THE UNIVERSITY OF WESTERN ONTARIO
BIOLOGICAL AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: October 14, 2010
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).

Completed forms are to be returned to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190) 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

PRINCIPAL INVESTIGATOR	S. Jeffrey Dixon
DEPARTMENT	Physiology and Pharmacology
ADDRESS	Dental Sciences Building, Rm 0075
PHONE NUMBER	83769
EMERGENCY PHONE NUMBER(S)	519-472-3690 (home) 519-777-6919 (cell)
EMAIL	jeff.dixon@schulich.uwo.ca

Location of experimental work to be carried out: Building(s): Dental Sciences_ Room(s): 0078, 0078a____

*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_____
GRANT TITLE(S): Ion transport and signaling in skeletal cells: P2 nucleotide receptor function in bone _
_____

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

Name	UWO E-mail Address	Date of Biosafety Training
Elizabeth Pruski	Elizabeth.Pruski@schulich.uwo.ca	2006/06/22
Karen Ann Bridge	KarenAnn.Bridge@schulich.uwo.ca	2009/01/20
Matthew Grol	Matthew.Grol@schulich.uwo.ca	2006/09/25
Kim Beaucage	Kim.Beaucage@schulich.uwo.ca	2008/10/7
Tom Chrones	tchrones@uwo.ca	2006/07/14
Ben Wheal	bwheal@uwo.ca	2009/09/16
Alexey Pereverzev	alexey.pereverzev@schulich.uwo.ca	2006/07/13

Please explain the biological agents and/or biohazardous substances used and how they will be stored, used and disposed of. Projects without this description will not be reviewed.				
Biohazard/Biological Agent		**How is it Stored?**	**How is it Used?**	**How is it Disposed of?**
Microorganisms	Escherichia Coli	In freezer (-80) for long term storage or in Incubator when in use. This applies to agents listed below	Kept in culture for carrying out molecular biology	Bleached. Materials that come in contact are autoclaved
	Recombinant Adenovirus - Non replicating		Cells are infected with modified adenovirus for expression of selected proteins	
The following molecular reagents are used for expression of various markers and reporters in cultured cells				
Vectors/ Plasmids	pcDNA3			
	pEGFP			
	PEYFP			
	pAD/CMV/V5-DEST			
	pCEP4YPet-MAMM YPET			
	pGL4.74[hRluc/TK]			
	pGL3BARL			
	pGL3fuBARL			
	pGL3-Control			
	pRL-SV40			
	pCORON1000-EGFP-NFATc1			
	pGL3-NFAT luciferase			
Cells	Human (established): HEK 293	All cell lines are maintained in liquid nitrogen, with aliquots thawed for expansion in culture		Bleached. Materials that come in contact are autoclaved
	Rodent (established): RAW 264.7, MC3T3-E1, UMR-106, ROS17/2.8			
	CHO			
	Non-human primate (established): COS			
	Rodent (primary)	Primary cells are prepared freshly and may be maintained in short-term culture		
Toxins	Pertussis Toxin	Freezer	Is prepared as stock solution then added to cell culture dishes for short-term treatment for blocking cell signaling. Pertussis toxin catalyzes the ADP-ribosylation of the αi	Is used in extremely low volumes and concentrations. The remaining solutions are diluted and are then bleached.

			subunits of heterotrimeric G proteins. This prevents the G proteins from interacting with G protein-coupled receptors on the cell membrane, thus interfering with intracellular communication. Pertussis toxin is widely used as a biochemical tool to ADP-ribosylate GTP-binding proteins in the study of signal transduction.	

Please include a one page research summary or teaching protocol.

Ion transport and signaling in skeletal cells: P2 nucleotide receptor function in bone

Background: Studies by us and others have shown that extracellular nucleotides (such as ATP) interact with mammalian and human bone cells through multiple subtypes of P2 cell-surface nucleotide receptors. P2X receptor family members are ligand-gated cation channels; whereas, P2Y family members are G protein-coupled receptors. Since nucleotides are released from cells in response to mechanical stimuli and trauma, they may serve as autocrine/paracrine regulators during mechanotransduction and wound healing in bone. In collaboration with Pfizer, we reported a unique skeletal phenotype in the P2X7 receptor knockout mouse – diminished periosteal bone formation and excessive trabecular bone resorption. Our mechanistic studies revealed that P2X7 receptors enhance osteoblast differentiation in a cell-autonomous manner, in part through production of the potent lipid mediator lysophosphatidic acid. Interestingly, prevalent loss-of-function polymorphisms in the human P2X7 receptor gene (P2RX7) have recently been associated with accelerated bone loss and increased fracture risk in postmenopausal women. However, critical questions remain regarding P2X7 signaling and function in bone. Our overall hypothesis is that P2X7 receptors regulate skeletal remodeling through direct effects on osteoblasts and osteoclasts, and indirectly through a novel mechanism of intercellular signaling in bone.

The following **specific hypotheses** will be tested:

1) Stimulation of osteogenesis by P2X7 requires activation of the transcription factor NFATc1 in osteoblasts.

2) Nucleotides, acting through P2X7 receptors on osteoblasts, induce the rapid release of membrane vesicles that contain bioactive molecules capable of regulating osteoblast and osteoclast activity.

3) Activation of P2X7 receptors on osteoclasts suppresses bone resorption via disruption of the actin cytoskeleton and induction of apoptosis.

4) Skeletal healing and mechanotransduction are impaired in mice lacking the P2X7 receptor.

Research plan: We will use calvarial cells and osteoclasts from neonatal rats, P2rx7-/- and wild-type mice. P2X7 receptor activity and function will be assessed by patch clamp and calcium fluorescence. Nuclear translocation of NFATc1 in response to P2X7 agonists will be monitored in real-time by imaging of osteoblasts expressing NFAT-EGFP fusion proteins, permitting unique insights into signaling. Effects of NFATc1 loss-of-function will be assessed by quantifying expression of marker genes using real-time PCR, and osteogenesis using the bone nodule formation assay. A novel mechanism for intercellular communication in bone – P2X7-induced vesicle shedding from osteoblasts – will be characterized by time-lapse, confocal and electron microscopy, and electrophysiology. Released bioactive compounds will be identified by mass spectrometry and immunodetection, and their roles assessed using complementary in vitro and in vivo assays. The effects of nucleotides on osteoclast motility and cytoskeletal dynamics will be studied using live-cell imaging of cells expressing actin-EGFP. Survival and apoptosis will be monitored using light and fluorescence microscopy, and confirmed by TUNEL. Resorptive activity will be quantified using pit formation assays. Skeletal healing and mechanotransduction will be examined in wild-type and P2rx7-/- mice.

Significance: Nucleotides may regulate bone remodeling by stimulating P2X7 on osteoblasts and osteoclasts during mechanotransduction and early stages of wound healing. Elucidation of downstream signaling pathways and functions of P2X7 will add to our understanding of osteopenia and impaired skeletal healing in patients with common loss-of-function P2RX7 polymorphisms. P2X7 receptors represent an attractive therapeutic target for inhibiting osteoclast activity while simultaneously promoting bone formation by osteoblasts.

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? X YES O 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? O YES X 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:

_____
_____
_____

Please attach the CFIA permit.
Please describe any CFIA permit conditions:

_____
_____
_____

1.2 Please complete the table below:

Name of Biological agent(s)*	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
Escherichia Coli (TOP 10 competent cells-Invitrogen)	x Yes O No	x Yes O No	x Yes O No	1.5		x 1 O 2 O 2+ O 3
Recombinant Adenovirus - Non replicating	O Yes x No	O Yes x No	O Yes x No	0.15	Invitrogen	O 1 x 2 O 2+ O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 2+ O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 2+ O 3

*Please attach a Material Safety Data Sheet or equivalent from the supplier.

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? x YES O 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	O Yes x No		Not applicable
Rodent	x Yes O No	Rats, Mice, Rabbit	2008-043-06
Non-human primate	O Yes x No		
Other (specify)	O Yes x No		

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)*	Supplier / Source
Human	x Yes O No	HEK 293	Invitrogen, ATCC
Rodent	x Yes O No	RAW 264.7, CHO, MC3T3-E1, UMR-106, ROS17/2.8	ATCC
Non-human primate	x Yes O No	COS-1	ATCC
Other (specify)	O Yes x No		

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

2.3 For above named cell types(s) indicate PHAC or CFIA containment level required O 1 x 2 O 2+ O 3

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? O YES x 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/NO**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid		O Yes O Unknown		O 1 O 2 O 2+ O 3
Human Blood (fraction) or other Body Fluid		O Yes O Unknown		O 1 O 2 O 2+ O 3
Human Organs or Tissues (unpreserved)		O Yes O Unknown		O 1 O 2 O 2+ O 3
Human Organs or Tissues (preserved)		Not Applicable		Not Applicable

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? x YES O NO If no, please proceed to Section 5.0

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

Bacteria Used for Cloning *	Plasmid(s) **	Source of Plasmid	Gene Transfected	Describe the change that results from transformation or transfection
Escherichia Coli (TOP 10 competent cells-Invitrogen)	pcDNA3 pEGFP PEYFP pCEP4YPet-MAMM YPET pGL4.74[hRluc/TK] pGL3BARL	Invitrogen Invitrogen Invitrogen addgene Promega Dr. R. T. Moon (Univ Washington)	Backbone expression vector eGFP eYFP (GFP variant) YPet Renilla luciferase Beta-catenin reporter (firefly	These plasmids are used to express fluorescently labeled proteins or luciferase reporter constructs in the transfected cells. Cells are appropriately destroyed after every experiment.

			luciferase)	
	pGL3fuBARL	Dr. R. T. Moon (Univ Washington)	Negative control for beta-catenin reporter(firefly luciferase)	
	pGL3-Control	Promega	Vector control for firefly luciferase	
	pRL-SV40	Promega	Renilla luciferase	
	pCORON1000-EGFP-NFATc1	GE Healthcare	EGFP-NFATc1 fusion	
	pGL3-NFAT luciferase	addgene	NFAT reporter(firefly luciferase)	

* Please attach a Material Data Sheet or equivalent if available.
** Please attach a plasmid map.

4.3 Will genetic modification(s) involving viral vectors be made? x YES, complete table below O NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results from transduction
human adenovirus type 5 (Ad5)	pAd/CMV/V5-DEST	Invitrogen, Cat# V493-20	human beta actin-EGFP fusion; EGFP	Transient expression of human beta actin-EGFP fusion and EGFP proteins that can be visualized by fluorescence microscopy. Cells are appropriately destroyed after every experiment.

* Please attach a Material Safety Data Sheet or equivalent.

4.4 Will genetic sequences from the following be involved?

- HIV O YES, please specify _____ x NO
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens O YES, specify _____ x NO
- SV 40 Large T antigen x YES O NO
- E1A oncogene x YES O NO
- Known oncogenes O YES, please specify _____ x NO
- Other human or animal pathogen and or their toxins O YES, please specify _____ x NO

4.5 Will virus be replication defective? x YES O NO

4.6 Will virus be infectious to humans or animals? O YES x NO

4.7 Will this be expected to increase the containment level required? O YES x NO

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials be conducted involving a biological agent? O YES x NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 6.0

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

5.2 Will the biological agent be able to replicate in the host? O YES O NO

5.3 How will the biological agent be administered? _____

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

5.5 Has human ethics approval been obtained? O YES, number: _____ O NO O PENDING

6.0 Animal Experiments

6.1 Will live animals be used? O YES x NO If no, please proceed to section 7.0

6.2 Name of animal species to be used_____

6.3 AUS protocol # _____

6.4 Will any of the agents listed in section 4.0 be used in live animals O YES, specify: _____ O NO

6.5 Will the agent(s) be shed by the animal: O YES O NO, please justify:

_____

_____

7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any animals with zoonotic hazards or their organs, tissues, lavages or other body fluids including blood be used (see list below)? O YES x No If no, please proceed to section 8.0

7.2 Please specify the animal(s) used:

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

8.0 Biological Toxins

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

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

8.3 What is the LD_{50} (specify species) of the toxin__18 ug/kg_____

8.4 How much of the toxin is handled at one time*? __100 ng_____

8.5 How much of the toxin is stored*? _50 ug

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

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

9.0 Insects

9.1 Do you use insects? O YES x NO If no, please proceed to Section 10.0

9.2 If YES, please give the name of the species. _____

9.3 What is the origin of the insect? _____

9.4 What is the life stage of the insect? _____

9.5 What is your intention? O Initiate and maintain colony, give location: _____
O "One-time" use, give location: _____

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

_____

_____

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

_____

_____

10.0 Plants

10.1 Do you use plants? O YES x 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 plant? _____

10.4 What is the form of the plant (seed, seedling, plant, tree...)? _____

10.5 What is your intention? O Grow and maintain a crop O "One-time" use

10.6 Do you do any modifications to the plant? O YES O NO
If yes, please describe: _____

_____

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

_____

_____

_____

10.8 Is the CFIA permit attached? O YES O NO
If YES, Please attach the CFIA permit & describe any CFIA permit conditions:

_____

_____

11.0 Import Requirements

11.1 Will any of the above agents be imported? O YES, please give country of origin_____ x NO
If no, please proceed to Section 12.0

11.2 Has an Import Permit been obtained from HC for human pathogens? O YES O NO

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

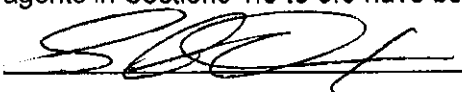
11.4 Has the import permit been sent to OHS? O YES, please provide permit # _____ O NO

12.0 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.

SIGNATURE 

13.0 Containment Levels

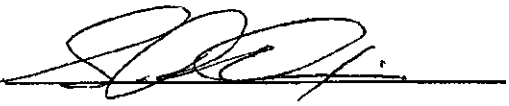
13.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. O 1 x 2 O 2+ O 3

13.2 Has the facility been certified by OHS for this level of containment?

x YES, permit # if on-campus_BIO-UWO-0096_____
O NO, please certify
O NOT REQUIRED for Level 1 containment

14.0 Procedures to be Followed

14.1 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.wph.uwo.ca/

SIGNATURE  Date: 24 Jan 2012

14.2 Please describe additional risk reduction measures will be taken beyond containment level 1, 2, 2+ or 3 measures, that are unique to this agent.

__Our lab has a Biohazard level 2 designation and all members are trained to use universal Precautions. We still encouraged all to seek help when starting new projects that use cell lines, toxins or chemicals that they are not entirely familiar with. _____

_____

14.3 Please outline what will be done if there is an exposure to the biological agents listed, such as a needlestick injury:

_In the unlikely event of exposure to one of the agents listed above the priority will be the affected person. First aid and medical attention will be given immediately followed by the completion of an incident report. The incident will also be examined in the presence of other lab members to investigate how it occurred and how it can be prevented in the future. _____

15.0 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:_____
Date:_____

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

Special Conditions of Approval:

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

Info on cell Line(s)

MSDS FOR ATCC MICROBIAL CULTURES (Biosafety Level 1)

ATCC cultures are not hazardous as defined by OSHA 1910.1200. However, as living microorganisms they are potential biohazards.

ATCC Emergency Telephone: (703) 365-2710 (24 hours)
Chemtrec: (800) 424-9300
To be used only in the event of an emergency involving a spill, leak, fire, exposure or accident.

Description

ATCC microbial cultures consist of all bacteria, fungi, plant and animal viruses, and molecular biology materials such as hosts, vectors, clones and libraries.

Either frozen, freeze-dried or growing cells shipped on solid or liquid 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).

SECTION I
Hazardous Ingredients

Frozen cultures may contain 5 to 10% Dimethyl sulfoxide (DMSO).

SECTION II
Physical data

Liquid or solid suspensions; frozen liquid suspensions; freeze-dried.

SECTION III
Health hazards

This culture is not known to cause disease in healthy human adults or animals.

SECTION IV
Fire and explosion

Not applicable

SECTION V
Reactivity data

Stable. Hazardous polymerization will not occur.

SECTION VI
Method of disposal

Spill: Contain the spill and decontaminate using suitable disinfectants such as chlorine bleach or 70% ethyl or isopropyl alcohol.

Waste disposal: Dispose of cultures and exposed materials by autoclaving at 121°C for 20 minutes. Dispose of sealed vials of freeze-dried material by dry heat sterilization at 170°C for four hours.

Follow all Federal, State and local regulations.

SECTION VII
Special protection information

For Biosafety Level 1 Microbial Cultures

Handle as a potentially biohazardous material under at least Biosafety Level 1 containment.

SECTION VIII
Special precautions or comments

ATCC recommends that all ATCC microbial cultures be handled by qualified microbiologists using appropriate safety procedures and precautions. Detailed discussions of laboratory safety procedures are provided in **Laboratory Safety: Principles and Practice** (Fleming et al., ASM Press, Washington, DC, 1995), and in the U.S. Government Publication, **Biosafety in Microbiological and Biomedical Laboratories** (CDC, 1999). This publication is available in its entirety in the Center for Disease Control Office of Health and Safety's web site at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

Information on the classification of human etiologic agents on the basis of hazard can be found as Appendix B in the NIH **Guidelines for Research Involving Recombinant DNA Molecules** at http://grants.nih.gov/grants/policy/recombinentdnaguidelines.htm.

THE ABOVE INFORMATION IS CORRECT TO THE BEST OF OUR KNOWLEDGE. ALL MATERIALS AND MIXTURES MAY PRESENT UNKNOWN HAZARDS AND SHOULD BE USED WITH CAUTION. THE USER SHOULD MAKE INDEPENDENT DECISIONS REGARDING THE COMPLETENESS OF THE INFORMATION BASED ON ALL SOURCES AVAILABLE. ATCC SHALL NOT BE HELD LIABLE FOR ANY DAMAGE RESULTING FROM HANDLING OR CONTACT WITH THE ABOVE PRODUCT.

February 2002

ATCC®

MATERIAL SAFETY DATA SHEET

MSDS FOR GENOMIC DNA

ATCC genomic DNA products are not hazardous as defined by OSHA 1910.1200.

ATCC Emergency Telephone: (703) 365-2710 (24 hours)
Chemtrec: (800) 424-9300
To be used only in the event of an emergency involving a spill, leak, fire, exposure or accident.

SECTION 1. Product Identification
Product name: Genomic DNA.

SECTION 2: Composition/Information on Ingredients
CAS #: None

SECTION 3: Hazards Identification
These products are not known to be hazardous.

SECTION 4: First Aid Measures
Not applicable

SECTION 5: Fire Fighting Measures
Stable. Hazardous polymerization will not occur.

SECTION 6: Accidental Release Measures
Contain the spill and dispose of the material appropriately.

SECTION 7: Handling and Storage
Store intact at -70°C.

SECTION 8: Exposure Controls/Personal Protection
Special protection not required under normal usage. Use product in accordance with good laboratory practices.

SECTION 9: Physical and Chemical Properties
Frozen suspension.

SECTION 10: Stability and Reactivity
This product is stable.

SECTION 11: Toxicological Information
Not available.

SECTION 12: Ecological Information
Not available.

SECTION 13: Disposal Considerations
Not available.

SECTION 14: Transport Information
Not regulated.

SECTION 15: Regulatory Information
Not regulated in the United States.

SECTION 16: Other Information

THE ABOVE INFORMATION IS CORRECT TO THE BEST OF OUR KNOWLEDGE. ALL MATERIALS AND MIXTURES MAY PRESENT UNKNOWN HAZARDS AND SHOULD BE USED WITH CAUTION. THE USER SHOULD MAKE INDEPENDENT DECISIONS REGARDING THE COMPLETENESS OF THE INFORMATION BASED ON ALL SOURCES AVAILABLE. ATCC SHALL NOT BE HELD LIABLE FOR ANY DAMAGE RESULTING FROM HANDLING OR CONTACT WITH THE ABOVE PRODUCT.

March 2002

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

ATCC cultures are not hazardous as defined by OSHA 1910.1200. However, as live cells they are potential biohazards.

ATCC Emergency Telephone: (703) 365-2710 (24 hours)
Chemtrec: (800) 424-9300
To be used only in the event of an emergency involving a spill, leak, fire, exposure or accident.

Description

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).

SECTION I
Hazardous Ingredients

Frozen cultures may contain 5 to 10% Dimethyl sulfoxide (DMSO)

SECTION II
Physical data

Pink or red aqueous liquid

SECTION III
Health hazards

For Biosafety Level 1 Cell Lines

This cell line is not known to harbor an agent known to cause disease in healthy adult humans. This cell line has **NOT** been screened for Hepatitis B, human immunodeficiency viruses or other adventitious agents. Handle as a potentially biohazardous material under at least Biosafety Level 1 containment.

For Biosafety Level 2 Cell Lines

This cell line is known to contain an agent that requires handling at Biosafety Level 2 containment [U.S. Government Publication **Biosafety in Microbiological and Biomedical Laboratories** (CDC, 1999)]. These agents have been associated with human disease. This cell line has **NOT** been screened for Hepatitis B, human immunodeficiency viruses or other adventitious agents. Cell lines derived from primate lymphoid tissue may fall under the regulations of 29 CFR 1910.1030 Bloodborne Pathogens.

SECTION IV
Fire and explosion

Not applicable

SECTION V
Reactivity data

Stable. Hazardous polymerization will not occur.

SECTION VI
Method of disposal

Spill: Contain the spill and decontaminate using suitable disinfectants such as chlorine bleach or 70% ethyl or isopropyl alcohol.
Waste disposal: Dispose of cultures and exposed materials by autoclaving at 121°C for 20 minutes. Follow all Federal, State and local regulations.

SECTION VII
Special protection information

For Biosafety Level 1 Cell Lines

Handle as a potentially biohazardous material under at least Biosafety Level 1 containment. Cell lines derived from primate lymphoid tissue may fall under the regulations of 29 CFR 1910.1030 Bloodborne Pathogens.

For Biosafety Level 2 Cell Lines

Handle as a potentially biohazardous material under at least Biosafety Level 2 containment. Cell lines derived from primate lymphoid tissue may fall under the regulations of 29 CFR 1910.1030 Bloodborne Pathogens.

SECTION VIII
Special precautions or comments

ATCC recommends that appropriate safety procedures be used when handling all cell lines, especially those derived from human or other primate material. Detailed discussions of laboratory safety procedures are provided in **Laboratory Safety: Principles and Practice** (Fleming, et al., 1995) the ATCC manual on quality control (Hay, et al., 1992), the Journal of Tissue Culture Methods (Caputo, 1988), and in the U.S. Government Publication, **Biosafety in Microbiological and Biomedical Laboratories** (CDC, 1999). This publication is available in its entirety in the Center for Disease Control Office of Health and Safety's web site at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

THE ABOVE INFORMATION IS CORRECT TO THE BEST OF OUR KNOWLEDGE. ALL MATERIALS AND MIXTURES MAY PRESENT UNKNOWN HAZARDS AND SHOULD BE USED WITH CAUTION. THE USER SHOULD MAKE INDEPENDENT DECISIONS REGARDING THE COMPLETENESS OF THE INFORMATION BASED ON ALL SOURCES AVAILABLE. ATCC SHALL NOT BE HELD LIABLE FOR ANY DAMAGE RESULTING FROM HANDLING OR CONTACT WITH THE ABOVE PRODUCT.

February 2002

Cell Biology

ATCC® Number:	**CRL-1573™** Order this Item	Price:	**$279.00**

Designations:	293 [HEK-293]
Depositors:	FL Graham
Biosafety Level:	2 [CELLS CONTAIN ADENOVIRUS]
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens*
Morphology:	epithelial 
Source:	**Organ:** embryonic kidney
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Restrictions:	These cells are distributed for research purposes only. 293 cells, their products, or their derivatives may not be distributed to third parties.
Applications:	efficacy testing transfection host viruscide testing
Receptors:	vitronectin, expressed
Tumorigenic:	YES
DNA Profile (STR):	Amelogenin: X CSF1PO: 11,12 D13S317: 12,14 D16S539: 9,13 D5S818: 8,9 D7S820: 11,12 THO1: 7,9.3 TPOX: 11 vWA: 16,19

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Product Information Sheet

This is a hypotriploid human cell line. The modal chromosome

Cell Biology

ATCC® Number:	**TIB-71™**	Order this Item	Price:	**$279.00**

Designations:	RAW 264.7
Depositors:	WC Raschke
Biosafety Level:	2
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Mus musculus* monocyte/macrophage
Morphology:	  
Source:	**Strain:** BALB/c **Tissue:** ascites **Disease:** Abelson murine leukemia virus-induced tumor **Cell Type:** macrophage; Abelson murine leukemia virus transformed
Cellular Products:	lysozyme
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Applications:	Biological response transfection host
Receptors:	complement (C3) [1207]
Antigen Expression:	H-2d
Age:	adult
Gender:	male

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Cell Micrograph

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BioProducts

Cell, microbial and molecular genomics products for the life sciences

This line was established from a tumor induced by Abelson murine

Cell Biology

ATCC® Number:	**CCL-61™**	Order this Item	Price:	**$279.00**

Designations:	CHO-K1
Depositors:	TT Puck
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Cricetulus griseus*
Morphology:	epithelial-like 
Source:	**Organ:** ovary
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Isolation:	**Isolation date:** 1957
Applications:	transfection host
Virus Resistance:	poliovirus 2; modoc virus; Button Willow virus
Cytogenetic Analysis:	Chromosome Frequency Distribution 50 Cells: 2n = 22. Stemline number is hypodiploid.
Gender:	female
Comments:	The CHO-K1 cell line was derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary of an adult Chinese hamster by T. T. Puck in 1957. The cells require proline in the medium for growth.
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated F-12K Medium, Catalog No. 30-2004. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Temperature:** 37.0°C

Protocol:

Related Links

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Product Information Sheet

Cell Biology

ATCC® Number:	**CRL-2593™** Order this Item	Price:	**$329.00**

Designations:	MC3T3-E1 Subclone 4
Depositors:	RT Franceschi
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Mus musculus*
Morphology:	fibroblast
Source:	**Organ:** bone **Strain:** C57BL/6 **Tissue:** calvaria **Cell Type:** preosteoblast;
Cellular Products:	collagen [51540]
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Applications:	These cell lines are good models for studying in vitro osteoblast differentiation, particularly ECM signaling. They have behavior similar to primary calvarial osteoblasts. The MC3T3-E1 Subclone 4 (ATCC CRL-2593) and the MC3T3 Subclone 14 (ATCC CRL-2594) lines exhibit high levels of osteoblast differentiation after growth in ascorbic acid and 3 to 4 mM inorganic phosphate.
Tumorigenic:	Yes
Age:	newborn

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Product Information Sheet

A series of subclones were isolated from the cloned but

Cell Biology

ATCC® Number:	**CRL-1661™** Order this Item	Price:	**$329.00**

Designations:	UMR-106
Depositors:	AE Bogden
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Rattus norvegicus* deposited as *Rattus* sp.
Morphology:	epithelial
Source:	**Organ:** bone **Strain:** Sprague-Dawley **Disease:** osteosarcoma
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Receptors:	parathyroid hormone (PTH); 1-25(OH)2D3 (bone resorbing steroid hormone)
Comments:	The UMR-106 cell line is a clonal derivative of a transplantable rat osteosarcoma that had been induced by injection of radiophosphorous (32P). The cells are responsive to PTH, prostaglandins and bone resorbing steroids. The PTH responsiveness of UMR-106 is greater than that of the related cell line UMR-108 (ATCC CRL-1663). Activation of protein kinase C inhibits ATP induced increases in intracellular calcium levels. Both the original sarcoma and the cloned line were developed by T.J. Martin at the University of Sheffield.
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Temperature:** 37.0°C

Subcultivation Ratio: A subcultivation ratio of 1:4 to 1:8 is

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Cell Biology

ATCC® Number:	**CRL-1650™** Order this Item	Price:	**$279.00**

Designations:	COS-1
Depositors:	Y Gluzman
Biosafety Level:	2 [Cells Contain PAPOVAVIRUS]
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Cercopithecus aethiops*
Morphology:	fibroblast
Source:	**Organ:** kidney **Cell Type:** SV40 transformed
Cellular Products:	T antigen
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Applications:	transfection host
Comments:	This is an African green monkey kidney fibroblast-like cell line suitable for transfection by vectors requiring expression of SV40 T antigen. This line contains T antigen, retains complete permissiveness for lytic growth of SV40, supports the replication of ts A209 virus at 40C, and supports the replication of pure populations of SV40 mutants with deletions in the early region. The line was derived from the CV-1 cell line (ATCC ® CCL-70) by transformation with an origin defective mutant of SV40 which codes for wild type T antigen. The cells contain a single integrated copy of the complete early region of the SV40 genome.
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Temperature:** 37.0°C

Protocol:

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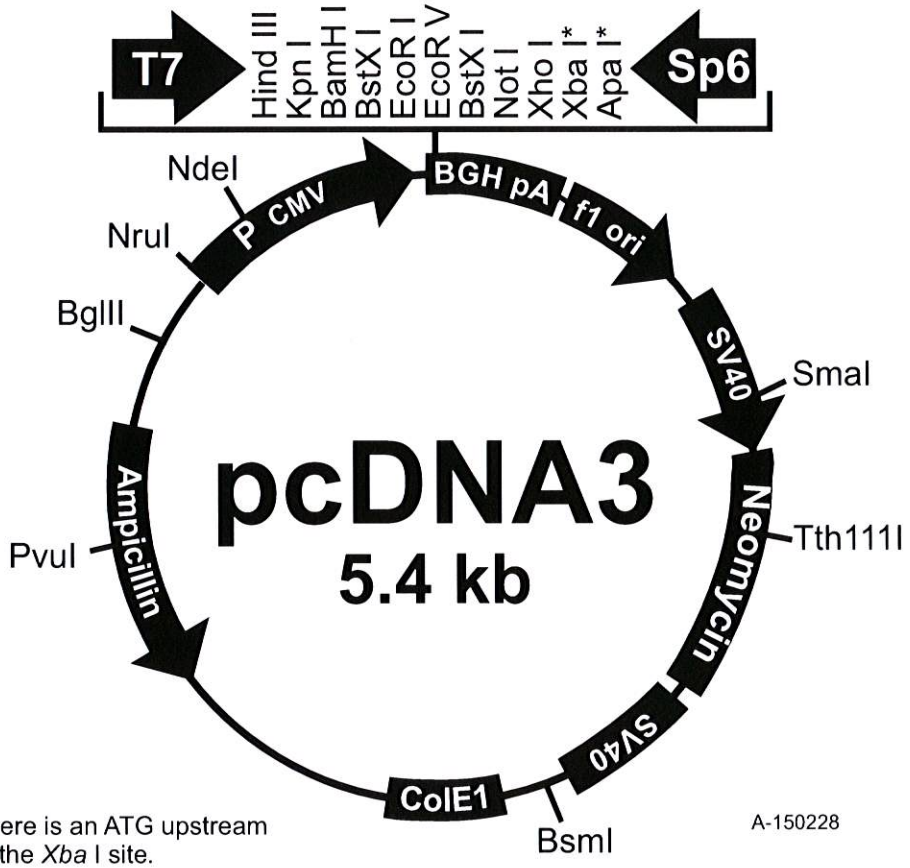
Cell, microbial and molecular genomics products for the life sciences

BioServices

Bio-materials management; basic repository to complex partnership-level services

Comments for pcDNA3:
5446 nucleotides

CMV promoter: bases 209-863
T7 promoter: bases 864-882
Polylinker: bases 889-994
Sp6 promoter: bases 999-1016
BGH poly A: bases 1018-1249
SV40 promoter: bases 1790-2115
SV40 origin of replication: bases 1984-2069
Neomycin ORF: bases 2151-2945
SV40 poly A: bases 3000-3372
ColE1 origin: bases 3632-4305
Ampicillin ORF: bases 4450-5310



* There is an ATG upstream of the *Xba* I site.

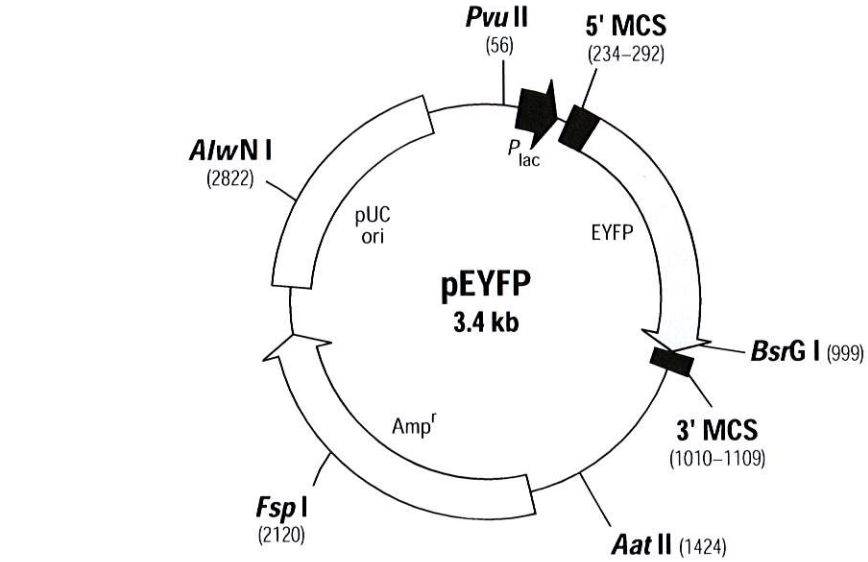
The sequence of pcDNA3 has been compiled from information in sequence databases, published sequences, and other sources. This vector has not yet been completely sequenced. If you suspect an error in the sequence, please contact Invitrogen's Technical Services Department.

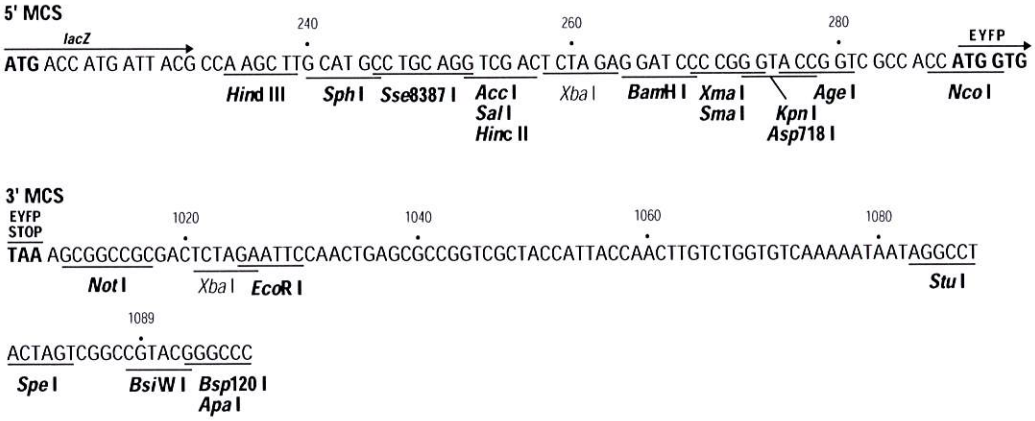
Vector Information

pEYFP Vector Information

PT3175-5

Catalog #6004-1





Restriction map and multiple cloning site (MCS) of pEYFP. Unique restriction sites are in bold. The *Xba* I sites in the 5' and 3' MCSs can be used together to excise the EYFP gene.

Description:

pEYFP encodes an enhanced yellow-green variant of the *Aequorea victoria* green fluorescent protein (GFP). The EYFP gene contains the four amino acid substitutions previously published as GFP-10C (1): Ser-65 to Gly; Val-68 to Leu; Ser-72 to Ala; and Thr-203 to Tyr. The fluorescence excitation maximum of EYFP is 513 nm, and the emission spectrum has a peak at 527 nm (in the yellow-green region). When excited at 513 nm, the E_m of EYFP is 36,500 $cm^{-1}M^{-1}$ and the fluorescent quantum yield is 0.63 (1), resulting in a bright fluorescent signal. The fluorescence observed from EYFP is roughly equivalent to that from EGFP.

A mixture of EYFP- and EGFP-expressing cells can be sorted by flow cytometry using a single excitation wavelength (i.e., 488 nm). EYFP emission is detected using a 525-nm dichroic shortpass mirror and a 530/30-nm bandpass filter; EGFP emission is detected using a 510/20-nm bandpass filter.

In addition to the chromophore mutations, EYFP contains >190 silent mutations that create an open reading frame comprised almost entirely of preferred human codons (2). Furthermore, upstream sequences flanking EYFP have been converted to a Kozak consensus translation initiation site (3). These changes increase the translational efficiency of the EYFP mRNA and consequently the expression of EYFP in mammalian and plant cells.

(PR29943; published 03 October 2002)

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The EYFP gene is flanked at the 5' and 3' ends by the two MCSs of the pUC19 derivative pPD16.43 (4). Thus, the EYFP coding sequence can be easily excised from the vector or amplified by PCR. In *E. coli*, EYFP is expressed from the *lac* promoter as a fusion with several additional amino acids, including the first five amino acids of the LacZ protein. Note, however, that if you excise the EYFP coding sequence using a restriction site in the 5' MCS, the resulting fragment will encode the native (i.e., nonfusion) EYFP protein. The pUC19 backbone of EYFP provides a high-copy-number origin of replication and an ampicillin resistance gene for propagation and selection in *E. coli*.

Location of features:

- *lac* promoter: 95–178
  - CAP binding site: 111–124
  - –35 region: 143–148; –10 region: 167–172
  - Transcription start point: 179
  - *lac* operator: 179–199
- *lacZ*–EYFP fusion protein expressed in *E. coli*
  - Ribosome binding site: 206–209
  - Start codon (ATG): 217–219; Stop codon: 1006–1008
- 5' multiple cloning site: 234–281
- Enhanced yellow fluorescent protein (EYFP) gene
  - Kozak consensus translation initiation site: 282–292
  - Start codon (ATG): 289–291; stop codon: 1006–1008
  - Insertion of Val at position 2: 292–294
  - GFP-10C mutations (Ser-65 to Gly: 484–486; Val-68 to Leu: 493–495; Ser-72 to Ala: 505–507; Thr-203 to Tyr: 898–900)
  - His-231 to Leu mutation (A→T): 983
- 3' multiple cloning site: 1010–1109
- Ampicillin resistance gene
  - Promoter: –35 region: 1485–1490; –10 region: 1508–1513
  - Transcription start point: 1520
  - Ribosome binding site: 1543–1547
  - β-lactamase coding sequences
    - Start codon (ATG): 1555–1557; stop codon: 2413–2415
    - β-lactamase signal peptide: 1555–1623
    - β-lactamase mature protein: 1624–2412
- pUC plasmid replication origin: 2563–3206

Primer location:

- EGFP-N Sequencing Primer (#6479-1): 355–334
- EGFP-C Sequencing Primer (#6478-1): 942–963

Propagation in *E. coli*:

- Recommended host strain: JM109
- Selectable marker: plasmid confers resistance to ampicillin (100 µg/ml) to *E. coli* hosts
- *E. coli* replication origin: pUC
- Copy number: ~500
- Plasmid incompatibility group: pMB1/ColE1

References:

1. Ormö, M. *et al.* (1996) *Science* **273**:1392–1395.
2. Haas, J., *et al.* (1996) *Curr. Biol.* **6**:315–324.
3. Kozak, M. (1987) *Nucleic Acids Res.* **15**:8125–8148.
4. Fire, A., *et al.* (1990) *Gene* **93**:189–198.

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 BD Biosciences Clontech. This vector has not been completely sequenced.

Notice to Purchaser

Use of BD Biosciences Clontech's Living Colors™ products containing DNA sequences coding for mutant *Aequorea victoria* green fluorescent protein (GFP) variants or proteins thereof requires a license from Amersham Biosciences under U.S. Patent Nos. 5,625,048; 5,777,079; 6,054,321 and other pending U.S. and foreign patent applications. In addition, certain BD Biosciences Clontech products are made under U.S. Patent No. 5,804,387 licensed from Stanford University.

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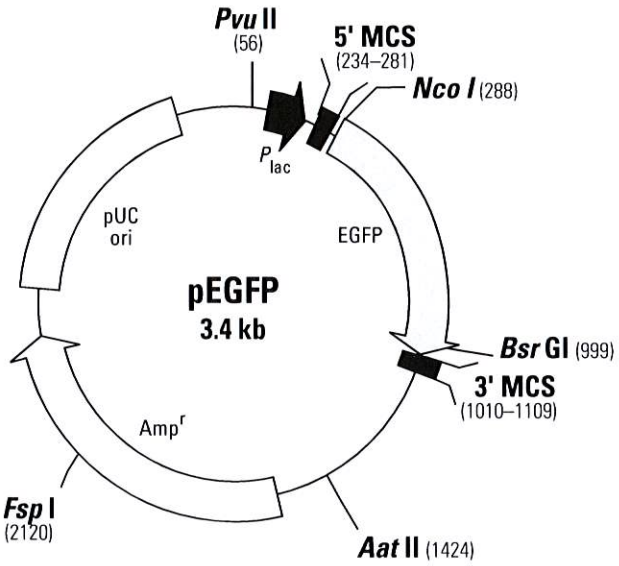
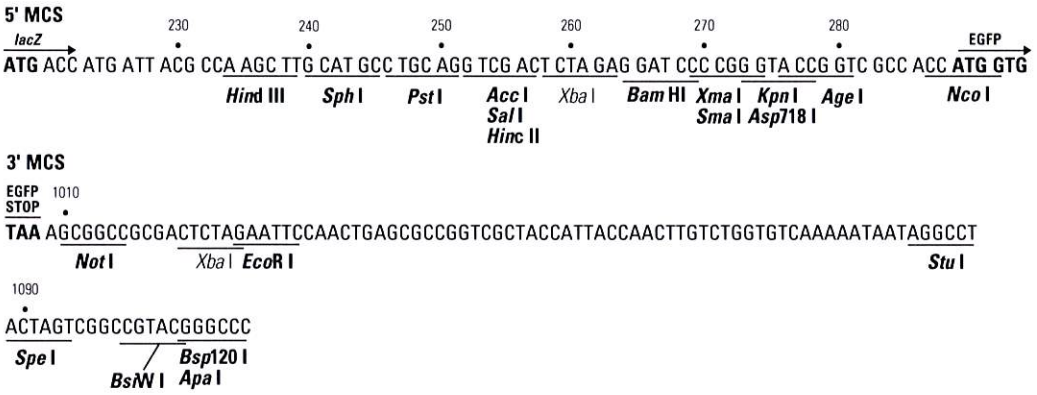
Vector Information

pEGFP Vector Information

PT3078-5

Catalog #6077-1





Restriction Map and Multiple Cloning Site (MCS) of pEGFP Vector. Unique restriction sites are in bold. The *Xba* I sites in the MCS can be used together to excise the EGFP gene.

Description:

pEGFP carries a red-shifted variant of wild-type green fluorescent protein (GFP) which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nm; emission maximum = 507 nm.) pEGFP encodes the GFPmut1 variant (1) which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences (2). Upstream sequences flanking EGFP have been converted to a Kozak consensus translation initiation site (3) to further increase the translation efficiency in eukaryotic cells.

The EGFP gene was cloned between the two MCS of the pUC19 derivative pPD16.43 (4). The EGFP coding sequence is flanked by separate MCS at the 5' and 3' ends, so the EGFP gene can be easily excised from pEGFP. Alternatively, the EGFP coding sequence can be amplified by PCR. The EGFP gene was inserted in frame with the *lacZ* initiation codon from pUC19 so that a EGFP fusion protein is expressed from the *lac* promoter in *E. coli.* Note, however, that if you excise the EGFP coding sequence using a restriction site in the 5' MCS, the resulting fragment will encode the native (i.e., non-fusion) EGFP protein. The pUC backbone of EGFP provides a high-copy-number origin of replication and an ampicillin resistance gene for propagation and selection in *E. coli.*

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E-mail: tech@clontech.com
www.clontech.com

(PR29965; published 03 October 2002)

Location of features:

- *lac* promoter: 95–178
  - CAP binding site: 111–124
  - –35 region: 143–148; –10 region: 167–172
  - Transcription start point: 179
  - *lac* operator: 179–199
- *lacZ*–EGFP fusion protein expressed in *E. coli*
  - Ribosome binding site: 206–209
  - Start codon (ATG): 217–219; Stop codon: 1006–1008
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- Enhanced green fluorescent protein (EGFP) gene
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  - His-231 to Leu mutation (A→T): 983
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    - β-lactamase signal peptide: 1555–1623
    - β-lactamase mature protein: 1624–2412
- pUC plasmid replication origin: 2563–3206

Primer location:

- EGFP-N Sequencing Primer (#6479-1): 355–334
- EGFP-C Sequencing Primer (#6478-1): 942–963

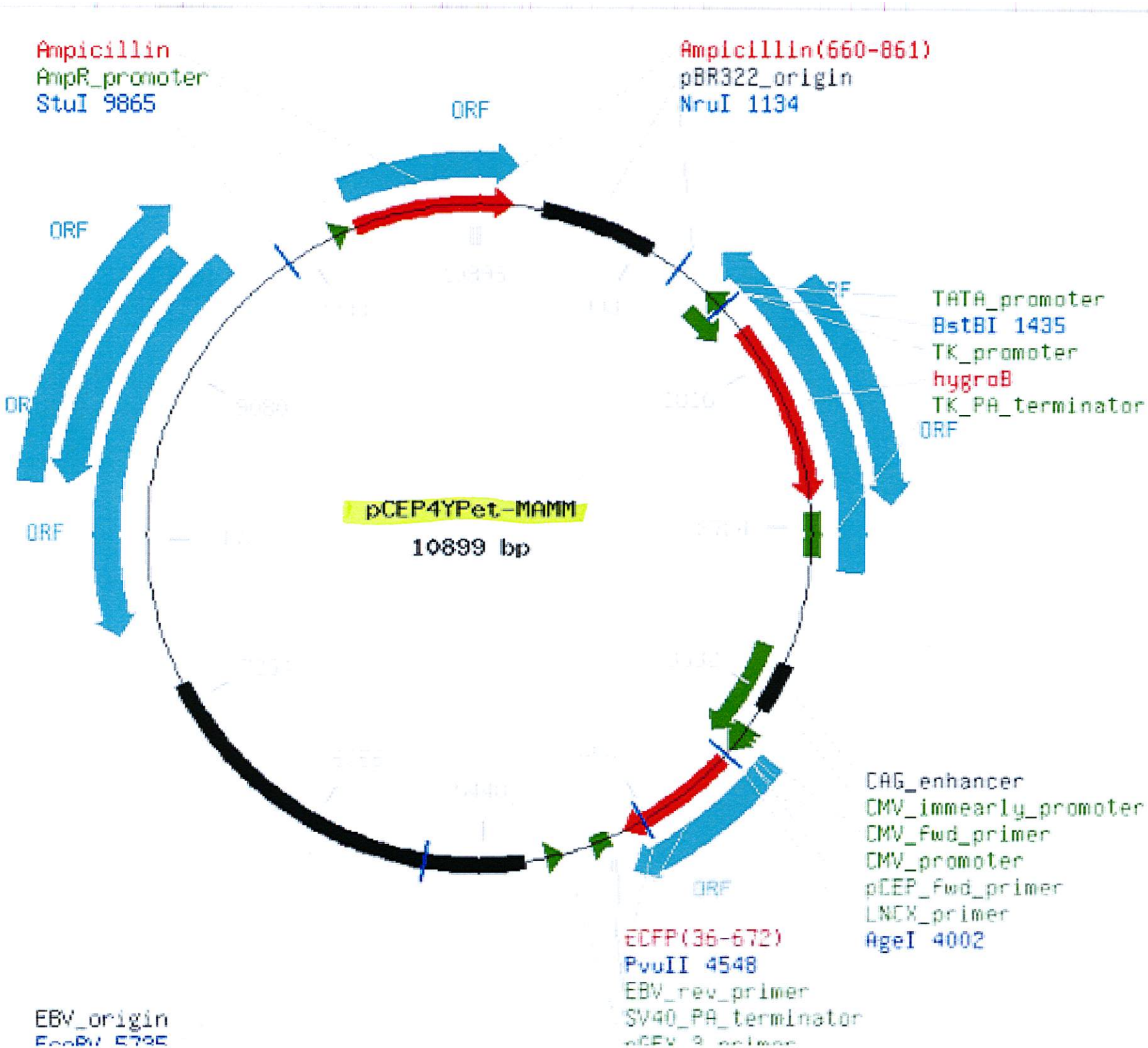
Propagation in *E. coli*:

- Recommended host strain: JM109
- Selectable marker: plasmid confers resistance to ampicillin (100 µg/ml) to *E. coli* hosts
- *E. coli* replication origin: pUC
- Copy number: ~500
- Plasmid incompatibility group: pMB1/ColE1

References:

1. Cormack, B., *et al.* (1996) *Gene* **173**:33–38.
2. Haas, J., *et al.* (1996) *Curr. Biol.* **6**:315–324.
3. Kozak, M. (1987) *Nucleic Acids Res.* **15**:8125–8148.
4. Fire, A., *et al.* (1990) *Gene* **93**:189–198.

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 BD Biosciences Clontech. This vector has not been completely sequenced.


Ampicillin
AmpR_promoter
StuI 9865
Amplcillin(660-861)
pBR322_origin
NruI 1134
ORF
ORF
ORF
ORF
ORF
ORF
TATA_promoter
BstBI 1435
TK_promoter
hygroB
TK_PA_terminator
pCEP4YPet-MAMM
10899 bp
CAG_enhancer
CMV_immearly_promoter
CMV_fwd_primer
CMV_promoter
pCEP_fwd_primer
LNCX_primer
AgeI 4002
ECFP(36-672)
PvuII 4548
EBV_rev_primer
SV40_PA_terminator
EBV_origin

Promega

Features List and Map for the pGL4.74 [*hRluc*/TK] Vector

The following features are present in the vector based on nucleotide sequence.

HSV-TK promoter	27–779
hRluc reporter gene	815–1750
SV40 late poly(A) signal	1784–2005
Reporter Vector primer 4 binding region	2071–2090
ColEI-derived plasmid replication origin	2330
Synthetic β-lactamase (Amp^r) coding region	3119–3979
Synthetic poly(A) signal/transcriptional pause site	4084–4237
Reporter Vector primer 3 binding region	4186–4205

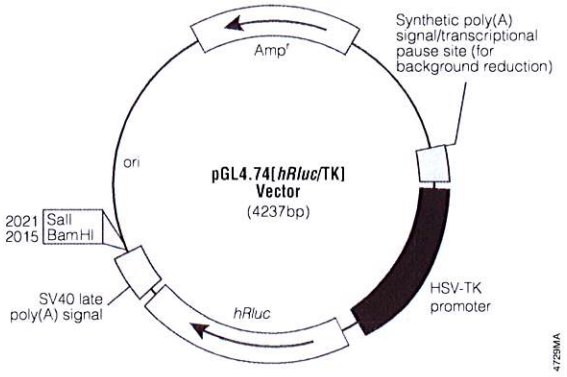


Figure 1. pGL4.74 [*hRluc*/TK] Vector circle map and sequence reference points.

Sequence information and restriction enzyme tables for the pGL4 Vectors are available online at: **www.promega.com/vectors/**
Further information on use of the pGL4 Vectors is available in Technical Manual #TM259 available online at: **www.promega.com/tbs/**

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Chapter 8

Assaying β-Catenin/TCF Transcription with β-Catenin/TCF Transcription-Based Reporter Constructs

Travis L. Biechele and Randall T. Moon

Abstract

Transcription-based reporters have been instrumental in characterizing the Wnt/β-catenin signaling pathway and will be essential in the search for therapeutics aimed at combating diseases linked to aberrant signaling. In this chapter, we introduce a new improved Wnt/β-catenin reporter system, β-catenin-activated reporter (BAR), and its accompanying control reporter system, found unresponsive BAR (fuBAR). Its enhanced sensitivity, increased dynamic range, and lentiviral platform provide a reporter system that will keep pace with the needs of scientists in the field.

Key words: Wnt, b-Catenin, Luciferase, Transcription, Reporter, BAR, TCF, LEF.

1. Introduction

The Wnt/β-catenin pathway is the best-studied Wnt pathway in part due to robust tools for measuring pathway activation both in vivo and in vitro. Among the earliest and still commonly used assays of Wnt/β-catenin signaling include phenotypic assays in *Drosophila (1)*, dorsal axis duplication in *Xenopus (2)*, and proliferation of C57MG mammary epithelial cells *(3)*. These assays were crucial for a substantial amount of the early characterization of the pathway. Much of the more recent characterization of the pathway has relied on the convenience of transcription-based reporter systems. The first transcription-based luciferase reporter of Wnt/β-catenin signaling, TOPFlash, was designed by Korinek et al. *(4)*. The TOPFlash reporter contains three TCF response elements (CCTTTGATC) upstream of a basal

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c-fos promoter while the control reporter, FOPFlash, contains three mutant TCF response elements (CCTTTGGCC). TOPFlash was later modified by Upstate Biotechnology to contain three TCF response elements upstream of a minimal thymidine kinase (TK) promoter. The TOPFlash reporter system provided a reliable assay of pathway activation and was crucial for the identification and characterization of several pathway components.

As the characterization of the Wnt/β-catenin pathway turned to identifying modifiers of the core pathway components, the need for a more sensitive reporter developed. This niche was filled by Ajamete Kaykas in the Moon lab with the construction of the SuperTOPFlash reporter. SuperTOPFlash contains eight TCF response elements upstream of Clontech's minimal TA promoter *(5)*. This modification greatly enhances the sensitivity and dynamic range of the reporter (**Fig. 8.1a**) providing a better tool for characterizing modifiers of the pathway as well as the ability to identify new components in *Drosophila* genome-wide RNA interference (RNAi) screens *(6)*. This was significant as SuperTOPFlash was the first Wnt/β-catenin reporter responsive to wingless in *Drosophila* cells.

The necessity to monitor Wnt/β-catenin signaling in non-transfectable cells and achieve even greater sensitivity for high

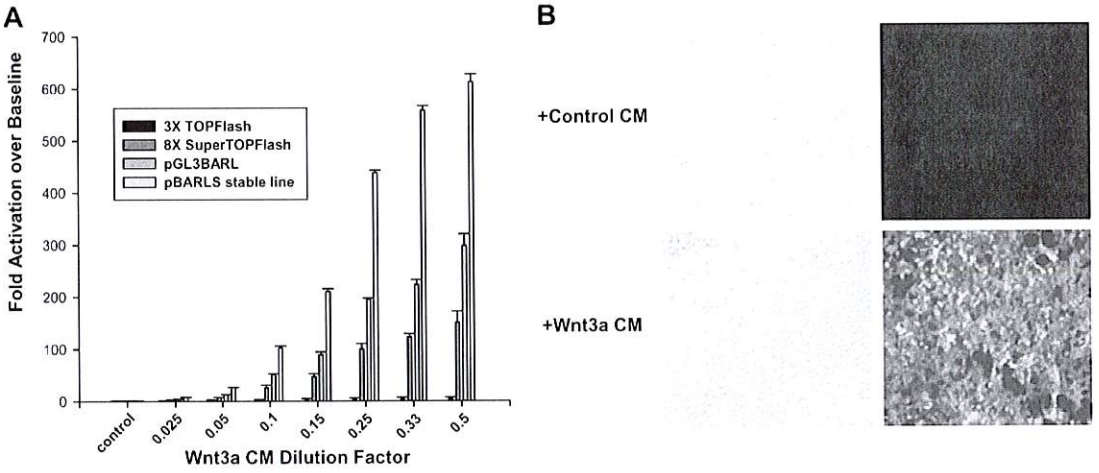

Fig. 8.1. The BAR system has enhanced sensitivity and dynamic range when directly compared with TOPFlash and SuperTOPFlash. **A** 10 ng of TOPFlash, SuperTOPFlash, or pGL3BARL were transfected along with 10 ng of pRLTK in HEK293T cells seeded in a 48-well plate. HEK293T cells stably expressing pBARLS were generated as described in Section 3.4. Cells were treated with specified doses of Wnt3a-conditioned media (*CM*) for 18 h. Luciferase activity was measured as described in Section 3.5.2 and data are presented as fold activation over control conditioned media-treated cells. **B** A monoclonal HEK293T cell line stably expressing pBARVS was generated as described in Section 3.4. Cells were treated with either control conditioned media or Wnt3a-conditioned media for 30 h.

throughput screening inspired the construction of the β-catenin activated reporter (BAR) system. The BAR system contains a concatemer of 12 TCF response elements separated by unique five-nucleotide linkers specifically designed to minimize recombination that can lead to loss of TCF binding sites. This series of TCF response elements is inserted upstream of Promega's minP minimal promoter, completing a functional promoter that drives the transcription of either Firefly luciferase (pBARL), renilla luciferase (pBARRen), or β-globin intron-linked Venus (pBARV) (Venus is a variant of EYFP *(7)*). These reporters were inserted between the long terminal repeats (LTRs) of a lentiviral-transducing plasmid. The result is a highly sensitive luciferase reporter with an unmatched dynamic range and Venus reporter that allows a spatial report of pathway activation (**Fig. 8.1**).

Control reporters, found unresponsive BAR (fuBAR), were constructed using the same strategy. They are identical to their respective parent reporter with the exception that each TCF DNA binding element contains a two-base substitution conferring a non-functional element (pfuBARL and pfuBARV). The essentially identical nature of the control reporters provides the most optimal experimental control, as well as allowing for identical lentiviral titer production when generated side by side with the responsive reporter.

A second version of the reporter constructs containing a PGK promoter driving a puromycin- or hygromycin-resistance gene was constructed for antibiotic selection in mammalian cells (pBARLS, pfuBARLS, pBARLHyg, pfuBARLHyg, pBARVS, pfuBARVS, pBARVHyg, and pfuBARLHyg). A third version containing a PGK or EF1α promoter driving dsRed (pBARVR and pfuBARVR) was constructed for visual detection of cells containing the reporter independent of reporter activation (**Fig. 8.2**).

It should be noted that although the Wnt/β-catenin reporters appear to be very specific readouts of signaling, they are in fact artificial promoters that may not faithfully reflect the activity of endogenous TCF/LEF response elements *(8)*. Therefore it is important to complement reporter data with a measure of the transcription profile of known Wnt/β-catenin target genes (see http://www.stanford.edu/~rnusse/pathways/targets.html and **Note 1** for target genes). It is also important to note that TCF/LEF-independent β-catenin-mediated transcriptional activation will not be detected with these reporter systems *(9)*.

In the following sections, we outline protocols for using BAR transiently, generating stable BAR cell lines, and measuring BAR luciferase activity.

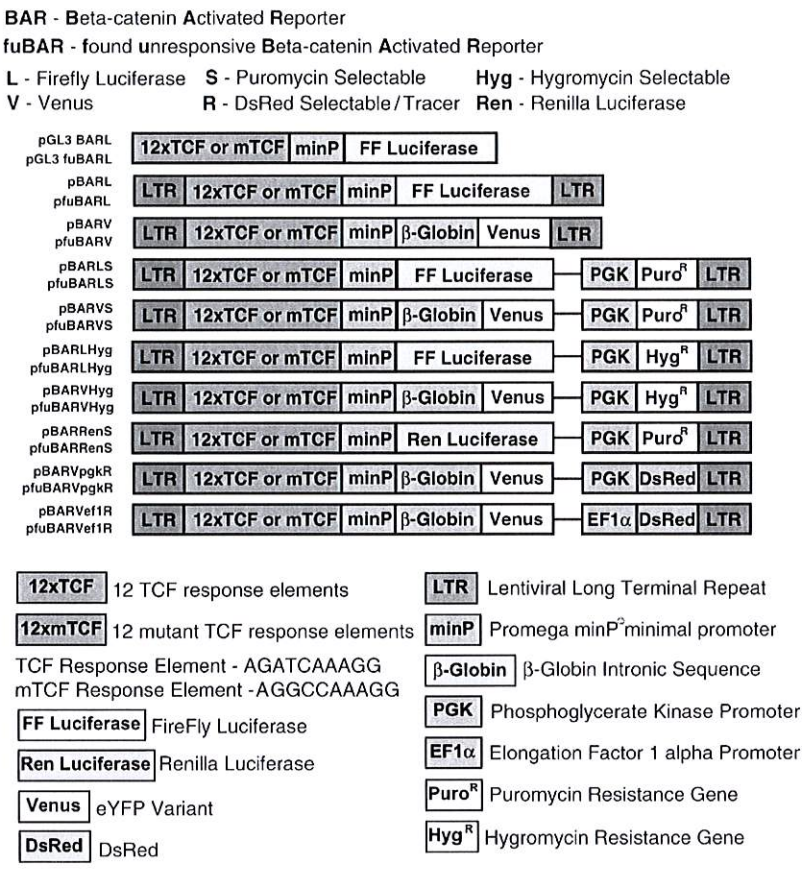

Fig. 8.2. Multiple platforms of the BAR system make it a versatile reporter system.

2. Materials

2.1. Transient Transfection of Reporter for Complementary DNA (cDNA) Overexpression or Small Interfering RNA (siRNA) Knockdown

1. 48-well cell culture plate.
2. HEK293T or other transfectable cells.
3. Lipofectamine 2000 (Invitrogen, Carlsbad, CA; cat. #11668-027) or transfection reagent of choice.
4. Optimem (Invitrogen; cat. #31985-088).
5. Plasmids (*see* **Notes 2 and 3**): pGL3BARL, pGL3fuBARL, pRLTK (Promega, Madison, WI; cat. #E2241), cDNA of interest.
6. siRNA, shRNA, and carrier plasmid (backbone of cDNA expression plasmid or empty vector).
7. L-cell control and Wnt3a-conditioned media or purified Wnt3a (*ref. (10)*; *see* **Chapter 2**; ATCC, Manassas, VA; CRL-2648 and CRL-2647) or (R&D Systems, Minneapolis, MN; cat. #1324-WN-002).

2.2. Lentivirus Production

1. HEK293T cells.
2. Media: DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin.
3. 2× HEPES-buffered saline (HBS) pH 7.1 (0.22-μm filtered). To prepare 40 mL of 2× HBS, pH 7.1, add 5.6 mL of 2 M NaCl, 4 mL of 0.5 M HEPES, pH 7, 60 μL of 1 M Na_2HPO_4, and 30.4 mL of dH_2O.
4. 2.5 M $CaCl_2$ (0.22-μm filtered).
5. Sterile water (0.22-μm filtered).
6. Plasmids (*see* **Note 3**)—pSL3, pSL4, pSL5, pSL9/rLuc, BAR, and fuBAR in lentiviral platform.

2.3. Lentivirus Concentration

1. 150 mL Millipore Stericup-GP PES filters (Millipore, Billerica, MA; cat. #SCGP U01 RE).
2. Beckman ultracentrifuge tubes (Beckman Coulter, Fullerton, CA; cat. #344058).
3. Beckman SW-28 swinging bucket rotor.
4. Pasteur pipettes.
5. 1× Tris-buffered saline (TBS): 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl. To prepare, dissolve 6.05 g Tris and 8.76 g NaCl in 800 mL of ddH_2O. Adjust pH to 7.5 with 1 M HCl and make volume up to 1 L with ddH_2O. TBS is stable at 4°C for 3 months.

2.4. Generating Stable Reporter Cell Lines

1. pBARLS and pfuBARLS or pBARLHyg and pfuBARLHyg virus.

2.4.1. Stable Luciferase Reporter Cell Line

2. pSL9/rLuc virus.
3. Puromycin or hygromycin.
4. 6-well and 48-well cell culture plates.
5. L-cell control and Wnt3a-conditioned media or purified Wnt3a (ref. *(10)*; see **Chapter 2**; ATCC, CRL-2648 and CRL-2647) or (R&D Systems; cat. #1324-WN-002).

2.4.2. Stable Venus Reporter Cell Line

1. pBARVS and pfuBARVS.
2. Puromycin.
3. 6-well and 100-mm cell culture plates.
4. L-cell control and Wnt3a-conditioned media or purified Wnt3a (ref. *(10)*; see Chapter 2; ATCC, CRL-2648 and CRL-2647) or (R&D Systems; cat. #1324-WN-002).

2.5. Luciferase Assay

1. 1× Passive lysis buffer.

2.5.1. Low-Throughput Assay

2. Firefly luciferase reagent.
3. Stop & Glo® reagent (Renilla luciferase substrate and Firefly luciferase antagonist; Promega).
4. 96-well plate with white wells.

2.5.2. High-Throughput Assay

1. Dual-Glo™ Firefly luciferase reagent (Promega).
2. Dual-Glo™ Stop & Glo® reagent (Renilla luciferase reagent and Firefly luciferase antagonist).

3. Methods

3.1. Transient Transfection of Reporter for cDNA Overexpression or siRNA Knockdown

Prior to the BAR system, the majority of Wnt/β-catenin luciferase reporter assays were performed by transiently transfecting cells with the Firefly luciferase reporter, a Renilla luciferase normalization plasmid, and cDNAs/siRNA/shRNA to be analyzed. Although the transient reporter assay has a decreased dynamic range compared with the stably integrated reporter, it is still very robust and alleviates the production of lentivirus. The following method is based on a 48-well plate format and can be modified for other plate formats by scaling based on the surface area of the well. Each experimental condition is performed in triplicate. Specific transfection details for the transfection reagent used should be followed according to manufacturer's specifications (Lipofectamine 2000 protocol: http://www.invitrogen.com/content/sfs/manuals/lipofectamine2000_man.pdf).

3.1.1. Transiently Transfecting Reporter with cDNA Expression Plasmids

1. Day 1: Plate cells at a density such that they will be 80% confluent the following day.
2. Day 2: Transfect cells with 10 ng pGL3BARL or 10 ng pGL3fuBARL, 10 ng pRLTK, your construct(s) of interest, and the appropriate amount of carrier plasmid using the manufacturer's protocol.
3. Day 3: If the cells will not be treated with a source of Wnt3a or other modulators, then proceed to **Section 3.5** to read luciferase activity. Otherwise, treat the cells with Wnt3a or other modulators (*see* **Note 4**).
4. Day 4: Proceed to **Section 3.5** for measuring luciferase activity.

3.1.2. Transiently Transfecting Reporter with shRNA or siRNA

1. Day 1: Plate cells at a density such that they will be 40% confluent the following day.
2. Day 2: Transfect cells with siRNA or shRNA using the manufacturer's protocol for the transfection reagent used (Lipofectamine 2000 protocol: http//www.invitrogen.com/content/sfs/manuals/lipofectamine2000_man.pdf).
3. Day 3: Transfect cells with 10 ng pGL3BARL or 10 ng pGL3fuBARL, 10 ng pRLTK, and the appropriate amount of carrier plasmid using the manufacturer's protocol.
4. Day 5: Treat cells with Wnt or other modulator if necessary.
5. Day 6: Proceed to section **Section 3.5** for measuring luciferase activity.

3.2. Production of Lentivirus-Containing BAR and Stable Cell Line Production

The transduction plasmid backbone used for the lentiviral-compatible BAR constructs and the lentiviral helper plasmids were provided by the Naldini lab, Vita-Salute San Raffaele University, Milan, Italy. This lentivirus is replication incompetent and does not carry oncogenic cDNAs, which makes it Biosafety Level 2. The virus is, however, competent for human infection, requiring the use of personal protective guidelines, including double gloving, the use of barrier tips, and collection of all liquids in a non-aspirating system for inactivation with 10% bleach. The following protocol will yield high-titer virus that can be used to generate stable reporter cell lines, assay Wnt/β-catenin signaling in cells that are difficult to transfect such as primary cultures, or assay signaling in vivo. BAR and fuBAR virus is made at the same time to ensure equal titer. The pSL9/rLuc plasmid can be used to generate lentivirus containing a constitutive EF1α promoter driving Renilla luciferase for reporter assay normalization.

1. Day 1: Seed a 100-mm dish with HEK 293T cells such that they will be 70–80% confluent the next day. If very high titer virus is needed, scale up production to several 150-mm dishes and adjust the transfection suggested guidelines based on dish surface area.
2. Day 2: Prepare DNA cocktails for transfection as in **Table 8.1**. Add 500 μL (1,250 μL for 150-mm dish) of 2× HBS drop-wise to the above cocktail and bubble with 10 strokes of your pipette. Add drop-wise to you cells, gently mix, and return to incubator.
3. Day 3: Remove media and dispose of media following proper procedures for inactivation in 10% bleach. Replace with fresh media.
4. Day 4: Collect media and centrifuge for 5 min at 3,000×*g* to remove cellular debris. This media may now be used to infect cells or can be concentrated to achieve higher viral titer.

Table 8.1
DNA cocktails for transfection

	100-mm Dish	150-mm Dish
ddH_2O	450 μL – volume of DNA	1,125 μL – volume of DNA
2.5 M $CaCl_2$	50 μL	125 μL
Transducing vector (e.g., pBARLS)	4 μg	10 μg
Packaging vector (pSL4)	8 μg	20 μg
Envelope (pSL3)	2 μg	5 μg
Rev (pSL5)	4 μg	10 μg

3.3. Lentivirus Concentration

There are two methods for concentrating virus. Concentrating virus with 30-kDa molecular weight cut-off centrifugation filters (Millipore Amicon Ultra cat. #UFC903024) is a simple approach to yield a 50× concentration. A limitation to this approach is concurrent concentration of other components in the media including serum and this may have deleterious affects on the cell line to be infected. A second approach involves pelleting the virus by ultracentrifugation. This technique is slightly more labor intensive but allows you to completely exchange the media and yield a 500× concentration.

1. Aliquot 30–35 mL of viral containing media into Beckman ultracentrifuge tubes, match tubes by weight (use fresh media to balance the tubes), and spin at 50,000×*g* for 2 h at 4°C in the SW28 swing bucket rotor.
2. Carefully decant the supernatant and invert the tube on a paper towel for 5 min (will have ~50 µL supernatant plus virus left in the tube).
3. Add 50 µL (or desired volume) of 1× TBS or 1× PBS to each tube, seal with paraffin, and leave at 4°C overnight with no shaking.
4. Pipette up and down three to five times and combine the resuspended virus from each tube. Filter pooled virus with a 0.45-µm filter.
5. Aliquot, snap-freeze in liquid nitrogen, and store at –80°C. Virus should only be freeze–thawed once, dictating the size of the aliquots.

3.4. Generating Stable Reporter Cell Lines

For assays that do not require stable cell lines or the DsRed tracer, the reporters without a selectable marker are recommended, as they will produce higher titer virus. In this section, we cover methods for generating stable luciferase reporter cell lines as well as stable Venus reporter cell lines. The volume of virus used will vary depending on the cell line and viral titer.

3.4.1. Stable Luciferase Reporter Cell Line

The following method describes the production of a polyclonal reporter line. We want to stress that this is a general protocol and variable factors such as a cell line's responsiveness to Wnt and the sensitivity of your luminometer will determine the amount of virus needed to generate a perfect reporter line. Infecting the reporter line with pSL9/rLuc virus provides constitutive expression of Renilla luciferase providing normalization for siRNA experiments or assays that do not involve transfection. To date, we have generated over 40 stable reporter lines in vastly different cell types. Although rare, we have found cell line exceptions in which the reporter is not responsive to pathway activation.

1. Day 1: Seed a 6-well plate such that the cells will be 50% confluent the following day.

2. Add three different doses of reporter virus and matching doses of control reporter virus to the 6-wells. We typically start with 200 μL, 50 μL, and 10 μL of virus that has been concentrated 50×.
3. Day 2: Replace the media with fresh media. As always, inactivate viral containing media in 10% bleach.
4. Day 3: Transfer cells from each well to a 100-mm dish containing the appropriate concentration of puromycin or hygromycin for selection.
5. Allow several days for selection and repopulation of the cells.
6. Test each reporter line by seeding each line in several wells of a 48-well cell culture plate. Treat each line with several doses of L-cell control or Wnt3a-conditioned media and measure luciferase activity the following day (*see* **Section 3.5**).
7. Choose the best reporter line and corresponding control reporter line based on the dynamic range, expand the cells, and freeze back several vials, as reporter activity has been found in some cases to diminish over several passages.
8. For constitutive Renilla luciferase expression, seed the reporter cells in a 6-well plate such that they will be 50% confluent the following day and treat the cells with different doses of pSL9/rLuc virus.
9. Repeat steps 6 and 7.

3.4.2. Stable Venus Reporter Cell Line

A stable polyclonal Venus reporter line can be generated using an identical approach as the stable luciferase reporter line. The only difference is that reporter activity is measured by fluorescence using a microscope or plate reader. The following protocol details the use of fluorescence-activated cell sorting (FACS) to refine the heterogeneity of the line. Briefly, a stable pBARVS virus infected cell line is generated. A monoclonal or polyclonal line with the highest possible dynamic range is generated with two rounds of FACS. In the first round, cells are stimulated with an EC50 dose of Wnt3a-conditioned and a population of high Venus-expressing cells are collected. The population is cultured for several days without Wnt3a-conditioned medium and then resorted for cells that are not expressing Venus. This protocol yields a reporter line with very low basal activity and robust response to pathway activation (**Fig. 8.1b**).

1. Perform steps 1–5 from **Section 3.4.1**.
2. Test each reporter line by seeding each individual line in several wells of a 48-well cell culture plate. Treat each line with several doses of L cell control or Wnt3a-conditioned media and visualize or measure Venus fluorescence the following day. Choose the best cell line based on dynamic range and determine the EC_{50} dose of the Wnt3a-conditioned media.

3. Seed a 100-mm culture dish with reporter cells such that they will be 70% confluent the next day.
4. The following day, treat the cells with the EC_{50} dose of conditioned media.
5. 18–24 h following treatment, sort the cells by FACS with a narrow gate of the highest Venus-expressing cells.
6. Replate the sorted cells in standard growth media for at least 4 days to allow Venus expression to return to baseline. Before proceeding to step 7, you can restimulate a fraction of the cells with the EC_{50} dose of Wnt3a-conditioned media to check the integrity of the FACS.
7. Repeat the FACS and collect a narrow window of the lowest expressing cells. The entire sorted population can be collected as a single population or plated individually in a 96-well plate to create a monoclonal line (**Fig. 8.1b**).
8. Expand the line(s) and freeze back several vials of cells.

3.5. Luciferase Assay

The sensitivity and robustness of the BAR reporter allows for measuring luciferase activity in a broad range of luminometers and plate formats. BAR activity has been measured in luminometers ranging from single-tube luminometers to high-throughput plate readers. A greater than 1,000-fold dynamic range was achieved in a 384-well plate format and it is foreseeable that this can be achieved in a 1,536-well format as well. The luciferase assay reagent to be used depends on throughput of the assay. The standard low-throughput reagent is Promega's Dual-Luciferase® reporter assay system (cat. #E1910). For high-throughput assays, Promega's Dual-Glo™ (cat. #E2940) is recommended. The robustness of the BAR reporter allows you to use a fraction of Promega's suggested volume of reagent. The following low-throughput method has been optimized for use on a Berthold Mitras LB940 luminometer and the high-throughput method has been optimized on the Perkin Elmer Envision plate reader.

3.5.1. Low-Throughput Assay

In the following method, the cells were plated and treated in a 48-well plate and the luciferase activity was measured in a 96-well plate.

1. Aspirate cell culture media from each well.
2. Add 50 µL of 1× passive lysis buffer and moderately rotate for 20 min at room temperature.
3. Transfer 5 µL of each sample in duplicate to a 96-well white well plate.
4. Program your luminometer with the following settings:
   (a) Inject 10 µL of Firefly luciferase reagent.
   (b) Read total luminescence.

(c) Inject 10 µL of Stop & Glo® reagent.

(d) Read total luminescence.

5. Express data as a ratio of Firefly relative light units to Renilla relative light units.

3.5.2. High-Throughput Assay

In the following method, the cells were plated, treated, and the luciferase activity measured in a 384-well plate. The volume of culture media in each well prior to measuring luciferase activity is 40 µL.

1. Add 10 µL of Dual-Glo™ Firefly luciferase reagent using a liquid dispenser and incubate for 10 min at room temperature (if using clear bottom 384-well plates, bare nuclei will can be visualized if lysis is complete) (*see* **Note 5**).
2. Read total luminescence.
3. Add 10 µL of Dual-Glo™ Stop & Glo® luciferase reagent using a liquid dispenser and incubate for 10 min at room temperature (*see* **Note 5**).
4. Read total luminescence.
5. Express data as a ratio of Firefly relative light units to Renilla relative light units.

4. Notes

1. Two common Wnt target genes are axin2 and lef1. Real-time PCR primers for analyzing the human transcripts are as follows:

   Axin2 forward: CTCCCCACCTTGAATGAAGA.
   Axin2 reverse: TGGCTGGTGCAAAGACATAG.
   Lef1 forward: GACGAGATGATCCCCTTCAA.
   Lef1 reverse: AGGGCTCCTGAGAGGTTTGT.

2. The reporters in the lentiviral backbones cannot be used for transient reporter assays as the episomal form contains a constitutive promoter upstream of the TCF response elements that drives transcription independent of Wnt/β-catenin signaling.
3. All reporter plasmids and lentiviral helper plasmids can be obtained from the Moon lab by contacting either author.
4. The dose of Wnt3a-conditioned media and incubation time will vary based on the potency of the conditioned media. We have treated cells with Wnt3a-conditioned media for as little as 4 h and measured reporter activity above baseline. Common incubation times are 12–24 h.

5. The 10 µL of Dual-Glo™ luciferase and Stop & Glo® reagent used in Section 3.5.2 may be reduced even further. The only concern is incomplete cell lysis, which may be overcome by supplementing the Firefly reagent with Promega's passive lysis buffer.

Acknowledgments

We thank the Howard Hughes Medical Institute for funding.

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III.D. pGL3-Control Vector

The pGL3-Control Vector contains SV40 promoter and enhancer sequences, resulting in strong expression of *luc*+ in many types of mammalian cells. This plasmid is useful in monitoring transfection efficiency, in general, and is a convenient internal standard for promoter and enhancer activities expressed by pGL3 recombinants.

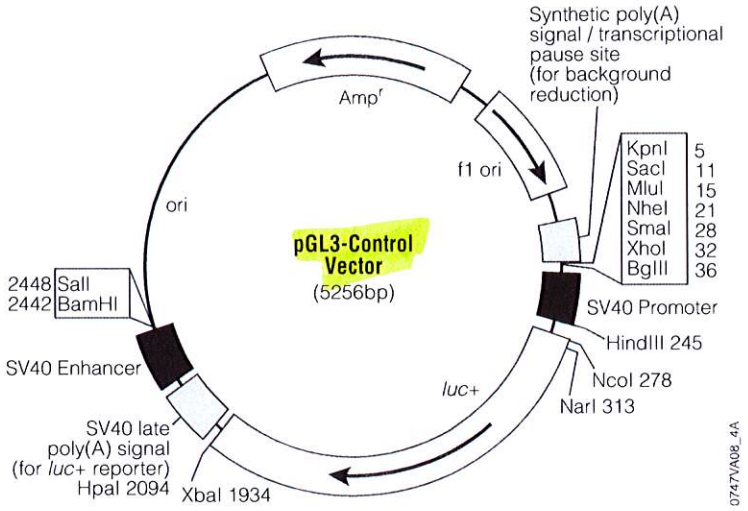


Figure 4. pGL3-Control Vector circle map. Additional description: *luc*+, cDNA encoding the modified firefly luciferase; Amp^r, gene conferring ampicillin resistance in *E. coli*; f1 ori, origin of replication derived from filamentous phage; ori, origin of plasmid replication in *E. coli*. Arrows within *luc*+ and the Amp^r gene indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA strand synthesis.

pGL3-Control Vector Sequence Reference Points:

Multiple cloning region	1–41
Promoter	48–250
Luciferase gene (*luc*+)	280–1932
GLprimer2 binding site	281–303
SV40 late poly(A) signal	1964–2185
Enhancer	2205–2441
RVprimer4 binding site	2499–2518
ColE1-derived plasmid replication origin	2756
β-lactamase gene (Amp^r)	3518–4378
f1 origin	4510–4965
upstream poly(A) signal	5096–5249
RVprimer3 binding site	5198–5217

II. Product Components

Product	Size	Cat.#
pRL-SV40 Vector	20µg	E2231

All pRL Vectors are supplied in TE buffer (pH 7.4) and are provided with a glycerol stock of bacterial strain JM109. The JM109 cells do not contain vector and are not competent cells.

Storage Conditions: Store vector DNA at –20°C and the glycerol stock of JM109 cells at –70°C.

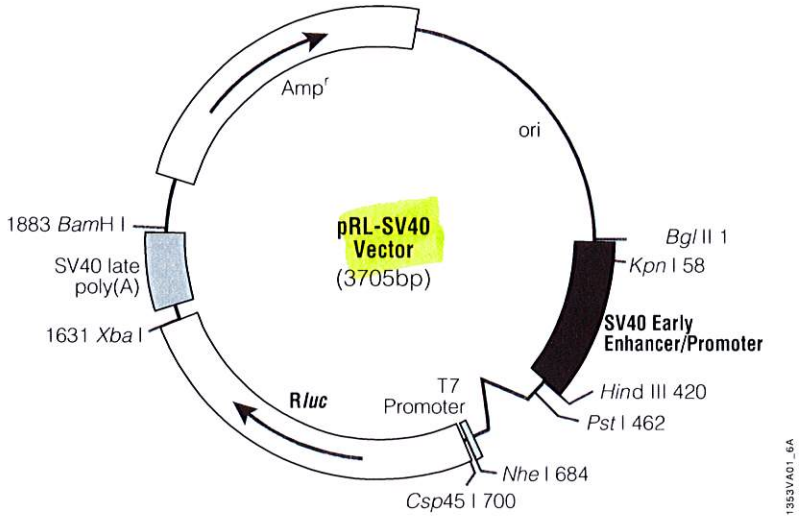


Figure 1. The pRL-SV40 Vector circle map and sequence reference points.

Sequence reference points:	
SV40 enhancer and early promoter	7–418
chimeric intron	486–622
T7 RNA polymerase promoter (–17 to +2)	666–684
T7 RNA polymerase transcription initiation site	683
R*luc* reporter gene	694–1629
SV40 late polyadenylation signal	1671–1872
β-lactamase (Amp^r) coding region	2019–2879

In addition:

- –^– indicates the position of the intron.
- R*luc* is the cDNA encoding the *Renilla* luciferase enzyme.
- Amp^r indicates the gene encoding ampicillin resistance in *E. coli.*
- ori is the origin of replication in *E. coli.*
- The arrows within the R*luc* and Amp^r genes indicate the direction of transcription.

3. Product contents

3.1. Components summary

- U-2 OS derived cells expressing the EGFP-NFATc1 fusion protein (two vials, each containing 1 x 10^6 cells), in 1 ml fetal calf serum and 10% (v/v) DMSO - NIF2029
- pCORON1000-EGFP- NFATc1 expression vector (one vial containing 10 µg DNA, at a concentration of 250 µg/ml, supplied in TE buffer: 10 mM Tris, 1 mM EDTA pH8.0) - NIF2030
- User manual

3.2. U-2 OS derived cell line expressing EGFP-NFATc1 fusion protein - NIF2029

3.2.1. U-2 OS derived parental cell line

The parental cell line U-2 OS (ATCC HTB-96) was derived from a moderately differentiated sarcoma of the tibia of a 15 year old girl (11). The U-2 OS cell line is choromosomally highly alerted, with chromosome counts in the hypertriploid range, and expresses the insulin-like growth factor I and II receptors.

3.2.2. U-2 OS derived EGFP-NFATc1 expressing cell line

U-2 OS cells were transfected with the pCORON1000 EGFP-NFATc1 vector (supplied) using the FuGENE 6 transfection method according to the manufacturer's instructions. A stable clone expressing the recombinant fusion protein was selected using 500 µg/ml Geneticin for approximately two weeks. The stable cell line was cloned and sorted using a FACS machine to obtain a uniform cell line. The passage number was set to one after FACS. Following sorting, the cells were grown for a further 8 passages before freezing. The cells tested negative for mycoplasma, bacterial and yeast contamination (testing details are available upon request).

3.3. EGFP-NFATc1 expression vector - NIF2030

The 8.6 kb plasmid, pCORON1000-EGFP-NFATc1, contains a bacterial ampicillin resistance gene and a mammalian neomycin resistance gene (see Fig 3.1.) The sequence of the construct is available on a CD, upon request.

A detailed restriction map is shown in chapter 11, appendix A.

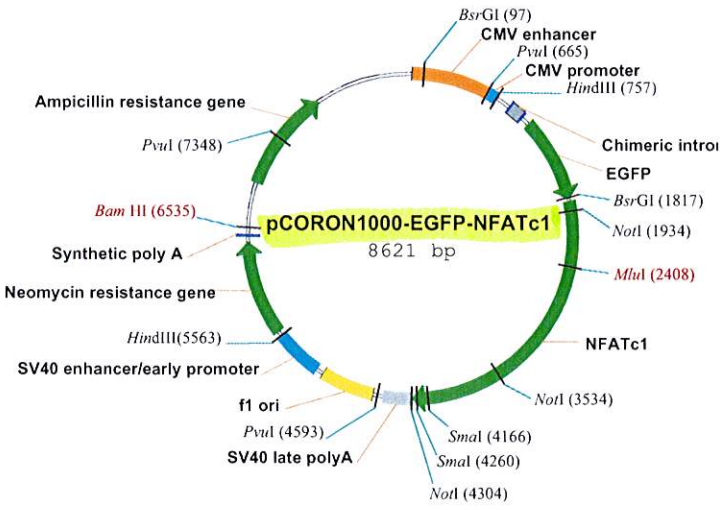


Fig 3.1.: Vector map of the supplied EGFP-NFATc1 expression vector.

GE Healthcare

Material Safety Data Sheet

Canada
English

Section 1. Chemical product and company identification

Product name **EGFP-NFAT vector; part of 'EGFP-NFAT Assay 12 Mth AEL'**

Catalogue Number 25-8010-45

Component Number NIF2030

Material uses Industrial applications: Analytical chemistry. Research.

Product type Liquid.

Validation date 10 November 2008

Print date 10 November 2008

Supplier GE Healthcare UK Ltd
Amersham Place
Little Chalfont
Buckinghamshire HP7 9NA
England
+44 0870 606 1921

In case of emergency			
	US	ChemTrec (US)	1-800-424-9300
	Canada	ChemTrec (US)	1-703-527-3887

2. Hazards identification

Physical state Liquid.

Odor Odorless.

Emergency overview No specific hazard.

NOT EXPECTED TO PRODUCE SIGNIFICANT ADVERSE HEALTH EFFECTS WHEN THE RECOMMENDED INSTRUCTIONS FOR USE ARE FOLLOWED.

No known significant effects or critical hazards. Avoid prolonged contact with eyes, skin and clothing.

Potential acute health effects

Eyes No known significant effects or critical hazards.

Skin No known significant effects or critical hazards.

Inhalation No known significant effects or critical hazards.

Ingestion No known significant effects or critical hazards.

Potential chronic health effects

Chronic effects No known significant effects or critical hazards.

Carcinogenicity No known significant effects or critical hazards.

Mutagenicity No known significant effects or critical hazards.

Teratogenicity No known significant effects or critical hazards.

Developmental effects No known significant effects or critical hazards.

Fertility effects No known significant effects or critical hazards.

Target organs Not available.

Inhalation No specific data.

Ingestion No specific data.

Skin No specific data.

Eyes No specific data.

Medical conditions aggravated by over-exposure None known.

See toxicological information (section 11)

3. Composition/information on ingredients

There are no 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.

Section 4. First aid measures

Eye contact	In case of contact with eyes, rinse immediately with plenty of water. Get medical attention if irritation occurs.
Skin contact	Wash with soap and water. Get medical attention if symptoms appear.
Inhalation	If inhaled, remove to fresh air. Get medical attention if symptoms appear.
Ingestion	Do not ingest. Get medical attention if symptoms appear.
Protection of first-aiders	No action shall be taken involving any personal risk or without suitable training.

Section 5. Fire fighting measures

Flammability of the product	In a fire or if heated, a pressure increase will occur and the container may burst.
Extinguishing media	
Suitable	Use an extinguishing agent suitable for the surrounding fire.
Not suitable	None known.
Special exposure hazards	Promptly isolate the scene by removing all persons from the vicinity of the incident if there is a fire. No action shall be taken involving any personal risk or without suitable training.
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.

Section 6. Accidental release measures

Personal precautions	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).
Environmental precautions	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	Stop leak if without risk. Move containers from spill area. Prevent entry into sewers, water courses, basements or confined areas. Wash spillages into an effluent treatment plant or proceed as follows. Contain and collect spillage with non-combustible, absorbent material e.g. sand, earth, vermiculite or diatomaceous earth and place in container for disposal according to local regulations (see section 13). Dispose of via a licensed waste disposal contractor. Note: see section 1 for emergency contact information and section 13 for waste disposal.
Small spill	Stop leak if without risk. Move containers from spill area. Dilute with water and mop up if water-soluble or absorb with an inert dry material and place in an appropriate waste disposal container. Dispose of via a licensed waste disposal contractor.

Section 7. Handling and storage

Handling	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.
Storage	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.

Section 8. Exposure controls/personal protection

Consult local authorities for acceptable exposure limits.

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	No special ventilation requirements. Good general ventilation should be sufficient to control worker exposure to airborne contaminants. If this product contains ingredients with exposure limits, use process enclosures, local exhaust ventilation or other engineering controls to keep worker exposure below any recommended or statutory limits.
Hygiene measures	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.

Section 9. Physical and chemical properties

Physical state	Liquid.
Color	Colorless.
Odor	Odorless.
Volatility	0% (v/v)
VOC	0 (g/l).
Solubility	Easily soluble in the following materials: cold water and hot water.

Section 10. Stability and reactivity

Stability	The product is stable.
Materials to avoid	No specific data.
Hazardous polymerization	Under normal conditions of storage and use, hazardous polymerization will not occur.
Conditions of reactivity	Non-flammable in the presence of the following materials or conditions: open flames, sparks and static discharge, heat, shocks and mechanical impacts, oxidizing materials, reducing materials, combustible materials, organic materials, metals, acids, alkalis and moisture. Non-explosive in the presence of the following materials or conditions: open flames, sparks and static discharge, heat, shocks and mechanical impacts, oxidizing materials, reducing materials, combustible materials, organic materials, metals, acids, alkalis and moisture.

Section 11. Toxicological information

Acute toxicity

Product/ingredient name	Result	Species	Dose	Exposure
Not available.				

Conclusion/Summary Very low toxicity to humans or animals.

Classification

Product/ingredient name	ACGIH	IARC	EPA	NIOSH	NTP	OSHA
Not available.						

Synergistic products Not available.

Section 12. Ecological information

Environmental effects	No known significant effects or critical hazards.
Octanol/water partition coefficient	Not available.
Bioconcentration factor	Not available.
Other adverse effects	No known significant effects or critical hazards.

Section 13. Disposal considerations

Waste disposal	The generation of waste should be avoided or minimized wherever possible. Empty containers or liners may retain some product residues. This material and its container must be disposed of in a safe way. 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. Avoid dispersal of spilled material and runoff and contact with soil, waterways, drains and sewers.
RCRA classification	Not available.

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 CONTROLS/PERSONAL PROTECTION for additional handling information and protection of employees.

Section 14. Transport information

International transport regulations

Not classified.

-

Section 15. Regulatory information

WHMIS (Canada)	Not controlled under WHMIS (Canada).
Canadian lists	**CEPA Toxic substances:** None of the components are listed. **Canadian ARET:** None of the components are listed. **Canadian NPRI:** None of the components are listed. **Alberta Designated Substances:** None of the components are listed. **Ontario Designated Substances:** None of the components are listed. **Quebec Designated Substances:** None of the components are listed.
Canada inventory	All components are listed or exempted.

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

EU regulations

Hazard symbol or symbols

Risk phrases	This product is not classified according to EU legislation.
Safety phrases	Not applicable.

International regulations

International lists	**Australia inventory (AICS):** All components are listed or exempted. **China inventory (IECSC):** All components are listed or exempted. **Japan inventory (ENCS):** All components are listed or exempted. **Japan inventory (ISHL):** Not determined. **Korea inventory (KECI):** All components are listed or exempted. **New Zealand Inventory of Chemicals (NZIoC):** All components are listed or exempted. **Philippines inventory (PICCS):** All components are listed or exempted.

Section 16. Other information

The customer is responsible for determining the PPE code for this material.

Indicates information that has changed from previously issued version.

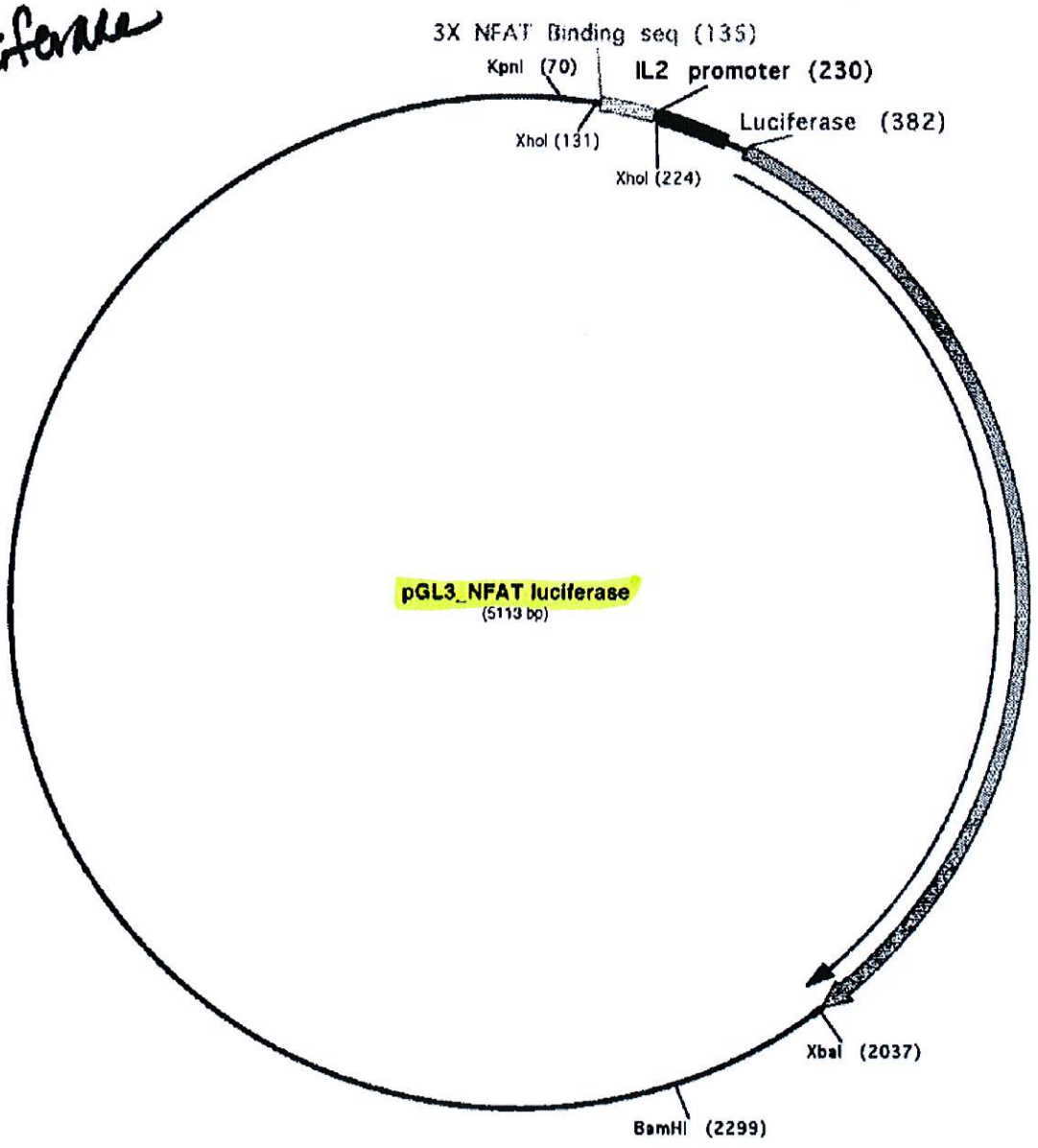
History

Date of printing	10 November 2008	**Date of previous issue**	01 February 2006
Date of issue	10 November 2008	**Version**	3

Notice to reader

To the best of our knowledge, the information contained herein is accurate. However, neither the above-named supplier, nor any of its subsidiaries, assumes any liability whatsoever for the accuracy or completeness of the information contained herein.
Final determination of suitability of any material is the sole responsibility of the user. All materials may present unknown hazards and should be used with caution. Although certain hazards are described herein, we cannot guarantee that these are the only hazards that exist.

luciferase


3X NFAT Binding seq (135)
KpnI (70)
IL2 promoter (230)
Luciferase (382)
XhoI (131)
XhoI (224)
pGL3_NFAT luciferase
(5113 bp)
XbaI (2037)
BamHI (2299)

invitrogen™

Material Safety Data Sheet

Revision Date: 21-Jan-2006

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

Product code V49320
Product name pAd/CMV/V5-DEST™ Gateway® Vector

Contact manufacturer
INVITROGEN CORPORATON
1600 FARADAY AVENUE
PO BOX 6482
CARLSBAD, CA 92008
760-603-7200

INVITROGEN CORPORATION
2270 INDUSTRIAL STREET
BURLINGTON, ONT
CANADA L7P 1A1
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
suspension

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

4. FIRST AID MEASURES

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

5. FIRE-FIGHTING MEASURES

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

6. ACCIDENTAL RELEASE MEASURES

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

7. HANDLING AND STORAGE

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

8. EXPOSURE CONTROLS / PERSONAL PROTECTION

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

9. PHYSICAL AND CHEMICAL PROPERTIES

General Information

Form	suspension

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

10. STABILITY AND REACTIVITY

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

11. TOXICOLOGICAL INFORMATION

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

12. ECOLOGICAL INFORMATION

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

13. DISPOSAL CONSIDERATIONS

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
Not regulated

Clean Air Act, Section 112 Hazardous Air Pollutants (HAPs) (see 40 CFR 61)
This product contains the following HAPs:

U.S. State Regulations

California Proposition 65
This product contains the following Proposition 65 chemicals:

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

LIST BIOLOGICAL LABORATORIES, INC.

540 DIVISION STREET ▪ CAMPBELL ▪ CALIFORNIA 95008-6906 ▪ USA
408-866-6363 ▪ 800-726-3213 ▪ FAX 408-866-6364 ▪ EMAIL info@listlabs.com
WEBSITE www.listlabs.com

Product #179A, 179B

MATERIAL SAFETY DATA SHEET
Pertussis Toxin
In Glycerol

Ingredients:

Each vial contains 50.0 µg or 200.0 µg of pertussis toxin (islet-activating protein) at a concentration of 0.2 mg/ml in 50% glycerol, 0.05 M Tris, 0.01 M glycine, 0.5 M sodium chloride, pH 7.5.

Health Hazard Data:

The LD_{50} of pertussis toxin in mice is 18 µg/kg i.p. There is no LD_{50} information for humans.

The excipients have a low hazard for normal industrial uses.

Emergency Procedures:

Pertussis toxin is degraded by the low pH in the gut and is not absorbed. If swallowing occurs, induce vomiting.

If skin pricking should occur, induce bleeding and flush with copious amounts of water. If i.v. or i.m. injection should occur, consult a physician. Attempt to obtain hyperimmune globulin to pertussis from the CDC. In an adult immunized versus whooping cough, no long term ill effects are likely to result.

Handling:

Pertussis toxin, in spite of its name, is not considered hazardous. However, as with any biochemical, it should be handled by trained personnel using good laboratory technique. Observe the following practices when working with pertussis toxin: Special care should be taken when working in conjunction with hypodermic needles. Wear protective gloves, avoid contact with cuts or wounds, avoid inhalation, do not mouth pipet, and flush thoroughly any area of the body that comes in contact with this product. Only individuals who were immunized in childhood against whooping cough should work with this product. This product is intended for research purposes only.

Stability:

Stable for months when stored at -20°C.

Deactivation:

Boil at 100°C for 15 to 30 minutes.

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	Pertussis toxin
Proposed Use Dose:	0.1 µg
Proposed Storage Dose:	50 µg
LD_{50} (species):	18 µg

Calculation:

18 µg/kg x 50 kg/person

Dose per person based on LD_{50} in µg = 900

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = 90

Comments/Recommendations: