Modification Form for Permit BIO-UWO-0081

Permit Holder: Gregory Kelly

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

Leanne Sandieson

Jason Hwang

Additional Personnel
(Please list additional personnel here)

Gregory Golenia

	Please stroke out any approved Biological Agent(s) to be removed	Write additional Biological Agent(s) for approval below. Give the full name
Approved Microorganisms	E.coli Top 10	
Approved Primary and Established Cells	Human [established]: U87MG. Rodent [established]: F9 cells. Non-human primate [established]: Cos 7.	
Approved Use of Human Source Material		
Approved Genetic Modifications (Plasmids/Vectors)	[plasmids]: pCMV sport6, pcDNA3.1+. SV 40 Large T antigen.	pEX-YFP-hLC3WT pEX-YFP-hLC3ΔG pAd-Easy® - mCherry-Tubulin LamP1-RFP
Approved Use of Animals	Danio rerio	
Approved Biological Toxin(s)		
Approved Gene Therapy		
Approved Plants and Insects		

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

As the Principal Investigator, I have ensured that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.shs.uwo.ca/workplace/newposition.htm

Signature of Permit Holder: _____

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

Date of Last Biohazardous Agents Registry Form: Nov 15, 2010

Date of Last Modification (if applicable): _____

BioSafety Officer(s): _____

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

The plasmids requested will be stored at -20°C. They post no health hazard to humans and will be used according to established protocols, employed and approved in the Kelly lab, to transfect F9 cells and Cos 7 cells.

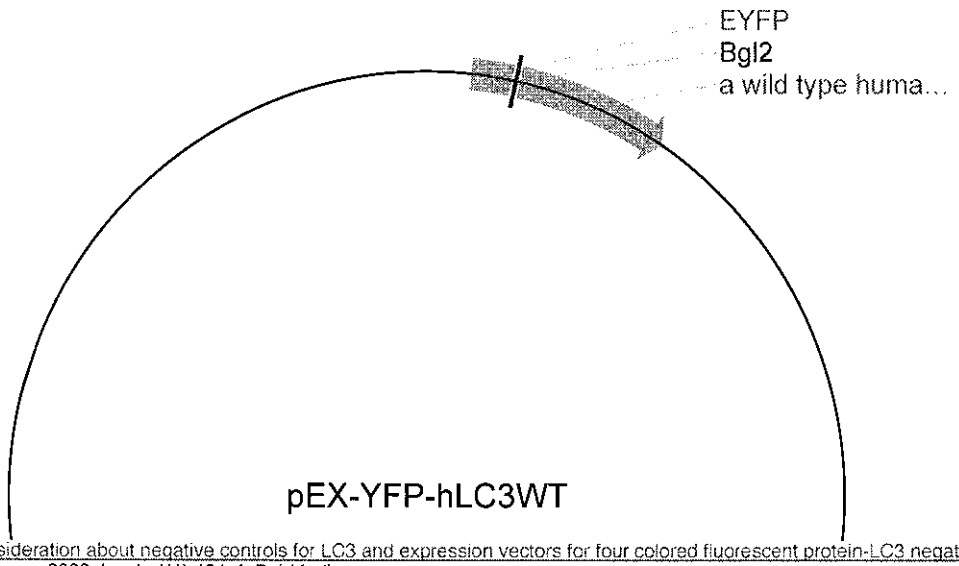
addgene
A better way to share plasmids

Browse > Isei Tanida > Tanida et al > pEX-YFP-hLC3WT

Plasmid 24989: pEX-YFP-hLC3WT

Gene/insert name:	a wild type human MAP1-LC3B fused with EYFP
Insert size:	378
Species:	H. sapiens (human)
Fusion protein or tag:	EYFP
Terminal:	N terminal on backbone
Vector backbone:	pCXN2 (Search Vector Database)
Vector type:	Mammalian Expression
Backbone size w/o insert:	6000
Cloning site 5':	Bgl2
Site destroyed during cloning:	No
Cloning site 3':	SalI/XhoI
Site destroyed during cloning:	Yes
5' sequencing primer:	GFP-C List of Sequencing Primers
Bacterial resistance:	Ampicillin
Growth strain:	DH5alpha
Growth temperature (°C):	37
High or low copy:	High Copy
Selectable markers:	Neomycin
Sequence:	View sequences (2)
Map:	View map
Principal Investigator:	Isei Tanida
Terms and Licenses:	MTA

Addgene has sequenced a portion of this plasmid for verification. Click here for the sequencing result.



Article: Consideration about negative controls for LC3 and expression vectors for four colored fluorescent protein-LC3 negative controls. Tanida et al (Autophagy. 2008 Jan 1. 4(1):131-4. PubMed)

Please acknowledge the principal investigator and cite this article if you use this plasmid in a publication. Also, please include the text "Addgene plasmid 24989" in your Materials and Methods section.

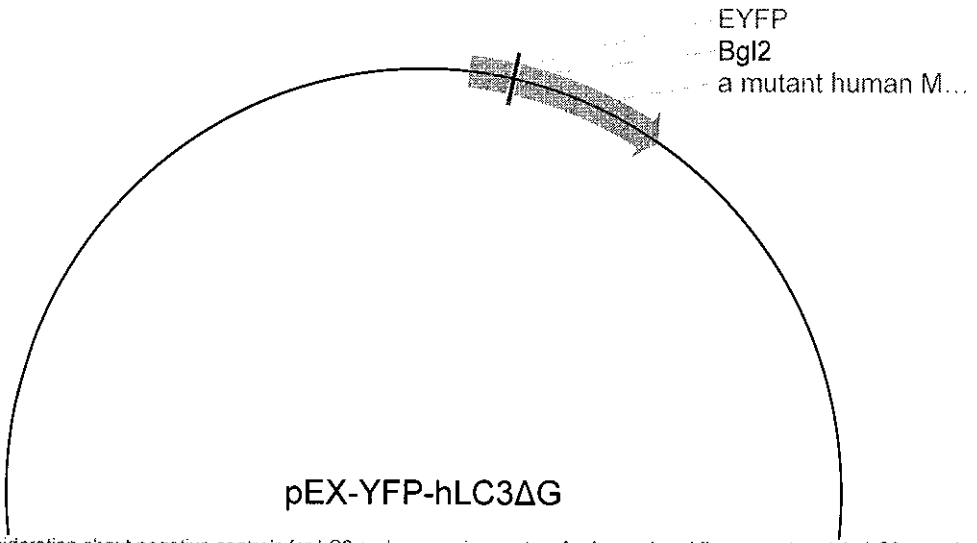
addgene
A better way to share plasmids

Browse > Isei Tanida > Tanida et al > pEX-YFP-hLC3ΔG

Plasmid 24990: pEX-YFP-hLC3ΔG

Gene/insert name:	a mutant human MAP1-LC3B fused with EYFP
Insert size:	375
Species:	H. sapiens (human)
Fusion protein or tag:	EYFP
Terminal:	N terminal on backbone
Mutation:	deleted Gly at 121
Vector backbone:	pCXN2 (Search Vector Database)
Vector type:	Mammalian Expression
Backbone size w/o insert:	6000
Cloning site 5':	Bgl2
Site destroyed during cloning:	No
Cloning site 3':	SalI/XhoI
Site destroyed during cloning:	Yes
5' sequencing primer:	GFP-C List of Sequencing Primers
Bacterial resistance:	Ampicillin
Growth strain:	DH5alpha
Growth temperature (°C):	37
High or low copy:	High Copy
Selectable markers:	Neomycin
Sequence:	View sequences (2)
Map:	View map
Principal Investigator:	Isei Tanida
Terms and Licenses:	MTA

Addgene has sequenced a portion of this plasmid for verification. Click here for the sequencing result.



Article: Consideration about negative controls for LC3 and expression vectors for four colored fluorescent protein-LC3 negative controls. Tanida et al (Autophagy. 2008 Jan 1. 4(1):131-4. PubMed)

Please acknowledge the principal investigator and cite this article if you use this plasmid in a publication. Also, please include the text "Addgene plasmid 24990" in your Materials and Methods section.

addgene
A better way to share plasmids

Browse > Torsten Wittmann > Matov et al > pAdEasy® mCherry-Tubulin

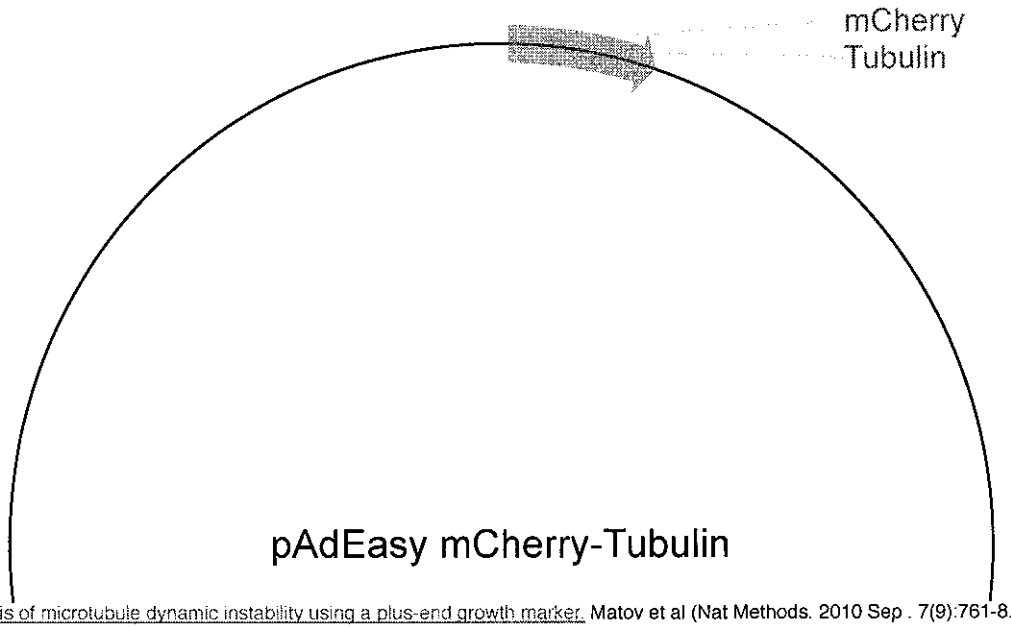
Plasmid 26767: pAdEasy® mCherry-Tubulin

Gene/insert name:	Tubulin
Alt name:	TUBA1B
Insert size:	1600
Species:	H. sapiens (human)
Entrez Gene:	TUBA1B (K-ALPHA-1)
Fusion protein or tag:	mCherry
Terminal:	N terminal on insert
Vector backbone:	pAdEasy®-1 (Search Vector Database)
Backbone manufacturer:	Stratagene, ATCC
Vector type:	Mammalian Expression
Backbone size w/o insert:	33414
5' sequencing primer:	CMV-F, mCherry-F List of Sequencing Primers
Bacterial resistance:	Kanamycin
Growth strain:	DH5alpha
Growth temperature (°C):	37
High or low copy:	Low Copy
Sequence:	View sequences (1)
Principal Investigator:	Torsten Wittmann
Terms and Licenses:	MTA Clontech Limited Use Label License

Comments: mCherry-Tubulin was cloned into pShuttle-CMV between KpnI and NotI then recombined into pAdEasy®-1

AdEasy® is a registered trademark of the Johns Hopkins University.

Addgene has sequenced a portion of this plasmid for verification. Click here for the sequencing result.



Article: Analysis of microtubule dynamic instability using a plus-end growth marker. Matov et al (Nat Methods. 2010 Sep . 7(9):761-8. PubMed)

Please acknowledge the principal investigator and cite this article if you use this plasmid in a publication. Also, please include the text "Addgene plasmid 26767" in your Materials and Methods section.

addgene
A better way to share plasmids

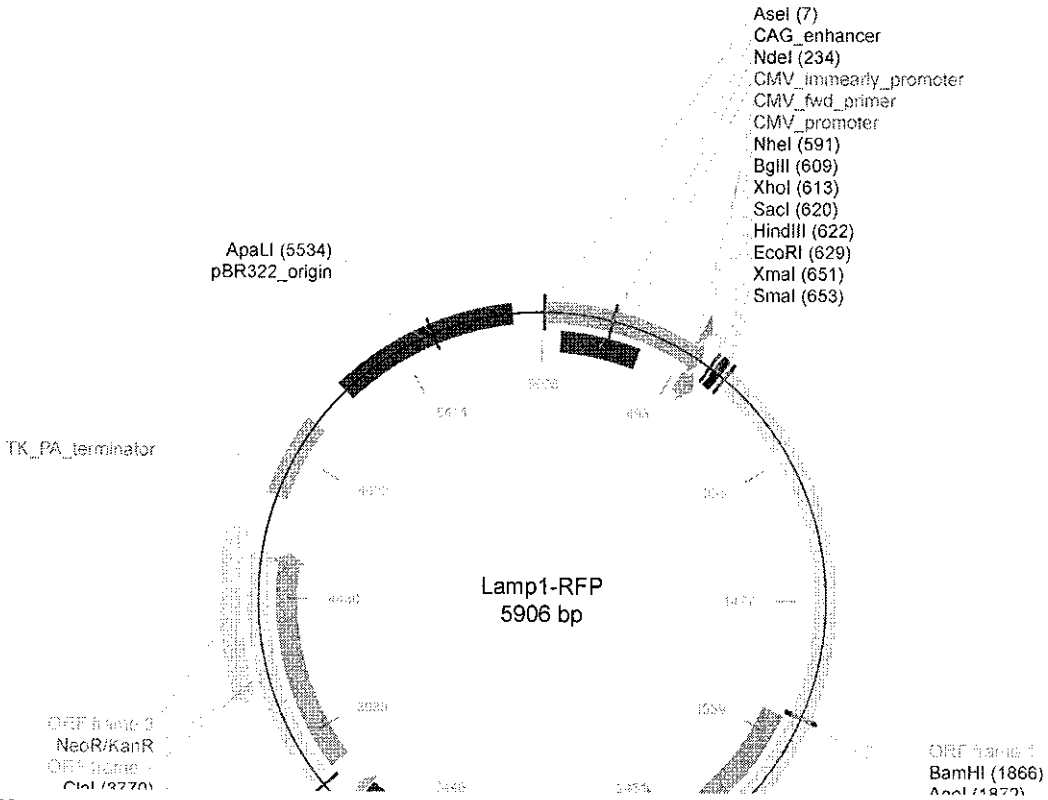
Browse > Walther Mothes > Sherer et al > Lamp1-RFP

Plasmid 1817: Lamp1-RFP

Gene/insert name:	lysosome associated membrane protein 1
Alt name:	Lamp-1
Insert size:	1200
Species:	R. norvegicus (rat)
Entrez Gene:	Lamp1 (LGP120)
Fusion protein or tag:	RFP
Terminal:	C terminal on backbone
Mutation:	D50E
Vector backbone:	Modified Clontech Plasmid (Search Vector Database)
Vector type:	Mammalian Expression
Backbone size w/o insert:	4700
Cloning site 5':	EcoRI
Site destroyed during cloning:	No
Cloning site 3':	BamHI
Site destroyed during cloning:	No
5' sequencing primer:	CMV Forward List of Sequencing Primers
3' sequencing primer:	DsRed1-N
Bacterial resistance:	Kanamycin
Growth strain:	DH5alpha
Growth temperature (°C):	37
High or low copy:	High Copy
Selectable markers:	Neomycin
Person or lab that originally cloned the gene/insert:	Norma Andrews provided Lamp-1
Sequence:	View sequences (3)
Principal Investigator:	Walther Mothes
Terms and Licenses:	MTA Clontech Limited Use Label License

Comments: Lamp-1 sequence contains a D50E mutation, which is not important for function of plasmid. There is also a silent mutation at bp#715 compared to GenBank NM_012857.1

Addgene has sequenced a portion of this plasmid for verification. Click here for the sequencing result.



Feature Name	Start	End
CMV_immearly_promoter	10	562
CAG_enhancer	65	352
CMV_fwd_primer	519	539
CMV_promoter	520	589
mRFP1	1888	2559
I197 (mTangerine)	2461	2487
dsRed1_C_primer	2477	2500
EBV_rev_primer	2785	2804
f1_origin	3260	2954
AmpR_promoter	3339	3367
pBABE_3_primer	3453	3433
SV40_enhancer	3654	3439
SV40_promoter	3451	3719
SV40_origin	3618	3695
SV40pro_F_primer	3680	3699
NeoR/KanR	3805	4593
TK_PA_terminator	4771	5040
pBR322_origin	5188	5807

ORF	Start	End
ORF frame 1	643	2571
ORF frame 1	3802	4596
ORF frame 3	4647	4111

Enzyme Name	Cut
AseI	7

Enzyme Name	Cut
NdeI	234
NheI	591
BglII	609
XhoI	613
SacI	620
HindIII	622
EcoRI	629
SmaI	653
XmaI	651
BamHI	1866
AgeI	1872
XbaI	2584
HpaI	2693
AflII	2812
ClaI	3770
ApaLI	5534

Article: Visualization of retroviral replication in living cells reveals budding into multivesicular bodies. Sherer et al (Traffic 2003 Nov;4(11):785-801. PubMed)

Please acknowledge the principal investigator and cite this article if you use this plasmid in a publication. Also, please include the text "Addgene plasmid 1817" in your Materials and Methods section.

THE UNIVERSITY OF WESTERN ONTARIO
BIOLOGICAL AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: April 9, 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 biohazardous 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 biohazards 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 Biohazard 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: Dr G.M. Kelly
DEPARTMENT: Biology
ADDRESS: 359 WSC
PHONE NUMBER: 83121
EMERGENCY PHONE NUMBER(S): 661-7535
EMAIL: gkelly@uwo.ca

Location of experimental work to be carried out: Building(s) WSC Room(s) 355

*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 12.0, Approvals).

FUNDING AGENCY/AGENCIES: NSERC
GRANT TITLE(S): Cell Signaling crosstalk in Development

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED.

Names of all personnel working under Principal Investigators supervision in this location:
Jason Huang - Ph.D student
Shaun Symons - M.Sc Student
Leanne Sandieson - M.Sc student

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? Ø 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 Ø 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
E. coli TOP10 N.C.	O Yes Ø No	O Yes Ø No	O Yes Ø No	1. l. tre	Invitrogen	Ø 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
	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? Ø 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 Ø No		Not applicable
Rodent	O Yes Ø No		
Non-human primate	O Yes Ø No		
Other (specify)	O Yes Ø No		

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

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Supplier / Source
Human	✓Yes O No	U87mG	UWO
Rodent	O Yes O No	F9 cells	ATCC
Non-human primate	✓Yes O No	COS-7	ATCC
Other (specify)	O Yes ✓No		

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

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required ✓1 ✓level 2s (COS-7 cells) 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 ✓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 ✓No O Unknown		O 1 O 2 O 2+ O 3
Human Blood (fraction) or other Body Fluid		O Yes ✓No O Unknown		O 1 O 2 O 2+ O 3
Human Organs or Tissues (unpreserved)		O Yes ✓No 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? ✓YES O NO If no, please proceed to Section 5.0

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

Bacteria Used for Cloning *	Plasmid(s) *	Source of Plasmid	Gene Transfected	Describe the change that results
E. coli	pCMV SPORT6 pcDNA3.1+	Origene	Gata6, Foxa2, Fzd7	plasmid propagation

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

See E-mail

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

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

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results

* Please attach a Material Safety Data Sheet or equivalent.

4.4 Will genetic sequences from the following be involved?

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

4.5 Will virus be replication defective? ⊘YES O NO

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

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

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials be conducted involving a biological agent? O YES ⊘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? ⊘YES O NO If no, please proceed to section 7.0

6.2 Name of animal species to be used Danio rerio

6.3 AUS protocol # 2007-109

6.4 Will any of the agents listed in section 4.0 be used in live animals ⊘YES, specify: injected into embryos. O NO

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

See attached e-mail.

* DESCRIPTION MUST BE ATTACHED TO THIS FOR

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 ✓O No If no, please proceed to section 8.0

7.2 Please specify the animal(s) used:

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

8.0 Biological Toxins

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

8.2 If YES, please name the toxin(s)_____
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_____

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

8.5 How much of the toxin is stored*? _____

8.6 Will any biological toxins be used in live animals? O YES, Please provide details:_____ O 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 ✓O 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:
_____
_____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

10.0 Plants

10.1 Do you use plants? O YES ⊘NO If no, please proceed to Section 11.0

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

10.3 What is the origin of the 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_____ ⊘NO
If no, please proceed to Section 12.0

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

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

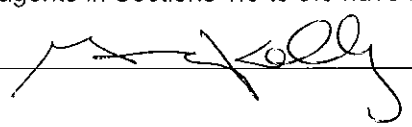
11.4 Has the import permit been sent to OHS? O YES, please provide permit # _____ ⊘NO

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 biohazardous agents in Sections 1.0 to 9.0 have been trained.

SIGNATURE 

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

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 ☑ 2 O 2+ O 3

13.2 Has the facility been certified by OHS for this level of containment?
☑ YES, permit # if on-campus BIO-UWO-0081
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: July 12/10

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.

_____
_____
_____

14.3 Please outline what will be done if there is an exp
such as a needlestick injury:

See E-mail

_____
_____

15.0 Approvals

1) UWO Biohazard Subcommittee: SIGNATURE: _____
Date: 15 Nov 10

2) Safety Officer for the University of Western Ontario
SIGNATURE: J Stanley
Date: NOV 12/10

3) Safety Officer for Institution where experiments will take place (if not UWO):
SIGNATURE: _____
Date: _____

Approval Number: BIO-UWO-0081 Expiry Date (3 years from Approval): 14 Nov 2013

Special Conditions of Approval:

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

-------- Original Message --------
Subject: Re: Biological Agents Registry Form: Kelly
Date: Wed, 03 Nov 2010 12:33:55 -0400
From: Greg Kelly <gkelly@uwo.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>

E-mail

Hi Jennifer:

I found 14.3

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

We introduce plasmids using the standard heat shock approach applied to calcium competent E. coli. The scientist wears gloves and no needles or sharp objects are used. The E. coli is a lab strain that poses no pathological problems to humans.

I hope that is sufficient. If not, can you tell me what others do for cloning with E. coli and I will amend my protocol to fit with SOPs.

Thanks,

Greg

--
Dr. G.M. Kelly
Professor of Biology
Adjunct Professor of Paediatrics & Associated Scientist, CHRI
University of Western Ontario, London, ON CANADA N6A 5B7

Phone:(519) 661-3121
Fax: (519) 661-3935

------- Original Message --------
Subject: Re: FW: Biological Agents Registry Form: Kelly
Date: Tue, 12 Oct 2010 11:07:15 -0400
From: Jason Hwang <jhwang5@uwo.ca>
To:jstanle2@uwo.ca

E-mail

Hi Jennifer,

Greg FWD the email to me and I guess I will be addressing the changes.

1. Sorry about the mix-up; COS7 are Level 2.
Gene transfected: sFRP5
Changes that result: as far as we know, the cells will just overproduce the gene/protein.

14.2. Nothing unique to the agent, normal risk reduction measures will suffice.
14.3 Standard First Aid measures apply, your own body's immune system will take care of the rest.

Please let me know if you have any other questions.

Jason

n 8/12/2010 8:09 AM, Greg Kelly wrote:
> Hi Jennifer:
>
>
> I didn't make a copy so can you please tell me the name of the plasmid
> in 4.2. We did include the MSDS on the plasmids we routinely used, but not
> certain about the one(s) you mentioned. As for #2, we inject those plasmids
> into embryos, but they are for transient expression analysis, i.e., they are
> not incorporated into the fish's genome.
> Let me know if there is anything else.
>
> Cheers,
>
> Greg

Section 6.0

invitrogen

Material Safety Data Sheet

Revision Date: 27-Dec-2006

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

Product code C505003
Product name ONE SHOT TOP10 P3 CHEMICALLY COMPETENT E COLI

Contact manufacturer
INVITROGEN CORPORATON
1600 FARADAY AVENUE
PO BOX 6482
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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

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

Cell Biology

ATCC® Number:	**HTB-14™**	Order this Item	Price:	**$256.00**

Designations:	U-87 MG
Depositors:	J Ponten
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens* (human)
Morphology:	epithelial 
Source:	**Organ:** brain **Tumor Stage:** classified as grade IV as of 2007 **Disease:** glioblastoma; astrocytoma
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 (Nucleofection technology from Lonza Roche FuGENE® Transfection Reagents)
Tumorigenic:	Yes
Antigen Expression:	Blood Type A, Rh+
DNA Profile (STR):	Amelogenin: X CSF1PO: 10,11 D13S317: 8,11 D7S820: 8,9 D5S818: 11,12 D16S539: 12 vWA: 15,17 THO1: 9.3 TPOX: 8

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BioProducts

Cell, microbial and molecular genomics products for the life sciences

BioServices

Bio-materials management; basic

References:

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Cell Biology

ATCC® Number:	**CRL-1720™**	Order this Item	Price:	**$365.00**

Designations:	F9
Depositors:	S Strickland
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Mus musculus* (mouse)
Morphology:	epithelial
Source:	**Organ:** testis **Strain:** 129 **Disease:** embryonal carcinoma; testicular teratoma
Cellular Products:	plasminogen activator; laminin; type IV collagen
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 (Roche FuGENE® Transfection Reagents)
Age:	embryo
Comments:	F9 cells can be stimulated to differentiate into parietal endoderm in the presence of retinoic acid and dibutyryl cyclic AMP (cAMP). Differentiating cells synthesize plasminogen activator, laminin and type IV collagen. cAMP is active only on cells that have been treated with retinoic acid. The cells maintain three copies of the beta 1 integrin gene. Tested and found negative for ectromelia virus (mousepox).
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

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BioProducts

Cell, microbial and molecular genomics products for the life sciences

BioServices

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

BioStandards

Biological Reference

References:

1160: Strickland S, et al. Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. Cell 21: 347-355, 1980. PubMed: 6250719

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Cell Biology

ATCC® Number:	**CRL-1651™**	Order this Item	Price:	**$269.00**

Designations:	COS-7
Depositors:	Y Gluzman
Biosafety Level:	2 [Cells Contain SV-40 viral DNA sequences]
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 (Nucleofection technology from Lonza Roche FuGENE® Transfection Reagents)
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.
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

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Product Information Sheet

8794326
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CELL SIGNALING CROSSTALK IN DEVELOPMENT

Overview: Signal transduction affects every facet of cell physiology and with the myriad of biochemical processes responding to a signal, the task of teasing apart a particular pathway is daunting. My research goal is to elucidate how signaling pathways are modified and modulated by the cell. In particular, I am interested in Wnt proteins, which are involved in almost every aspect of development and at the centre of numerous diseases[1]. When a Wnt, of which there are 19 in mammals, is secreted from a cell a signaling cascade is initiated when it binds to at least one of 10 different Frizzled (Fz) receptors or to a non-Fz receptor. Ligand-receptor selectivity acting through Dishevelled (Dvl) diverts the signal toward inducing changes in gene expression or toward the restructuring of the cytoskeleton.

We reported recently on Wnt6 in a cell model that recapitulates extraembryonic endoderm formation in the mouse embryo[2]. In chick and *Xenopus* embryos Wnt6 signals to both Dvl-directed pathways[3-5] and its up-regulation in murine stem cells correlates to self-renewal[6]. *Wnt6* and *Wnt6a* are present in the zebrafish genome[7], but there has been no definitive report on their expression or function. Many unanswered questions remain regarding the role of Wnt6 in mammalian and Wnt6 and 6a in zebrafish development and a series of experiments to address this are proposed within this application.

Background Literature: Wnts are secreted lipoglycoproteins that signal through G-protein coupled Fz receptors and LDL-receptor related protein (LRP) co-receptors[8,9]. Three individual signaling pathways have been described as canonical Wnt/β-catenin, non-canonical Wnt/cGMP/Ca^{2+} and Wnt/c-Jun N-terminal kinase (JNK)/planar cell polarity (PCP), but their individual nature is in question. Common players exist in each pathway, some forming scaffolds comprised of the proteins Dvl, Axin and β-arrestin[10,11]. In the canonical pathway, Wnt bound to Fz and LRP5/6 uses Dvl-1, -2 or -3 to dismantle a complex that includes the APC tumour suppressor, glycogen synthase kinase-3 (GSK-3β), Axin and β-catenin[11]. Without the complex β-catenin translocates to the nucleus where it acts with T-cell factor (Tcf) to regulate the expression of target genes[8]. Activation of the PCP pathway causes Dvl to signal to Rho and Rho-kinase or through Rac to JNK, which reorganizes the cytoskeleton effecting changes in cell polarity and motility[12]. Activating these multi-functional proteins has more extensive ramifications. For instance, Rac1 controls actin polymerization, but it also affects gene expression by JNK and p38 MAPK signaling, generates reactive oxygen species (ROS) by NOX activation and facilitates nuclear translocation of β-catenin via JNK[13-15]. Thus, an extensive network forms as a result of Wnt signaling through Dvl(s) to numerous downstream effectors.

Dissecting the Wnt network has been difficult, partly as a result of having a number of ligands in excess of available receptors. In fact, multiple Wnts bind to a given Fz. For example, in zebrafish embryos Wnt11 binds to Fz7 for PCP signaling, whereas in chick embryos Wnt6 binds to Fz7 to initiate canonical signaling[5,16]. In contrast, Wnt5a activation of PCP is mediated by Fz2 in zebrafish embryos, whereas Wnt5a signals via Fz3 in melanoma cells[17,18]. Thus, caution must be exercised when assigning definitive pathways to a ligand or receptor. With over 20 suspected Dvl-interacting proteins[11] the potential for extensive crosstalk from other signaling pathways only adds to the confusion. Crosstalk converges on Wnt/Dvl signaling networks at many places and is not limited to protein:protein interactions. ROS for instance, including superoxides, hydroxyl radicals and H_2O_2, are active participants in signaling and like Wnts are linked to proliferation and differentiation in normal development and tumour progression[19,20]. ROS are by-products of aerobic respiration, but are also produced in response to G-protein coupled receptor signaling from $G\alpha_{12/13}$ subunits leading to Rac1 and NOX activation[21]. ROS activation of the β-catenin and PCP pathways is mediated in part by the direct oxidation and subsequent release of nucleoredoxin from Dvl[22,23]. H_2O_2 also inactivates the PTEN tumour suppressor leading to increased Akt activity[24], which negatively regulates GSK-3 causing gastrulation defects in zebrafish[25]. Finally, PTEN and GSK-3 negatively regulate Wnt target genes required for mitosis[26]. However, Wnt activation of Akt[27], which acts directly on GSK-3 or indirectly by activating the Rac1/NOX axis to produce ROS, can also inhibit PTEN.

$G\alpha_{13}$ signaling and ROS and their involvement with Wnt pathways have been overlooked. In contrast, $G\alpha_{13}$ is well known for its ability to signal through RhoA, Rac1 and Cdc42 to JNK, which affects cytoskeletal changes during cell proliferation and migration. The importance of $G\alpha_{13}$ in development is evident by the response of F9 cells to retinoic acid (RA), which recapitulates a step in primitive endoderm (PrE) formation in mouse[28,29]. RA, a potent morphogen in vertebrate embryos, has pleiotropic effects ranging from inducing the GATA and forkhead box (Fox) transcription factors required for PrE formation to stimulating ERK1/2 and PI3K activities through non-genomic mechanisms[30,31]. In F9 cells, RA signaling from $G\alpha_{13}$ through p115RhoGEF to JNK is necessary for PCP and is accompanied by Akt activation[32,33]. In zebrafish, $G\alpha_{13}$ signaling acts in parallel to PCP signaling and is required to coordinate cell movements during gastrulation[34]. Details coupling $G\alpha_{13}$ activity with agonist-bound receptors are unclear, but evidence exists that RA increases $G\alpha_{13}$ activity[28]. RA also up-regulates GATA-6 and Foxa2 in F9 cells[35] and in silico analysis reveals putative GATA-6 and Foxa2 binding sites in the mouse Wnt6 promoter. Wnt6 activates PCP and Wnt/β-catenin pathways during chick and *Xenopus* organogenesis[4,5,36] and although it signals to β-catenin in RA-treated F9 cells[2], we are proposing it also participates in PCP in these cells. In F9 cells there is precedence for a one Wnt/one receptor/two pathway response, where Wnt3a signaling to ectopically expressed rat Fz1 and the $G\alpha_{q/o}$ subunits, stimulates β-catenin and PCP pathways[37]. One caveat, however, is that the application of Wnt3a-conditioned media (CM) to untransfected RA-treated F9 cells blocks activation of RA-responsive genes[38]! Nevertheless, the ability of F9 cells to respond to RA by activating Wnt/β-catenin, Wnt/PCP and PI3K/Akt signaling pathways, while expressing endo-A intermediate filaments, which are criteria for PrE differentiation, provides a unique platform by which one can investigate the crosstalk that occurs between signaling pathways. Extending these studies to the zebrafish will reveal how common this crosstalk is in development. *My long term goal is to elucidate how crosstalk from other pathways converges on the Wnt6 network to influence how cells are induced in embryos.*

Progress & Research Activities: We use two models to investigate signaling events during embryonic development[2,25,29,39-42]. Cell movements in zebrafish embryos are affected following the knockdown of the myosin regulatory light chain interacting protein (Mir)[39]. Two-hybrid analysis revealed Mir and Annexin V act downstream of Wnt11/Fz7 in PCP signaling[39,40]. We have also used transient transgenesis and pharmacological inhibitors to identify an Akt-PTEN feedback mechanism that affects gastrulation[25]. Reduced levels of p-Akt allow GSK-3 to target PTEN for inactivation, whereas high levels inhibit GSK-3, allowing PTEN activity to increase, thereby resetting pro-proliferation and pro-apoptotic signaling required in gastrulating embryos.

In F9 cells we showed *Wnt6* is up-regulated by RA during PrE formation[2]. In the absence of RA, F9 cells expressing *pCMV-Wnt6* or treated with Wnt6-CM accumulate nuclear β-catenin. We cloned the mouse *Wnt6* minimal promoter into a firefly luciferase vector for a Dual Reporter Assay and are in the process of stably expressing it and a non-GATA responsive *Renilla* reporter in COS-7 cells. In our assay we will test GATA-6 and Foxa2 constructs, which induce PrE markers when expressed in F9 cells. A *Wnt6* cDNA is being cloned behind the *Wnt6* promoter and will be transfected in the presence or absence of the GATA-6/Foxa2 constructs in F9 cells stably expressing the Super8XTOPFLASH reporter to detect β-catenin/Tcf-dependent transcription. Luciferase activity will confirm that nuclear β-catenin accumulation seen in Wnt6-CM-treated F9 cells partakes in Tcf-dependent transcription. In the search to define the RA-Wnt6-$G\alpha_{13}$ signaling axis, we reported on the influence p115RGS, a dominant negative mutant of p115RhoGEF, has on blocking RA-$G\alpha_{13}$ signaling, and the ability of LiCl (a GSK-3 inhibitor) to promote PrE[42]. We also found RA up-regulates *Fz2* and *7*, but have not confirmed that they encode bona fide Wnt6 receptors. Finally, we found that H_2O_2 causes F9 cells to undergo morphological changes and express PrE markers, and in zebrafish embryos H_2O_2 causes gastrulation defects. Together, we will use the zebrafish embryo and F9 cell model to identify common and unique features of the Wnt6 network that are influenced by crosstalk between ROS and the PI3K/Akt/PTEN signaling axis.

Hypothesis & Objectives: I hypothesize that a Wnt6 signaling network is augmented and fine tuned by the activities of ROS and the coordinated regulation of PTEN by PI3K/Akt. To address this hypothesis we will find answers to the following questions:

1) What are the consequences of altering *Wnt6* expression on PrE formation?
2) What effect does altering Wnt6 expression have on zebrafish embryogenesis?
3) To what extent do ROS have on augmenting Wnt signaling?
4) How much influence does Akt/PTEN crosstalk have on the Wnt/β-catenin pathway?

Proposed Research Plan:

Aim 1: What are the consequences of altering Wnt6 expression on PrE formation?

Since Wnts signal through Gα subunits and *Wnt6* is up-regulated in response to RA, I hypothesize that the RA-Wnt6 signaling axis extends to $G\alpha_{13}$, activating β-catenin and PCP pathways for PrE formation. There is no precedent for Wnt signaling to $G\alpha_{13}$, which makes my programme very exciting yet controversial. To investigate how crosstalk influences RA-Wnt6 signaling, it is important to identify the effect on PrE when levels of Wnt6 and its bona fide receptor(s) are depleted. *J. Hwang* and *B. Cadesky* (M.Sc. yr. 2) will use a knockdown approach to address these questions. To begin, three siRNAs will be tested for their efficacy in suppressing *Wnt6* expression. Knockdown efficiency will be determined by RT-PCR. These studies will benefit from Wnt6 antibodies as the ones currently available are unreliable. Towards that end, antibodies will be made using a commercial facility and then tested on immunoblots and by immunocytochemistry. Cells transfected with *Wnt6* siRNAs or sham transfected will be treated with RA and assayed by immunocytochemistry for endo-A intermediate filaments and β-catenin (canonical signaling) and by immunoprecipitation and immunoblot analysis to measure JNK activity (EZ-Detect, Pierce) indicative of PCP signaling. Rescuing the Wnt6 knockdown will be achieved using Wnt6-CM. The absence of differentiation markers following knockdown will suggest Wnt6 is necessary for activating β-catenin and PCP pathways, whereas the absence of nuclear β-catenin alone will indicate RA induces an alternate pathway to stimulate PCP. Wnt5a is a candidate for its involvement in PCP and Wnt/cGMP/Ca^{2+} pathways, but there are conflicting reports about it being up-regulated by RA in F9 cells[43,44]. If Wnt5a is involved in PrE formation, the p115RGS results showing constraint at the $G\alpha_{13}$ branch point would suggest Wnt5a and 6 utilize the same receptor or different receptors, but each signaling to $G\alpha_{13}$. Eliminating crosstalk from other Gα subunits including $G\alpha_{i/o/i}$ will be done using pertussis toxin or YM-254890 (Astellas) to selectively inhibit $Ga_{q/11}$. The knockdown results and eliminating the involvement of other Gα subunits will influence our decision as to whether to first silence the putative Wnt6 receptors (Fz7/2) or Wnt5a. Fz7 silencing is expected to phenocopy the Wnt6 knockdown and to prevent nuclear β-catenin accumulation, but we have no preconceived notions for Fz2. Together, a systematic knockdown of Wnt and Fz will define what in the Wnt6 network participates in and is accessible to signaling crosstalk. I remain confident Wnt6 signals to two pathways via $G\alpha_{13}$, but in the event my hypothesis is wrong, progress on the grant will not be affected as the focus remains on crosstalk converging on the Wnt6 network, specifically the β-catenin component.

Aim 2: What effect does altering Wnt6 expression have on zebrafish embryogenesis?

If Wnt6 expression in zebrafish embryos is as widespread as it is in chick and *Xenopus*[3-5], disrupting its activity should cause a broad spectrum of defects. *H. Struthers* (M.Sc. yr. 1) will use RT-PCR and in situ hybridization to elucidate the temporal and spatial expression of the two *Wnt6* mRNAs in embryos. cDNAs encoding full-length FLAG-tagged Wnt6 and HA-tagged Wnt6a protein will be cloned into *pT7TS* for in vitro transcription of mRNA or into *pEGFP-N* for expression from injected DNA. *Wnt6/6a* mRNA or DNA will be injected into 1-2 cell embryos to evaluate the effects of ectopic protein levels before and after the onset of zygotic transcription. These experiments are straightforward providing insight into the pathway(s) and organ system(s) requiring Wnt6/6a, but potentially misleading

if one or both aberrantly activate pathways before the endogenous genes are expressed. Clarification will come with results from the *Wnt6/6a* knockdowns. Once expression profiles are known, embryos will be injected with translational or splice-site targeting *Wnt6* or *6a* morpholinos (MOs), or with a "scrambled" MO as control. Off-targeting effects will be determined by TUNEL assay and for rescue, embryos will be co-injected with MO-insensitive *Wnt6* mRNA and a *Wnt6* MO. Morphants are expected to have defects in many organs systems and preference to investigate one over another will depend on factors including, but not limited to, the availability of molecular markers, antibodies and the accessibility of tissue(s) to target *Wnt6/6a* and *Fz7/2* MOs or mRNAs to specific sites by in vivo electroporation[45]. Wnt antibodies would complement this and subsequent studies, prompting us to generate them in years 1 (Wnt6) and 2 (Wnt6a). I am confident these studies will yield novel results, especially for Wnt6a, and will expose players in the Wnt6 network that may have been overlooked in the F9 model. We have experience with microinjection/electroporation and have used MOs and shRNAs[29].

Aim 3: To what extent do ROS have on augmenting Wnt signaling?

N. Alkahlout (M.Sc. yr. 1) found H_2O_2-treated F9 cells express PrE markers. ROS activate MAPK pathways converging on Wnt networks, with p38 MAPK promoting β-catenin accumulation by negatively regulating GSK-3[46], and JNK facilitating β-catenin translocation to the nucleus[15]. In a ROS-independent manner Wnt3a activation of p38 MAPK, in F9 cells expressing rat Fz1, inhibits GSK-3 and promotes β-catenin signaling[47]. However, these authors note that this activation is not an integral step in β-catenin signaling as Tcf-dependent transcription occurs even when p38 MAPK activation is abolished[47]. With this evidence, I hypothesize that the changes seen in H_2O_2-treated F9 cells are due to ROS-crosstalk acting on JNK and p38 MAPKs to increase β-catenin levels. To test this, H_2O_2-treated or untreated cells will be assayed for endo-A intermediate filaments, nuclear vs. cytoplasmic levels of β-catenin (NE-PER, Pierce), and measuring JNK and p38 MAPK activities (EZ-Detect, Pierce).

If my hypothesis is correct and ROS play a role in PrE formation, then RA must trigger the increase endogenously. With the evidence for a RA/$G\alpha_{13}$/Rac1 axis, we will test whether or not Rac1 activation of NOX is responsible for producing ROS. ROS levels, in response to RA, DMSO, and Wnt6-CM or in cells transfected with constitutively active V12Rac1 or the empty vector, will be measured in live cells using (CM)H_2DCFDA and fluorometry. Rac1, JNK and p38 MAPK activation following the aforementioned stimuli, will be measured by immunoprecipitation-immunoblotting (Cytoskeleton; Pierce), as will p-GSK-3 (inactive) and nuclear vs. cytoplasmic β-catenin levels, to determine if a correlation exists between ROS levels and PrE formation. Identifying elevated levels of ROS and p-GSK-3 in response to RA or Wnt6-CM, and especially in V12-Rac1 expressing cells, would be novel. Likewise, if nuclear β-catenin levels in V12-Rac1-expressing cells exceed that in controls, this would support the notion of Rac1 crosstalk augmenting the β-catenin pathway. Based on a previous study, RA-treated P19 embryonal cells expressing dominant negative Rac1 do not express endo-A intermediate filaments or form PrE[32], or more specifically do not undergo PCP based on our criteria. Thus, it will be interesting to see if low JNK activity in RA- or Wnt6-CM-treated cells expressing dominant negative N17Rac1 correlates to low levels of ROS, p-GSK-3 and nuclear β-catenin relative to controls. The Super8XTOPflash assay will be used to measure Tcf-dependent transcription in cells expressing V12-Rac1 or N17Rac1. This assay will be repeated for RA-stimulated cells treated with SB203580 to inhibit JNK activity or SP600125 to block p38 MAPK activity, clarifying the role each MAPK has in response to RA. If MAPK crosstalk converges on the RA-Wnt6 network, Tcf-dependent transcription should be reduced when JNK activity is inhibited and severely compromised when Rac1 or p38 MAPK activity is blocked.

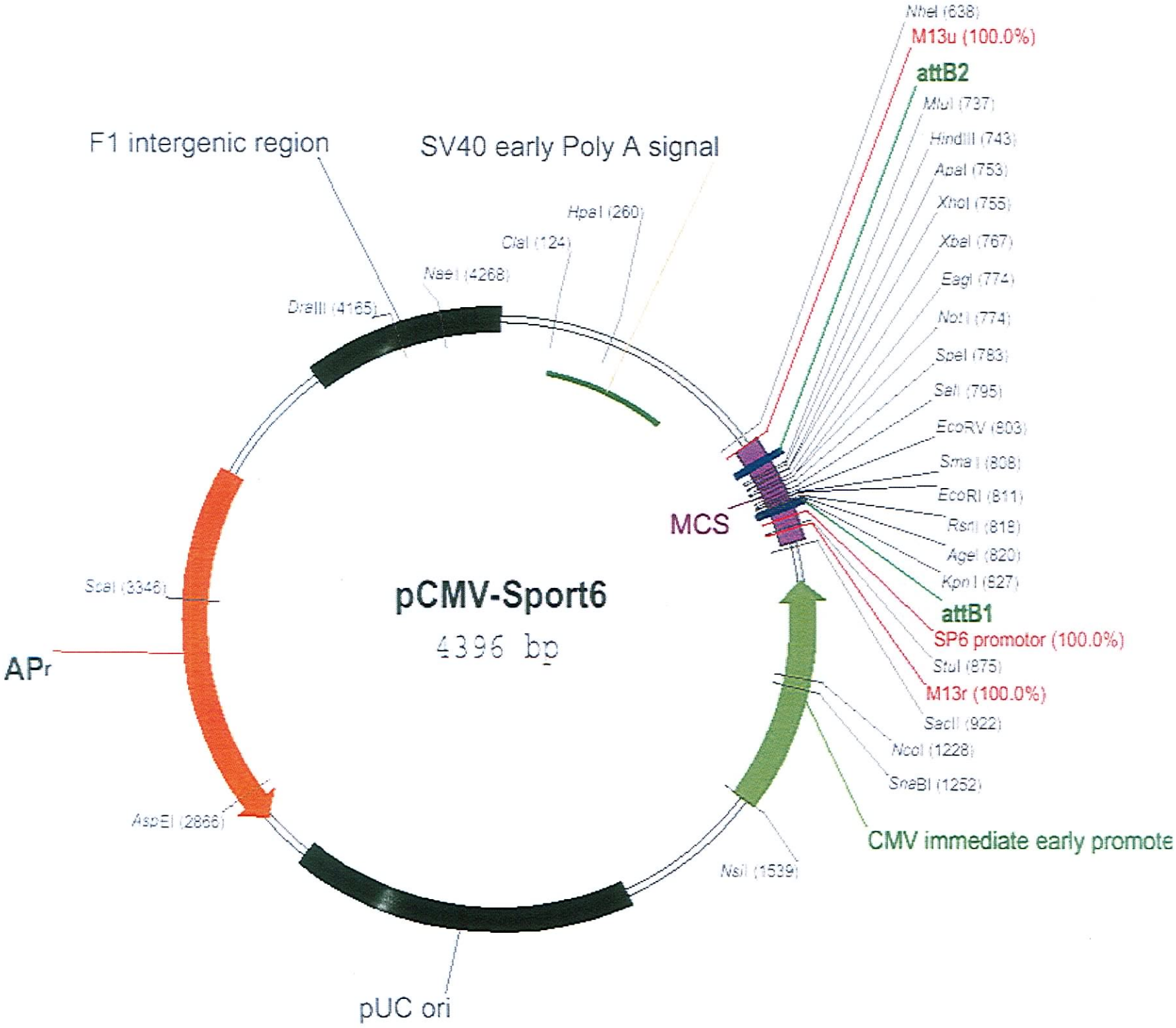
If my hypothesis that crosstalk from ROS on Wnt signaling influences differentiation, then treating cells with antioxidants should block PrE formation. To test this *J. Wen* (M.Sc. yr. 1) will treat F9 cells with Trolox or N-acetyl cysteine, followed by challenge with RA or Wnt6-CM. Levels of JNK and p38 MAPK activity will be measured on immunoblots, and immunocytochemistry used to assay for endo-A

intermediate filaments and β-catenin localization. ROS levels will be detected by (CM)H_2DCFDA and fluorometry. Results from the Super8XTOPflash assay will uncover the extent of β-catenin signaling. I expect RA- or Wnt6-CM-treated cells exposed to antioxidants will not have the elevated p38 MAPK or JNK activity seen in controls, but will show Tcf-dependent transcriptional activity for two reasons: 1) the Wnt6 negative regulation of GSK-3 is the obvious source; and 2) a ROS-independent mechanism involving PI3K/Akt (Aim 4). Results from these experiments will be very insightful and directional, providing the basis to explore ROS signaling in zebrafish. This is unchartered territory and although the prospect for discovery is great, at present it would be imprudent to propose experiments without clear hypotheses. To begin, however, we will use (CM)H_2DCFDA and fluorometry to measure ROS in embryos cultured under normal and hyperoxic conditions (assisted by R. Cumming, Biology, UWO) or in the presence of H_2O_2, which affects Akt/PTEN signaling and gastrulation. The relative transparency of the developing embryo/larva will allow us to use (CM)H_2DCFDA with confocal microscopy and digital imaging to identify relative levels of ROS in developing organs, pointing us toward a system in the Wnt6 project (Aim 2). Together, this information will provide background for a proposal to investigate the effects of environmental pollutants on the Wnt6 network and to promote zebrafish as a model for high throughput screening to identify environmental sources of oxidative stress.

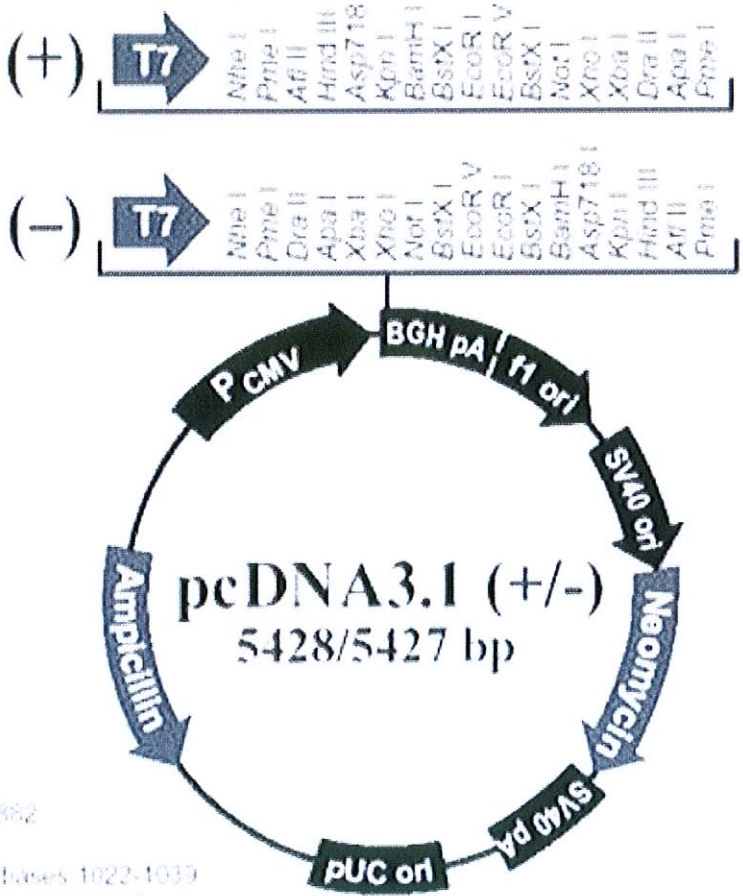
Aim 4: How much influence does Akt/PTEN crosstalk have on the Wnt/β-catenin pathway?

A. Finkielsztein (Ph.D. yr. 5) has shown GSK-3 activity decreases as Akt activity increases, allowing PTEN to counterbalance PI3K/Akt signaling[25]. This trend would explain why the sharp increase in Akt activity in RA-treated F9 cells is followed by a marked decrease in activity. The ability of Akt to negatively regulate GSK-3 and to stimulate the Rac1/NOX axis to produce ROS, places it as a "crosstalker" in Wnt signaling. I hypothesize that increasing Akt activity either directly or in response to Wnt6 leads to elevated Tcf-dependent transcription. To test this *Shaun Symons* (summer/M.Sc. student) will treat F9 cells with RA, Wnt6-CM, or with 740Y-P to specifically activate PI3K/Akt, then assay for changes in the levels of p-Akt (active), p-PTEN (inactive), p-GSK-3 (inactive) and Rac1 activity. Repeating these assays with lysates from Wnt6-silenced, RA-treated cells (Aim 1), will allow us to compare PI3K/Akt activity due specifically to Wnt6 signaling. The Super8XTOPflash reporter assay is required to compare PI3K/Akt and Tcf-transcriptional activities. If my hypothesis is correct, high p-GSK levels and elevated Rac1 activity under all treatments, especially for 740Y-P, should correlate to high levels of Tcf-dependent transcription. Likewise, if PI3K/Akt crosstalk augments β-catenin signaling, then we will expect to see a synergistic effect in Tcf-dependent transcriptional activity in Wnt6-CM/740Y-P-treated cells. Blocking PI3K activity with wortmannin or LY294002 will produce the opposite effect, reducing p-GSK3 and inactivating Rac1 thereby compromising Tcf-dependent transcription. Our transient transgenic and inhibitor studies have taught us a great deal about Akt/PTEN signaling in zebrafish, but we have not linked this information to a specific Wnt pathway. Thus, the results from the F9 model will be significant and extending these studies to zebrafish will provide the opportunity to test whether or not this crosstalk is a positive regulator on Wnt6 signaling in embryos. In 5 years I expect that we will know how ROS, MAPK and PI3K/Akt crosstalk converges on the RA/Wnt6/$G\alpha_{13}$ axis to effect PrE formation. Teasing apart the mechanism(s) and uncovering other sources of crosstalk in multicellular zebrafish and eventually mouse embryos will take longer.

***Significance & Training*:** This proposal addresses questions focused on the characterization of a complex Wnt signaling network. Wnt signaling is fundamental to almost every aspect of development and altering any facet of the circuitry has severe consequences in embryos and adults. We have made significant progress towards understanding how cells communicate and I am confident this new research will provide insight into how the Wnt6 signaling network influences cell differentiation under normal and disease conditions. Towards that end my research programme provides an environment adequately suited for training at all levels and allows trainees to blend together a range of cell, molecular, biochemical and genetic approaches to answer basic biological questions.


F1 intergenic region
SV40 early Poly A signal
NheI (638)
M13u (100.0%)
attB2
MluI (737)
HindIII (743)
ApaI (753)
XhoI (755)
XbaI (767)
EagI (774)
NotI (774)
SpeI (783)
SalI (795)
EcoRV (803)
SmaI (808)
EcoRI (811)
RsrII (818)
AgeI (820)
KpnI (827)
attB1
SP6 promotor (100.0%)
StuI (875)
M13r (100.0%)
SacII (922)
NcoI (1228)
SnaBI (1252)
CMV immediate early promote
NsiI (1539)
HpaI (260)
ClaI (124)
NaeI (4268)
DraIII (4165)
MCS
pCMV-Sport6
4396 bp
ScaI (3346)
APr
AspEI (2868)
pUC ori

Invitrogen
life technologies



Comments for pcDNA3.1 (+)
5428 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
pcDNA3.1 BGH reverse priming site: bases 1023-1039
BGH polyadenylation sequence: bases 1028-1252
f1 origin: bases 1298-1726
SV40 early promoter and origin: bases 1731-2074
Neomycin resistance gene (ORF): bases 2136-2930
SV40 early polyadenylation signal: bases 3104-3234
pUC origin: bases 3617-4287 (complementary strand)
Ampicillin resistance gene (bla): bases 4432-5428 (complementary strand)
ORF: bases 4432-5292 (complementary strand)
Ribosome binding site: bases 5300-5304 (complementary strand)
bla promoter (P3): bases 5327-5333 (complementary strand)