion hazard.
Explosion limits:	
Lower:	Not determined.
Upper:	Not determined.
Vapor pressure:	Not determined.
Density:	Not determined.
Relative density	Not determined.
Vapour density	Not determined.
Evaporation rate	Not determined.

(Contd. on page 4)

Material Safety Data Sheet
acc. to ISO/DIS 11014

Printing date 03/08/2011 Reviewed on 03/08/2011

Trade name: pGEM®-T Easy

(Contd. of page 3)

Solubility in / Miscibility with	
Water:	Not miscible or difficult to mix.
Segregation coefficient (n-octonol/water):	Not determined.
Viscosity:	
Dynamic:	Not determined.
Kinematic:	Not determined.
Solvent content:	
Organic solvents:	0.0 %
Water:	99.9 %
Solids content:	0.1 %
Other information	No further relevant information available.

10 Stability and reactivity

Thermal decomposition / conditions to be avoided: No decomposition if used according to specifications.
Incompatible materials: No further relevant information available.
Hazardous decomposition products: No dangerous decomposition products known.

11 Toxicological information

Acute toxicity:
LD/LC50 values that are relevant for classification: No data available
Primary irritant effect:
on the skin: No irritant effect.
on the eye: Irritating effect.
Sensitization: No sensitizing effects known.
Additional toxicological information:
The product is not subject to classification according to internally approved calculation methods for preparations:
When used and handled according to specifications, the product does not have any harmful effects according to our experience and the information provided to us.

*

12 Ecological information

Aquatic toxicity: Not harmful to the aquatic environment
Persistence and degradability Not available
Behavior in environmental systems:
Bioaccumulative potential Not known
Ecotoxical effects:
Remark: Not available
Additional ecological information:
General notes: Generally not hazardous for water

(Contd. on page 5)

Material Safety Data Sheet
acc. to ISO/DIS 11014

Printing date 03/08/2011 Reviewed on 03/08/2011

Trade name: pGEM®-T Easy

(Contd. of page 4)

*
13 Disposal considerations

Waste treatment methods
Recommendation:
Disposal should be in accordance with applicable regional, national and local laws and regulations.
Refer to Section 7: Handling and Storage and Section 8: Exposure Control/Personal Protection for additional handling information and protection of employees.

Uncleaned packagings:
Recommendation: Disposal must be made according to official regulations.

*
14 Transport information

Contact Promega Safety Department for additional transportation information

DOT regulations:	Not regulated
Hazard class:	-
Land transport ADR/RID (cross-border):	Not regulated
ADR/RID class:	-
UN-Number:	Not regulated
Maritime transport IMDG:	
IMDG Class:	-
Marine pollutant:	No
Air transport ICAO-TI and IATA-DGR:	Not regulated
ICAO/IATA Class:	-

*
15 Regulatory information

Sara

Section 355 (extremely hazardous substances):
None of the ingredients are listed.

Section 313 (Specific toxic chemical listings):
None of the ingredients are listed.

TSCA (Toxic Substances Control Act):
All ingredients are listed.

Proposition 65

Chemicals known to cause cancer:
None of the ingredients are listed.

Chemicals known to cause reproductive toxicity for females:
None of the ingredients are listed.

Chemicals known to cause reproductive toxicity for males:
None of the ingredients are listed.

Chemicals known to cause developmental toxicity:
None of the ingredients are listed.

(Contd. on page 6)

Material Safety Data Sheet
acc. to ISO/DIS 11014

Printing date 03/08/2011 Reviewed on 03/08/2011

Trade name: pGEM®-T Easy

(Contd. of page 5)

Cancerogenity categories

EPA (Environmental Protection Agency)
None of the ingredients are listed.

IARC (International Agency for Research on Cancer)
None of the ingredients are listed.

NTP (National Toxicology Program)
None of the ingredients are listed.

TLV (Threshold Limit Value established by ACGIH)
None of the ingredients are listed.

MAK (German Maximum Workplace Concentration)
None of the ingredients are listed.

NIOSH-Ca (National Institute for Occupational Safety and Health)
None of the ingredients are listed.

OSHA-Ca (Occupational Safety & Health Administration)
None of the ingredients are listed.

Product related hazard informations:
Observe the general safety regulations when handling chemicals.
The product is not subject to identification regulations according to directives on hazardous materials.

National regulations:

Water hazard class: Generally not hazardous for water.

*
16 Other information

This information is based on our present knowledge. However, this shall not constitute a guarantee for any specific product features and shall not establish a legally valid contractual relationship.

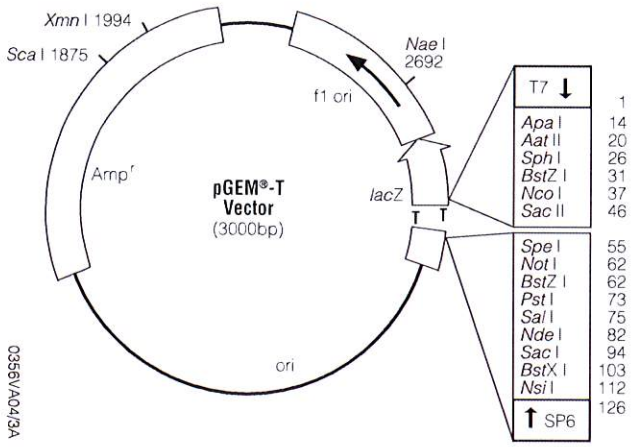
Department issuing MSDS:
Promega Corporation
Environmental Health and Safety Department
2800 Woods Hollow Road
Madison, WI
Ph:(608)274-4330
*** Data compared to the previous version altered.**

pGEM®-T and pGEM®-T Easy Vector Systems

INSTRUCTIONS FOR USE OF PRODUCTS A1360, A1380, A3600 AND A3610.

Quick PROTOCOL

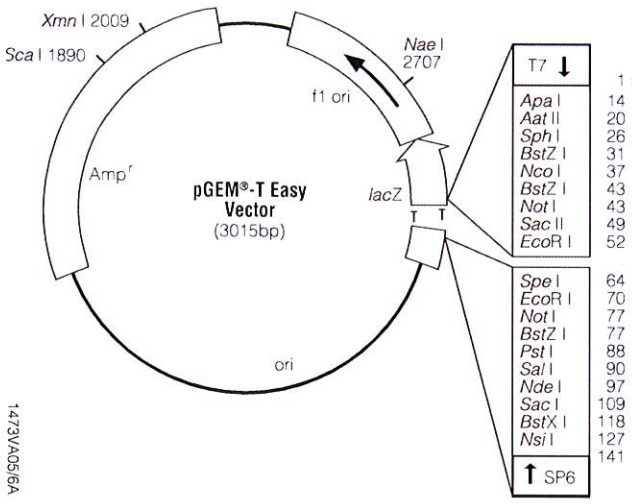
pGEM®-T Vector Circle Map and Sequence Reference Points



pGEM®-T Vector Sequence reference points:

T7 RNA Polymerase transcription initiation site	1
multiple cloning region	10–113
SP6 RNA Polymerase promoter (–17 to +3)	124–143
SP6 RNA Polymerase transcription initiation site	126
pUC/M13 Reverse Sequencing Primer binding site	161–177
lacZ start codon	165
lac operator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
lac operon sequences	2821–2981, 151–380
pUC/M13 Forward Sequencing Primer binding site	2941–2957
T7 RNA Polymerase promoter (–17 to +3)	2984–3

pGEM®-T Easy Vector Circle Map and Sequence Reference Points



pGEM®-T Easy Vector Sequence reference points:

T7 RNA Polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA Polymerase promoter (–17 to +3)	139–158
SP6 RNA Polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180
lac operator	200–216
ß-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA Polymerase promoter (–17 to +3)	2999-3

ORDERING/TECHNICAL INFORMATION:
www.promega.com • Phone 608-274-4330 or 800-356-9526 • Fax 608-277-2601

Printed in USA. Revised 3/09.
Part# 9FB033

MATERIAL SAFETY DATA SHEET

MSDS FOR ANIMAL CELL CULTURES (Biosafety Level 1 or 2)

MATERIAL SAFETY DATA SHEET

SECTION 1 - SUBSTANCE IDENTITY AND COMPANY INFORMATION

Product Name: Various Animal Cell Cultures at Biosafety Level 1 or 2
ATCC Catalog #: Various

COMPANY INFORMATION: AMERICAN TYPE CULTURE COLLECTION
PO BOX 1549
MANASSAS, VA 20108

FOR INFORMATION CALL: 800-638-6597 or 703-365-2700
AFTER-HOURS CONTACT: 703-365-2710
CHEMTREC EMERGENCY: 800-424-9300 or 703-527-3887

SECTION 2 - COMPOSITION/INFORMATION ON INGREDIENTS

Either frozen or growing cells shipped in liquid cell culture medium (a mixture of components that may include, but is not limited to: inorganic salts, vitamins, amino acids, carbohydrates and other nutrients dissolved in water). Frozen Cultures may also contain a 5%-10% solution of Dimethyl sulfoxide as a cryoprotectant.

SECTION 3 - HAZARD IDENTIFICATION

HMIS Rating:	Health: 0	Flammability: 0	Reactivity:0
NFPA Rating:	Health:0	Flammability:0	Reactivity:0

This substance is not hazardous as defined by OSHA 29CFR 1910.1200 however this product should be handled according to good lab practices, with proper personal protective equipment, proper engineering controls and within the parameters of the purchaser's safety program.

Health Hazards

For Biosafety Level 1 Cell Cultures
Handle as a potentially biohazardous material under at least Biosafety Level 1 containment.
This cell line is not known to cause disease in healthy adult humans. These cells have **NOT** been screened for Hepatitis B, human immunodeficiency viruses or other adventitious agents, unless otherwise reported on the Certificate of Analysis. Regardless of results reported on the Certificate of Analysis Universal Precautions according to 29 CFR 1910.1030 should be followed at all times when manipulating these cell lines.

See next page for Biosafety Level 2 cell cultures.

For Biosafety Level 2 Cell Cultures
Handle as a potentially biohazardous material under at least Biosafety Level 2 containment.
These cell lines are associated with human disease, hazards include: percutaneous injury, ingestion, mucous membrane exposure (U.S. Government Publication **Biosafety in Microbiological and Biomedical Laboratories)**. These cells have **NOT** been screened for Hepatitis B, human immunodeficiency viruses or other adventitious agents, unless otherwise reported on the Certificate of Analysis. Regardless of results reported on the Certificate of Analysis Universal Precautions according to 29 CFR 1910.1030 should be followed at all times when manipulating these cell lines.

SECTION 4 - FIRST AID MEASURES

Report to your Safety Office and Seek Medical Attention as Soon as Possible

Ingestion: If person is unconscious seek emergency medical attention; never give anything by mouth to an unconscious person. If the person is conscious wash mouth out with copious amounts of water and call a physician then administer three cupfuls of water. Do not induce vomiting unless directed to do so by a physician.

Inhalation: If person is unconscious seek emergency medical attention, if person is conscious remove to fresh air and call a physician.

Dermal exposure: Immediately wash skin with copious amounts of water followed by washing with soap and copious amounts of water. Remove all contaminated clothing.

Eye exposures: Flush eyes with copious amounts of water for at least 15 minutes with eyelids separated and call a physician.

SECTION 5 - FIRE FIGHTING MEASURES

Flammability: Data not available

Suitable Extinguishing Media: Water spray, carbon dioxide, dry chemical powder, Halon (where regulations permit), or appropriate foam.

Protective Equipment: Wear self-contained breathing apparatus and protective clothing to prevent inhalation, ingestion, skin and eye contact.

Specific Hazard(s): Responders should take into consideration the biohazard risk associated with responding to a fire in the area where the material may be stored or handled.

SECTION 6 - ACCIDENTAL RELEASE MEASURES

Procedure(s) of Personal Precaution(s): At a minimum use PPE listed in Section 8. Wear laboratory coat, gloves and eye protection. Avoid all contact.

Methods for Cleaning Up

Patient/Victim: Wash with soap and water. Work clothes should be laundered separately. Launder contaminated clothing before re-use. Do not take clothing home.

Equipment/Environment: Allow aerosols to settle; wearing protective clothing, gently cover spill with paper towel and apply 1% sodium hypochlorite, starting at perimeter and working towards the center; allow sufficient contact time before clean up (30 min).

Note: The use of additional PPE may be necessary for cleaning solutions.

SECTION 7 - HANDLING AND STORAGE

Handle and store according to instructions on product information sheet and label.
Special Requirements:
Follow established laboratory procedures when handling material.

SECTION 8 - EXPOSURE CONTROLS/PERSONAL PROTECTION

Use Personal Protective Equipment: Including Eye Protection, Chemical Resistant Gloves, and appropriate clothing to prevent skin exposure. In addition, a Respiratory protection program that complies with OSHA 29 CFR 1910.134 and ANSI Z88.2 requirements or European Standard EN 149 must be followed whenever workplace conditions warrant respirator use.

Engineering Controls: The use and storage of this material requires user to maintain and make available appropriate eyewash and safety shower facilities. Use fume hood or other appropriate ventilation method to keep airborne concentrations a low as possible.

Exposure Limits: No exposure limits for this material have been established by ACGIH, NIOSH, or OSHA.

SECTION 9 - PHYSICAL AND CHEMICAL PROPERTIES

Data Not Available

SECTION 10 - STABILITY AND REACTIVITY

Hazardous polymerization will not occur.

SECTION 11 - TOXICOLOGICAL INFORMATION

Route of Exposure

Eye Contact: Data not available. Avoid eye contact.
Skin Contact: Data not available. Avoid skin contact.
Skin Absorption: Data not available. Avoid skin absorption.
Inhalation: Data not available. Avoid inhalation.
Ingestion: Data not available. Avoid ingestion.
Parenteral Exposure: Data not available. Avoid parenteral exposure.

Sensitization
Skin: Data not available
Respiratory: Data not available

Target Organ(s) or System(s): Data not available

Signs and Symptoms of Exposure
Skin and Mucous Membranes: Data not available
Respiratory: Data not available
Gastrointestinal: Data not available

Toxicity Data: Data not available
Effects of Long Term or Repeated Exposure: Data not available
Chronic Exposure–Teratogen: Data not available
Chronic Exposure–Mutagen: Data not available
Chronic Exposure–Reproductive Hazard: Data not available

SECTION 12 - ECOLOGICAL INFORMATION

No ecological information available.

SECTION 13 - DISPOSAL CONSIDERATIONS

Decontaminate all wastes before disposal (steam sterilization, chemical disinfection, and/or incineration).

Dispose of in accordance with applicable regulations.

SECTION 14 - TRANSPORT INFORMATION

Contact ATCC for transport information.

SECTION 15 - REGULATORY INFORMATION

Contact ATCC for regulatory information.

SECTION 16 - OTHER INFORMATION

THE INFORMATION PRESENTED IN THIS DOCUMENT IS BELIEVED TO BE CORRECT BASED UPON DATA AVAILABLE TO ATCC. USERS SHOULD MAKE AN INDEPENDENT DECISION REGARDING THE ACCURACY OF THIS INFORMATION BASED ON THEIR NEEDS AND DATA AVAILABLE TO THEM. ALL SUBSTANCES AND MIXTURES MAY PRESENT UNKNOWN HAZARDS AND ALL NECESSARY SAFETY PRECAUTIONS SHOULD BE TAKEN. ATCC ASSUMES NO LIABILITY RESULTING FROM USING OR COMING IN CONTACT WITH THIS SUBSTANCE.

SIGMA-ALDRICH

MATERIAL SAFETY DATA SHEET

Date Printed: 12/02/2009
Date Updated: 09/14/2009
Version 1.7

Section 1 - Product and Company Information

Product Name	CHORIONIC GONADOTROPIN FROM HUMAN &
Product Number	C0684
Brand	SIGMA
Company	Sigma-Aldrich Canada, Ltd
Address	2149 Winston Park Drive Oakville ON L6H 6J8 CA
Technical Phone:	9058299500
Fax:	9058299292
Emergency Phone:	800-424-9300

Section 2 - Composition/Information on Ingredient

Substance Name	CAS #	SARA 313
CHORIONIC GONADOTROPIN FROM HUMAN PREGNANCY URINE	9002-61-3	No

Chemical Family Human source material.

Synonyms Ambinon * Antuitrin S * APL * APL (hormone) * Apoidina * Chorigon * Choriogonadotropin * Choriogonin * Chorionic gonadotrophin * Chorionic gonadotropic hormone * Chorionic gonadotropin * Chorulon * Coriantin * Follutein * Gonabion * Gonadex * HCG * Human chorionic gonadotropin * Korotrin * Physex * Praedyn * Pregnyl * Primogonyl * Randonos * Synaphorin

RTECS Number: MD6953000

Section 3 - Hazards Identification

EMERGENCY OVERVIEW

Biohazard.
Handle as if capable of transmitting infectious agents.

HMIS RATING

HEALTH: 0
FLAMMABILITY: 0
REACTIVITY: 0
SPECIAL HAZARD(S): Human Source

NFPA RATING

HEALTH: 0
FLAMMABILITY: 0
REACTIVITY: 0
SPECIAL HAZARD(S): Human Source

For additional information on toxicity, please refer to Section 11.

Section 4 - First Aid Measures

ORAL EXPOSURE

If swallowed, wash out mouth with water provided person is conscious. Call a physician immediately.

INHALATION EXPOSURE
If inhaled, remove to fresh air. If not breathing give artificial respiration. If breathing is difficult, give oxygen.

DERMAL EXPOSURE
In case of skin contact, flush with copious amounts of water for at least 15 minutes. Remove contaminated clothing and shoes. Call a physician.

EYE EXPOSURE
In case of contact with eyes, flush with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating the eyelids with fingers. Call a physician.

Section 5 - Fire Fighting Measures

FLASH POINT
N/A

AUTOIGNITION TEMP
N/A

FLAMMABILITY
N/A

EXTINGUISHING MEDIA
Suitable: Use extinguishing media appropriate to surrounding fire conditions.

FIREFIGHTING
Protective Equipment: Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.
Specific Hazard(s): Emits toxic fumes under fire conditions.

Section 6 - Accidental Release Measures

PROCEDURE(S) OF PERSONAL PRECAUTION(S)
Wear self-contained breathing apparatus, rubber boots, and heavy rubber gloves.

METHODS FOR CLEANING UP
Sweep up, place in a bag and hold for waste disposal. Avoid raising dust. Wash spill site with 10% bleach and ventilate area after material pickup is complete.

Section 7 - Handling and Storage

HANDLING
User Exposure: Avoid inhalation. Avoid contact with eyes, skin, and clothing. Avoid prolonged or repeated exposure.

STORAGE
Suitable: Keep tightly closed.
Store at -20°C

Section 8 - Exposure Controls / PPE

ENGINEERING CONTROLS
Safety shower and eye bath. Mechanical exhaust required.

PERSONAL PROTECTIVE EQUIPMENT

Respiratory: Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU). Where risk assessment shows air-purifying respirators are appropriate use a dust mask type N95 (US) or type P1 (EN 143) respirator.
Hand: Compatible chemical-resistant gloves.
Eye: Chemical safety goggles.

GENERAL HYGIENE MEASURES

Wash thoroughly after handling.

Section 9 - Physical/Chemical Properties

Appearance — Physical State: Solid

Property	Value	At Temperature or Pressure
pH	N/A	
BP/BP Range	N/A	
MP/MP Range	N/A	
Freezing Point	N/A	
Vapor Pressure	N/A	
Vapor Density	N/A	
Saturated Vapor Conc.	N/A	
Bulk Density	N/A	
Odor Threshold	N/A	
Volatile%	N/A	
VOC Content	N/A	
Water Content	N/A	
Solvent Content	N/A	
Evaporation Rate	N/A	
Viscosity	N/A	
Surface Tension	N/A	
Partition Coefficient	N/A	
Decomposition Temp.	N/A	
Flash Point	N/A	
Explosion Limits	N/A	
Flammability	N/A	
Autoignition Temp	N/A	
Refractive Index	N/A	
Optical Rotation	N/A	
Miscellaneous Data	N/A	
Solubility	N/A	

N/A = not available

Section 10 - Stability and Reactivity

STABILITY

Stable: Stable.
Materials to Avoid: Strong oxidizing agents.

HAZARDOUS DECOMPOSITION PRODUCTS

Hazardous Decomposition Products: Carbon monoxide, carbon dioxide, and nitrogen oxides.

HAZARDOUS POLYMERIZATION

Hazardous Polymerization: Will not occur

Section 11 - Toxicological Information

ROUTE OF EXPOSURE
Skin Contact: May cause skin irritation.
Skin Absorption: May be harmful if absorbed through the skin.
Eye Contact: May cause eye irritation.
Inhalation: Material may be irritating to mucous membranes and upper respiratory tract. May be harmful if inhaled.
Ingestion: May be harmful if swallowed.

SIGNS AND SYMPTOMS OF EXPOSURE
Potentially biohazardous material.

CHRONIC EXPOSURE - TERATOGEN

Species: Rat
Dose: 875 MG/KG
Route of Application: Subcutaneous
Exposure Time: (15-17D PREG)
Result: Effects on Embryo or Fetus: Extra embryonic structures (e.g., placenta, umbilical cord).

Species: Hamster
Dose: 60 MG/KG
Route of Application: Intraperitoneal
Exposure Time: (4-6D PREG)
Result: Effects on Embryo or Fetus: Fetal death.

CHRONIC EXPOSURE - REPRODUCTIVE HAZARD
Result: Overexposure may cause reproductive disorder(s) based on tests with laboratory animals.

Species: Woman
Dose: 36 MG/KG
Route of Application: Unreported
Exposure Time: (6D PRE)
Result: Effects on Fertility: Other measures of fertility

Species: Rat
Dose: 150 MG/KG
Route of Application: Intraperitoneal
Exposure Time: (1-3D PREG)
Result: Effects on Fertility: Other measures of fertility
Effects on Fertility: Pre-implantation mortality (e.g., reduction in number of implants per female; total number of implants per corpora lutea).

Species: Rat
Dose: 40 MG/KG
Route of Application: Intraperitoneal
Exposure Time: (10D MALE)
Result: Paternal Effects: Spermatogenesis (including genetic material, sperm morphology,motility, and count).

Species: Rat
Dose: 1250 UG/KG
Route of Application: Subcutaneous
Exposure Time: (1D PRE)
Result: Maternal Effects: Ovaries, fallopian tubes. Maternal Effects: Uterus, cervix, vagina.

Species: Rat
Dose: 5250 UG/KG

Route of Application: Subcutaneous
Exposure Time: (7-13D PREG)
Result: Effects on Fertility: Post-implantation mortality (e.g., dead and/or resorbed implants per total number of implants).

Species: Rat
Dose: 16 MG/KG
Route of Application: Subcutaneous
Exposure Time: (4D MALE)
Result: Paternal Effects: Other effects on male.

Species: Rat
Dose: 8890 UG/KG
Route of Application: Intravenous
Exposure Time: (1D PRE)
Result: Effects on Fertility: Pre-implantation mortality (e.g., reduction in number of implants per female; total number of implants per corpora lutea).

Species: Rat
Dose: 83 UG/KG
Route of Application: Intramuscular
Exposure Time: (9D PREG)
Result: Effects on Fertility: Post-implantation mortality (e.g., dead and/or resorbed implants per total number of implants).

Species: Rat
Dose: 125 MG/KG
Route of Application: Parenteral
Exposure Time: (4D PRE)
Result: Maternal Effects: Ovaries, fallopian tubes. Maternal Effects: Uterus, cervix, vagina.

Species: Rat
Dose: 420 MG/KG
Route of Application: Parenteral
Exposure Time: (7D MALE)
Result: Paternal Effects: Testes, epididymis, sperm duct.

Species: Rat
Dose: 200 MG/KG
Route of Application: Parenteral
Exposure Time: (1D MALE)
Result: Paternal Effects: Other effects on male.

Species: Rat
Dose: 1650 MG/KG
Route of Application: Unreported
Exposure Time: (1-22D PREG)
Result: Effects on Newborn: Growth statistics (e.g., reduced weight gain).

Species: Rat
Dose: 50 MG/KG
Route of Application: Unreported
Exposure Time: (4D PREG)
Result: Maternal Effects: Parturition.

Species: Rat
Dose: 21212 NG/KG
Route of Application: Unreported
Exposure Time: (7-13D PREG)

Result: Effects on Fertility: Post-implantation mortality (e.g., dead and/or resorbed implants per total number of implants).

Species: Rat
Dose: 12 MG/KG
Route of Application: Unreported
Exposure Time: (1D MALE)
Result: Paternal Effects: Other effects on male.

Species: Rat
Dose: 280 IU/KG
Route of Application: Unreported
Exposure Time: (2W MALE)
Result: Paternal Effects: Testes, epididymis, sperm duct.

Species: Mouse
Dose: 24 MG/KG
Route of Application: Intraperitoneal
Exposure Time: (6D PRE)
Result: Effects on Fertility: Pre-implantation mortality (e.g., reduction in number of implants per female; total number of implants per corpora lutea).

Species: Mouse
Dose: 400 IU/KG
Route of Application: Intraperitoneal
Exposure Time: (1D PRE)
Result: Maternal Effects: Oogenesis.

Species: Mouse
Dose: 560 MG/KG
Route of Application: Subcutaneous
Exposure Time: (7-13D PREG)
Result: Effects on Fertility: Post-implantation mortality (e.g., dead and/or resorbed implants per total number of implants).

Species: Mouse
Dose: 6 MG/KG
Route of Application: Intramuscular
Exposure Time: (3D PRE)
Result: Maternal Effects: Uterus, cervix, vagina.

Species: Monkey
Dose: 10 MG/KG
Route of Application: Subcutaneous
Exposure Time: (5D PRE)
Result: Maternal Effects: Menstrual cycle changes or disorders.

Species: Monkey
Dose: 224 MG/KG
Route of Application: Intramuscular
Exposure Time: (18-36D PREG)
Result: Maternal Effects: Menstrual cycle changes or disorders.

Species: Monkey
Dose: 30 MG/KG
Route of Application: Unreported
Exposure Time: (15D PRE)
Result: Maternal Effects: Parturition.

Species: Pig
Dose: 833 UG/KG

Route of Application: Intravenous
Exposure Time: (1D PRE)
Result: Effects on Fertility: Other measures of fertility

Species: Pig
Dose: 833 UG/KG
Route of Application: Intramuscular
Exposure Time: (1D PRE)
Result: Effects on Fertility: Other measures of fertility

Species: Hamster
Dose: 240 MG/KG
Route of Application: Intraperitoneal
Exposure Time: (1-3D PREG)
Result: Effects on Fertility: Pre-implantation mortality (e.g., reduction in number of implants per female; total number of implants per corpora lutea).

Species: Hamster
Dose: 280 MG/KG
Route of Application: Subcutaneous
Exposure Time: (7-13D PREG)
Result: Effects on Fertility: Post-implantation mortality (e.g., dead and/or resorbed implants per total number of implants).

Section 12 - Ecological Information

No data available.

Section 13 - Disposal Considerations

APPROPRIATE METHOD OF DISPOSAL OF SUBSTANCE OR PREPARATION
Contact a licensed professional waste disposal service to dispose of this material. Disposal should be made in accordance with existing disposal practices employed for infectious waste at your institution. Observe all federal, state, and local environmental regulations.

Section 14 - Transport Information

DOT
Proper Shipping Name: None
Non-Hazardous for Transport: This substance is considered to be non-hazardous for transport.

IATA
Non-Hazardous for Air Transport: Non-hazardous for air transport.

Section 15 - Regulatory Information

EU ADDITIONAL CLASSIFICATION
Symbol of Danger: B
Indication of Danger: Biohazard.

US CLASSIFICATION AND LABEL TEXT
Indication of Danger: Biohazard.
US Statements: Handle as if capable of transmitting infectious agents.

UNITED STATES REGULATORY INFORMATION
SARA LISTED: No

CANADA REGULATORY INFORMATION

WHMIS Classification: This product has been classified in accordance with the hazard criteria of the CPR, and the MSDS contains all the information required by the CPR.

DSL: No

NDSL: No

Section 16 - Other Information

DISCLAIMER

For R&D use only. Not for drug, household or other uses.

WARRANTY

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 Inc., 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.

SIGMA-ALDRICH

MATERIAL SAFETY DATA SHEET

Date Printed: 04/13/2007
Date Updated: 02/01/2006
Version 1.4

Section 1 - Product and Company Information

Product Name	GONADOTROPIN FROM PREGNANT MARES' SERUM
Product Number	G4877
Brand	SIGMA
Company	Sigma-Aldrich Canada, Ltd
Address	2149 Winston Park Drive Oakville ON L6H 6J8 CA
Technical Phone:	9058299500
Fax:	9058299292
Emergency Phone:	800-424-9300

Section 2 - Composition/Information on Ingredient

Substance Name	CAS #	SARA 313
GONADOTROPIN FROM PREGNANT MARE SERUM	9002-70-4	No

Synonyms: Anteron * Antex-490 * Antostab * Eleagol * Equine cyonin * Equine gonadotrophin * Equine gonadotropin * Gestyl * Gonadotraphon FSH * Gonadyl * Gorman * Lobulantina * PMS * PMSG * Predalon-S * Priatin * Primantron * Seragon * Seragonin * Serogan * Serotropin * Serum gonadotrophin * Serum gonadotropic hormone * Serum gonadotropin

RTECS Number: TU4517000

Section 3 - Hazards Identification

EMERGENCY OVERVIEW

Toxic.
Toxic if swallowed.
Target organ(s): Reproductive system.

HMIS RATING

HEALTH: 2*
FLAMMABILITY: 0
REACTIVITY: 0

NFPA RATING

HEALTH: 2
FLAMMABILITY: 0
REACTIVITY: 0

*additional chronic hazards present.

For additional information on toxicity, please refer to Section 11.

Section 4 - First Aid Measures

ORAL EXPOSURE

If swallowed, wash out mouth with water provided person is conscious. Call a physician immediately.

INHALATION EXPOSURE
If inhaled, remove to fresh air. If not breathing give artificial respiration. If breathing is difficult, give oxygen.

DERMAL EXPOSURE
In case of skin contact, flush with copious amounts of water for at least 15 minutes. Remove contaminated clothing and shoes. Call a physician.

EYE EXPOSURE
In case of contact with eyes, flush with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating the eyelids with fingers. Call a physician.

Section 5 - Fire Fighting Measures

FLASH POINT
N/A

AUTOIGNITION TEMP
N/A

FLAMMABILITY
N/A

EXTINGUISHING MEDIA
Suitable: Water spray. Carbon dioxide, dry chemical powder, or appropriate foam.

FIREFIGHTING
Protective Equipment: Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.
Specific Hazard(s): Emits toxic fumes under fire conditions.

Section 6 - Accidental Release Measures

PROCEDURE TO BE FOLLOWED IN CASE OF LEAK OR SPILL
Evacuate area.

PROCEDURE(S) OF PERSONAL PRECAUTION(S)
Wear self-contained breathing apparatus, rubber boots, and heavy rubber gloves.

METHODS FOR CLEANING UP
Sweep up, place in a bag and hold for waste disposal. Avoid raising dust. Ventilate area and wash spill site after material pickup is complete.

Section 7 - Handling and Storage

HANDLING
User Exposure: Do not breathe dust. Do not get in eyes, on skin, on clothing. Avoid prolonged or repeated exposure.

STORAGE
Suitable: Keep tightly closed.
Store at -20°C

Section 8 - Exposure Controls / PPE

ENGINEERING CONTROLS
Use only in a chemical fume hood. Safety shower and eye bath.

PERSONAL PROTECTIVE EQUIPMENT
Respiratory: Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU). Where risk assessment shows air-purifying respirators are appropriate use a full-face particle respirator type N99 (US) or type P2 (EN 143) respirator cartridges as a backup to engineering controls. If the respirator is the sole means of protection, use a full-face supplied air respirator.
Hand: Compatible chemical-resistant gloves.
Eye: Chemical safety goggles.

GENERAL HYGIENE MEASURES
Wash contaminated clothing before reuse. Wash thoroughly after handling.

Section 9 - Physical/Chemical Properties

Appearance	Color: White Form: Powder	
Property	Value	At Temperature or Pressure
pH	N/A	
BP/BP Range	N/A	
MP/MP Range	N/A	
Freezing Point	N/A	
Vapor Pressure	N/A	
Vapor Density	N/A	
Saturated Vapor Conc.	N/A	
SG/Density	N/A	
Bulk Density	N/A	
Odor Threshold	N/A	
Volatile%	N/A	
VOC Content	N/A	
Water Content	N/A	
Solvent Content	N/A	
Evaporation Rate	N/A	
Viscosity	N/A	
Surface Tension	N/A	
Partition Coefficient	N/A	
Decomposition Temp.	N/A	
Flash Point	N/A	
Explosion Limits	N/A	
Flammability	N/A	
Autoignition Temp	N/A	
Refractive Index	N/A	
Optical Rotation	N/A	
Miscellaneous Data	N/A	
Solubility	Solubility in Water:1 mg/ml H2O clear, very faintly yellow	

N/A = not available

Section 10 - Stability and Reactivity

STABILITY
Stable: Stable.
Materials to Avoid: Strong oxidizing agents.

HAZARDOUS DECOMPOSITION PRODUCTS
Hazardous Decomposition Products: Carbon monoxide, Carbon dioxide.

HAZARDOUS POLYMERIZATION
Hazardous Polymerization: Will not occur

Section 11 - Toxicological Information

ROUTE OF EXPOSURE
Skin Contact: May cause skin irritation.
Skin Absorption: Toxic if absorbed through skin.
Eye Contact: May cause eye irritation.
Inhalation: Material may be irritating to mucous membranes and upper respiratory tract. Toxic if inhaled.
Ingestion: Toxic if swallowed.

TARGET ORGAN(S) OR SYSTEM(S)
Reproductive system.

SIGNS AND SYMPTOMS OF EXPOSURE
Exposure can cause sensitivity reactions, abdominal discomfort, ovarian rupture, and intraperitoneal hemorrhage.

TOXICITY DATA

Oral
Mouse
120 mg/kg
LD50

CHRONIC EXPOSURE - TERATOGEN

Species: Rat Rat
Dose: 11700 UG/KG 11700 UG/KG
Route of Application: Intraperitoneal Intraperitoneal
Exposure Time: (5-7D PREG) (5-7D PREG)
Result: Effects on Embryo or Fetus: Fetal death. Effects on Embryo or Fetus: Fetal death.

Species: Rat Rat
Dose: 10 MG/KG 10 MG/KG
Route of Application: Subcutaneous Subcutaneous
Exposure Time: (1D PRE) (1D PRE)
Result: Effects on Embryo or Fetus: Fetotoxicity (except death, e.g., stunted fetus). Effects on Embryo or Fetus: Extra embryonic structures (e.g., placenta, umbilical cord). Effects on Embryo or Fetus: Extra embryonic structures (e.g., placenta, umbilical cord). Effects on Embryo or Fetus: Fetotoxicity (except death, e.g., stunted fetus).

Species: Mouse Mouse
Dose: 1 GM/KG 1 GM/KG
Route of Application: Subcutaneous Subcutaneous
Exposure Time: (1D PRE) (1D PRE)
Result: Specific Developmental Abnormalities: Craniofacial (including nose and tongue). Effects on Embryo or Fetus: Fetal death. Specific Developmental Abnormalities: Musculoskeletal system. Effects on Embryo or Fetus: Fetal death. Specific Developmental Abnormalities: Craniofacial (including nose and tongue). Specific Developmental Abnormalities: Musculoskeletal system.

Species: Hamster Hamster
Dose: 37500 UG/KG 37500 UG/KG
Route of Application: Intraperitoneal Intraperitoneal
Exposure Time: (4-6D PREG) (4-6D PREG)
Result: Effects on Embryo or Fetus: Fetal death. Effects on Embryo or Fetus: Fetal death.

CHRONIC EXPOSURE - MUTAGEN

Species: Mouse Mouse
Route: Intraperitoneal Intraperitoneal
Dose: 750 UG/KG 750 UG/KG
Mutation test: Cytogenetic analysis Cytogenetic analysis

CHRONIC EXPOSURE - REPRODUCTIVE HAZARD
Result: Overexposure may cause reproductive disorder(s) based on tests with laboratory animals.

Species: Rat Rat
Dose: 188 MG/KG 188 MG/KG
Route of Application: Intraperitoneal Intraperitoneal
Exposure Time: (1-3D PREG) (1-3D PREG)
Result: Effects on Fertility: Other measures of fertility Effects on Fertility: Other measures of fertility

Species: Rat Rat
Dose: 188 MG/KG 188 MG/KG
Route of Application: Intraperitoneal Intraperitoneal
Exposure Time: (5-7D PREG) (5-7D PREG)
Result: Effects on Fertility: Pre-implantation mortality (e.g., reduction in number of implants per female; total number of implants per corpora lutea). Effects on Fertility: Pre-implantation mortality (e.g., reduction in number of implants per female; total number of implants per corpora lutea).

Species: Rat Rat
Dose: 18750 UG/KG 18750 UG/KG
Route of Application: Subcutaneous Subcutaneous
Exposure Time: (1D PRE) (1D PRE)
Result: Maternal Effects: Parturition. Maternal Effects: Parturition.

Species: Rat Rat
Dose: 37500 UG/KG 37500 UG/KG
Route of Application: Subcutaneous Subcutaneous
Exposure Time: (18D PREG) (18D PREG)
Result: Effects on Fertility: Abortion. Effects on Fertility: Abortion.

Species: Rat Rat
Dose: 12500 UG/KG 12500 UG/KG
Route of Application: Subcutaneous Subcutaneous
Exposure Time: (5D PREG) (5D PREG)
Result: Effects on Fertility: Other measures of fertility Effects on Fertility: Post-implantation mortality (e.g., dead and/or resorbed implants per total number of implants). Effects on Fertility: Post-implantation mortality (e.g., dead and/or resorbed implants per total number of implants). Effects on Fertility: Other measures of fertility

Species: Rat Rat

Dose: 50 MG/KG 50 MG/KG
Route of Application: Subcutaneous Subcutaneous
Exposure Time: (1D PRE) (1D PRE)
Result: Maternal Effects: Ovaries, fallopian tubes. Maternal Effects: Uterus, cervix, vagina. Maternal Effects: Uterus, cervix, vagina. Maternal Effects: Ovaries, fallopian tubes.

Species: Rat Rat
Dose: 12500 UG/KG 12500 UG/KG
Route of Application: Subcutaneous Subcutaneous
Exposure Time: (2D PREG) (2D PREG)
Result: Effects on Fertility: Female fertility index (e.g., # females pregnant per # sperm positive females; # females pregnant per # females mated). Effects on Fertility: Female fertility index (e.g., # females pregnant per # sperm positive females; # females pregnant per # females mated).

Species: Rat Rat
Dose: 453 MG/KG 453 MG/KG
Route of Application: Intramuscular Intramuscular
Exposure Time: (7D PRE) (7D PRE)
Result: Maternal Effects: Uterus, cervix, vagina. Maternal Effects: Ovaries, fallopian tubes. Maternal Effects: Uterus, cervix, vagina. Maternal Effects: Ovaries, fallopian tubes.

Species: Rat Rat
Dose: 1120 IU/KG 1120 IU/KG
Route of Application: Unreported Unreported
Exposure Time: (2W MALE) (2W MALE)
Result: Paternal Effects: Testes, epididymis, sperm duct. Paternal Effects: Testes, epididymis, sperm duct.

Species: Mouse Mouse
Dose: 10 MG/KG 10 MG/KG
Route of Application: Subcutaneous Subcutaneous
Exposure Time: (2D PREG) (2D PREG)
Result: Effects on Fertility: Female fertility index (e.g., # females pregnant per # sperm positive females; # females pregnant per # females mated). Effects on Fertility: Female fertility index (e.g., # females pregnant per # sperm positive females; # females pregnant per # females mated).

Species: Mouse Mouse
Dose: 50 MG/KG 50 MG/KG
Route of Application: Subcutaneous Subcutaneous
Exposure Time: (8-11D PREG) (8-11D PREG)
Result: Effects on Fertility: Post-implantation mortality (e.g., dead and/or resorbed implants per total number of implants). Effects on Fertility: Abortion. Effects on Fertility: Post-implantation mortality (e.g., dead and/or resorbed implants per total number of implants). Effects on Fertility: Abortion.

Species: Mouse Mouse
Dose: 50 MG/KG 50 MG/KG
Route of Application: Subcutaneous Subcutaneous
Exposure Time: (12-15D PREG) (12-15D PREG)
Result: Maternal Effects: Parturition. Maternal Effects: Postpartum. Maternal Effects: Postpartum. Maternal Effects: Parturition.

Species: Mouse Mouse
Dose: 50 MG/KG 50 MG/KG

Route of Application: Subcutaneous Subcutaneous
Exposure Time: (3D PREG) (3D PREG)
Result: Effects on Fertility: Pre-implantation mortality (e.g., reduction in number of implants per female; total number of implants per corpora lutea). Effects on Fertility: Pre-implantation mortality (e.g., reduction in number of implants per female; total number of implants per corpora lutea).

Species: Hamster Hamster
Dose: 300 MG/KG 300 MG/KG
Route of Application: Intraperitoneal Intraperitoneal
Exposure Time: (1-3D PREG) (1-3D PREG)
Result: Effects on Fertility: Pre-implantation mortality (e.g., reduction in number of implants per female; total number of implants per corpora lutea). Effects on Fertility: Pre-implantation mortality (e.g., reduction in number of implants per female; total number of implants per corpora lutea).

Section 12 - Ecological Information

Section 13 - Disposal Considerations

APPROPRIATE METHOD OF DISPOSAL OF SUBSTANCE OR PREPARATION
Contact a licensed professional waste disposal service to dispose of this material. Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber. Observe all federal, state, and local environmental regulations.

Section 14 - Transport Information

DOT
Proper Shipping Name: Toxic solids, organic, n.o.s.
UN#: 2811
Class: 6.1
Packing Group: Packing Group III
Hazard Label: Toxic substances.
PIH: Not PIH

IATA
Proper Shipping Name: Toxic solid, organic, n.o.s.
IATA UN Number: 2811
Hazard Class: 6.1
Packing Group: III

Section 15 - Regulatory Information

EU ADDITIONAL CLASSIFICATION
Symbol of Danger: T
Indication of Danger: Toxic.
R: 25
Risk Statements: Toxic if swallowed.
S: 45
Safety Statements: In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

US CLASSIFICATION AND LABEL TEXT
Indication of Danger: Toxic.
Risk Statements: Toxic if swallowed.
Safety Statements: In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

US Statements: Target organ(s): Reproductive system.

UNITED STATES REGULATORY INFORMATION
SARA LISTED: No

CANADA REGULATORY INFORMATION
WHMIS Classification: This product has been classified in accordance with the hazard criteria of the CPR, and the MSDS contains all the information required by the CPR.
DSL: No
NDSL: No

Section 16 - Other Information

DISCLAIMER
For R&D use only. Not for drug, household or other uses.

WARRANTY
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 Inc., 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:	hCG
Proposed Use Dose:	1000 IU
Proposed Storage Dose:	500 IU/mL
LD $_{50}$ (species):	1800000 µg/kg

Calculation:

µg/kg x 50 kg/person

Dose per person based on LD $_{50}$ in µg = 0

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = ***0***

Comments/Recommendations: LD50 for chronic exposure not toxic dose.

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	eCG
Proposed Use Dose:	1000 IU
Proposed Storage Dose:	500 IU/mL
LD $_{50}$ (species):	N/A

Calculation:

µg/kg x 50 kg/person

Dose per person based on LD $_{50}$ in µg = 0

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = ***0***

Comments/Recommendations: No LD50 data found.

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	hCG
Proposed Use Dose:	20 IU/mL
Proposed Storage Dose:	10000 IU
LD_{50} (species):	µg

Calculation:

0 µg/kg x 50 kg/person

Dose per person based on LD_{50} in µg = 0

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = ***0***

Comments/Recommendations:

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	eCG
Proposed Use Dose:	20 IU/mL
Proposed Storage Dose:	10000 IU
LD_{50} (species):	µg

Calculation:

0 µg/kg x 50 kg/person

Dose per person based on LD_{50} in µg = 0

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = ***0***

Comments/Recommendations: