Modification Form for Permit BIO-UWO-0218

Permit Holder: Andrew Leask

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	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. Give the full name - do not abbreviate.
Approved Microorganisms	DH5 alpha, OmicsLink feline lentivirus	
Approved Primary and Established Cells	human (primary): foreskin, rodent (primary): mouse sla-, rodent (established): MH 3T3. HEK 293T	
Approved Use of Human Source Material		
Approved Genetic Modifications (Plasmids/Vectors)	pTEN	pGL3BARL, pGL3fuBARL, pSL3, pSL4, pSL5, pBARLS, pfuBARLSpSL9lrluc
Approved Use of Animals	Mouse	
Approved Biological Toxin(s)		

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

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

Signature of Permit Holder: _____

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

Date of Last Biohazardous Agents Registry Form: Aug 29, 2008

Date of Last Modification (if applicable): Sep 25, 2009

BioSafety Officer(s): _____

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

We wish to transfect the plasmid DNAs into fibroblasts to assess endogenous responses to wnt signals (they are wnt-responsive reporter plasmids)

MTA request for the following (From the University of Washington):

pGL3BARL

pGL3fuBARL

pSL3, pSL4

pSL5

pBARLS

pfuBARLS

pSL9/rLuc

--- Original Message --------
Subject: Re: Biohazard Permit Modification - Leask Lab
Date: Mon, 22 Nov 2010 10:41:46 -0500
From: Shangxi Liu <Shangxi.Liu@schulich.uwo.ca>
To: Andrew Leask <Andrew.Leask@schulich.uwo.ca>, jstanle2@uwo.ca

Hi, Jennifer,

Sorry for making confusion. These plasmids just contain Lentiviral-long terminal repeat. It is lentivirus replication incompetent and does not carry oncogenic cDNAs, which makes it Biosafety Level 2 (Section 3.2). We plan to use these palsmids to generate stable b-catenin reporter cell line (Section 3.2) to study wnt signaling in fibrosis. For this purpose, we must produce some plasmids as described in Section 2.2 first, then using these plasmids to transfect target cells.

Shangxi Liu

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

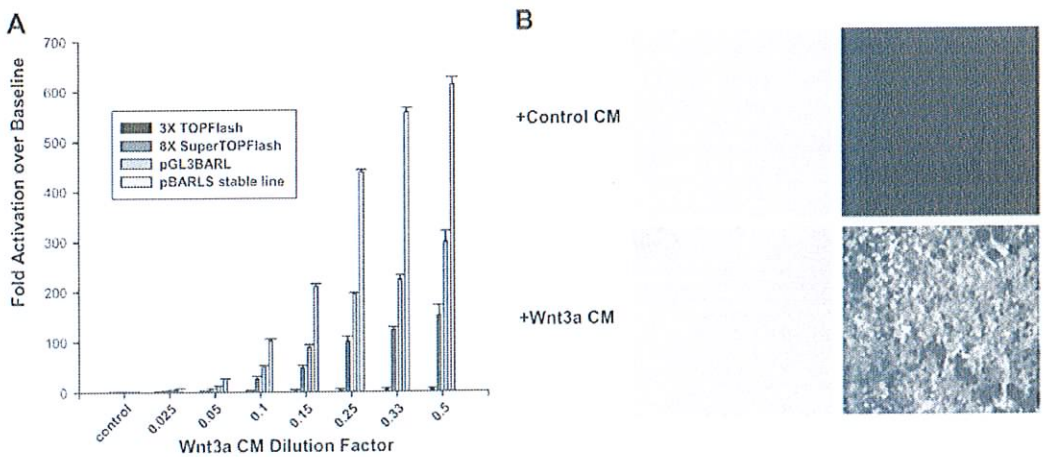

Fig. 8.1. The BAR system has enhanced sensitivity and dynamic range when directly compared with TOPFlash and SuperTOPFlash. **A** 10 ng of TOPFlash, SuperTOPFlash, or pGL3BARL were transfected along with 10 ng of pRLTK in HEK293T cells seeded in a 48-well plate. HEK293T cells stably expressing pBARLS 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.

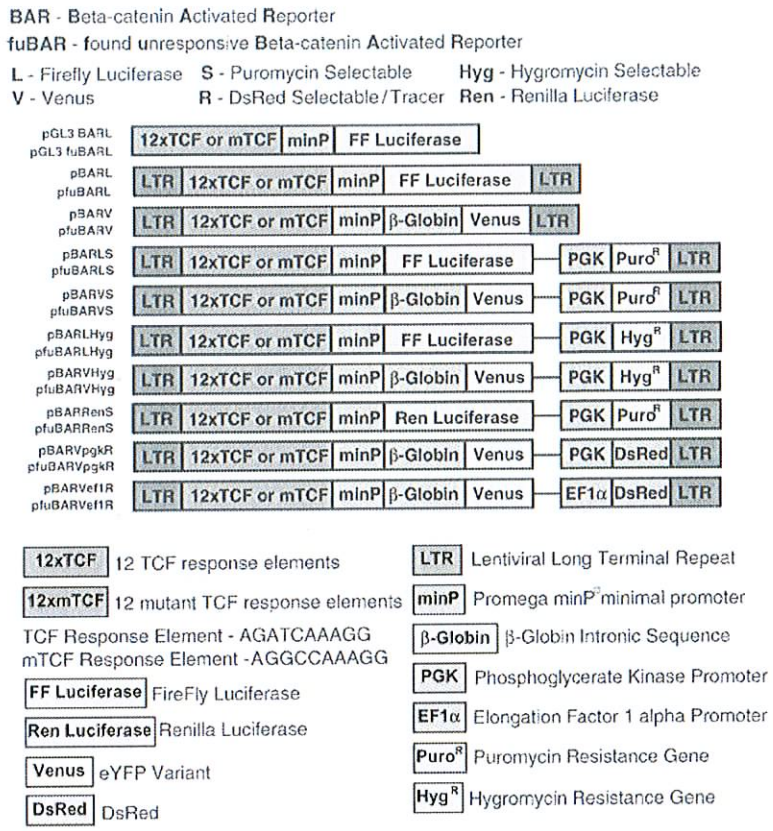

Fig. 8.2. Multiple platforms of the BAR system make it a versatile reporter system.

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. 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).

2.4.1. Stable Luciferase Reporter Cell Line

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

2.5.1. Low-Throughput Assay

1. 1× Passive lysis buffer.
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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Modification Form for Permit BIO-UWO-0218

Permit Holder: Andrew Leask

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

Matthew McCann

Kun Huh

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Mohit Kapoor

Additional Personnel
(Please list additional personnel here)

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms	DH5 alpha	Omneslink Feline lentivirus www.genecopoeia.com/product/lentiviral/
Approved Cells	human (primary): foreskin, rodent (primary): mouse sla-, rodent (established): MH 3T3. HEK 293T	
Approved Use of Human Source Material		
Approved GMO	pTEN	
Approved use of Animals	Mouse	

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

If not a submitt (per Biohazards Subcommittee meeting Sept 25/09)

Date of last Biohazardous Agents Registry Form Aug 29, 2008

Signature of Permit Holder: [signature]

BioSafety Officer(s): [signature] Sept 25/09

Chair,Biohazards Subcommittee: [signature]

If Follow Viral Vector Policy attached

Modification Form for Permit BIO UWO 0218

Permit Holder: Andrew Leask

Approved Toxin(s)		

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

Date of last Biohazardous Agents Registry Form Aug 29, 2008

Signature of Permit Holder: _____

BioSafety Officer(s): JL Tanney Sept 25/09

Chair,Biohazards Subcommittee: GM Kidder

-------- Original Message --------
Subject: Re: Leask Project involving Feline Lentivirus
Date: Fri, 07 Aug 2009 15:40:37 -0400
From: Andrew Leask <Andrew.Leask@schulich.uwo.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>
References:<4A7C591D.8000901@uwo.ca>

Dear Jennifer:

Thanks for the clarification; it is easy, then!

PTEN is a tumor suppressor, so overexpressing it wound not cause tumorigenesis; it would suppress tumorigenesis.

See below

http://ghr.nlm.nih.gov/gene=pten

"What is the normal function of the PTEN gene?
The PTEN gene provides instructions for making a protein that is found in almost all tissues in the body. This protein acts as a tumor suppressor, which means that it helps regulate the cycle of cell division by keeping cells from growing and dividing too rapidly or in an uncontrolled way....The PTEN enzyme acts as part of a chemical pathway that signals cells to stop dividing and triggers cells to undergo a form of programmed cell death called apoptosis. These functions prevent uncontrolled cell growth that can lead to the formation of tumors."

So, I think based on this, it should be a Level 2.

Andrew

>>> Jennifer Stanley <jstanle2@uwo.ca> 8/7/2009 12:41 PM >>>
Dr. Leask

I re-looked at the offical Committee's minutes, it states:

Based on revised viral vector policy, it is Level 2 plus unless researcher can demonstrate overexpression of PTEN does not cause tumourgenesis.**

Can you clarify this? Sorry that I missed this!

Jennifer

-------- Original Message --------
Subject: Re: Containment Level request: feline lentiviral vector
Date: Wed, 21 Jan 2009 15:15:47 -0500
From: Geneviève Lacroix <genevieve_lacroix@phac-aspc.gc.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>

Dear Ms. Stanley,

Based on the Human Pathogens Importation Regulations, to import this material you would need a containment level 2 permit for HEK 293 cells and a notice for a purified lentivirus vector.

Now, the actual containment and operational procedures related to the laboratory work done with this material is under your responsibility. Recombinant material risk assessments are usually more complexed considering that we are dealing with recombinant material. I can assist you with this risk assessment.

As a start HEK 293 cells alone are classified risk group 2 pathogens.

Lentiviral work is normally conducted in a containment level 2 physical laboratory with the addition of containment level 3 operational practices. However, feline immunodeficiency virus is a Risk Group 2 animal pathogens, so you might decide that containment level 2 physical and operational procedures are sufficient.

You also need to evaluate the risk of the transgene. Is PTEN an oncogene? Does it affect the cell cycle? Can it increase the risk group?

Also consider the work intent? Is it large scale or laboratory scale work? Will the infection be carried in vitro or in vivo? Which species and kind of cells are targeted by the pseudovirus?

You need to evaluate all these parameters and their risk levels in order to determine the physical and operational requirements. The lowest containment level requirements for this particular workdue to the HEK 293 cells is containment level 2 (physical and operational). You might evaluate that containment level 2 physical laboratory with the addition of containment level 3 operational practices or that containment level 3 (physical and operational) is more suitable. Personally, and this is based only on the information available to me at the moment, I would most probably go for containment level 2 physical laboratory with the addition of containment level 3 operational practices. But again, you have the final word and ultimately it is your responsibility.

I hope this information is clear enough. Do not hesitate to contact me if you need further assistance with this risk assessment.

Regards

Geneviève Lacroix
A/Head, Importation and Biosafety Program
Chef intérimaire, Importation et Services de biosécurité
Office of Laboratory Security / Bureau de la sécurité des laboratoires
Public Health Agency of Canada / Agence de la santé publique du Canada
[illegible] Colonnade Rd. AL: 6201A, Ottawa, Ontario, Canada, K1A 0K9
Tel: (613) 946-6861

------- Original Message --------
Subject: Re: Biohazardous Agents Registry Form Modification - Dr. Leask - FelineLentivirus
Date: Wed, 10 Dec 2008 14:50:06 -0500
From: Andrew Leask <Andrew.Leask@schulich.uwo.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>
References: <49401091.3030207@uwo.ca>
<493FD581.647C.0067.0@schulich.uwo.ca>
<49401C56.5000404@uwo.ca>

http://www.safety.rochester.edu/ibc/ibcvirus.htm

The above is a safety committee reference. I will send the other information as soon as I get it

>>> Jennifer Stanley <jstanle2@uwo.ca> 12/10/2008 2:45 PM >>>
Fabulous, thanks

Andrew Leask wrote:
> Dear Jennifer:
> Thank you for this note. I will be sending you a separate email (a brochure) that confirms that the virus is feline based.
>
> We wish to use the 293 cells as packaging cells to grow up a feline virus which will allow the overexpression of PTEN in primary rodent cells.
>
> I will send you the fax shortly.
> best wishes
>

PTEN (gene)

From Wikipedia, the free encyclopedia

edit

In the field of molecular biology, **phosphatase and tensin homolog** also known as **PTEN** is a protein which in humans is encoded by the *PTEN* gene.[2] *PTEN* acts as a tumor suppressor gene through the action of its phosphatase protein product. This phosphatase is involved in the regulation of the cell cycle, preventing cells from growing and dividing too rapidly. Mutations of this gene contribute to the development of certain cancers.[3]

This gene was identified as a tumor suppressor that is mutated in a large number of cancers at high frequency. The protein encoded this gene is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. It contains a tensin like domain as well as a catalytic domain similar to that of the dual specificity protein tyrosine phosphatases. Unlike most of the protein tyrosine phosphatases, this protein preferentially dephosphorylates phosphoinositide substrates. It negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a tumor suppressor by negatively regulating Akt/PKB signaling pathway.[4]

Contents

Function and structure

The corresponding PTEN protein is found in almost all tissues in the body. PTEN protein acts as a phosphatase to dephosphorylate phosphatidylinositol (3,4,5)-trisphosphate (PtdIns (3,4,5)P_3 or PIP_3). PTEN specifically catalyses the dephosporylation of the 3` phosphate of the inositol ring in PIP_3, resulting in the biphosphate product PIP_2 (PtdIns(4,5) P2). This dephosphorylation is important because it results in inhibition of the AKT signaling pathway.

Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)

Crystallographic structure of human PTEN. The N-terminal phosphatase domain is colored blue while the C-terminal C2 domain is colored red.[1]

Available structures: 1d5r

Identifiers

Symbols	PTEN; BZS; MGC11227; MHAM; MMAC1; PTEN1; TEP1
External IDs	OMIM: 601728 MGI: 109583 HomoloGene: 265
EC number	3.1.3.67

Gene ontology [show]

Orthologs

	Human	Mouse
Entrez	5728	19211
Ensembl	ENSG00000171862	ENSMUSG00000013663
Uniprot	P60484	Q3UFB0
Refseq	NM_000314 (mRNA) NP_000305 (protein)	NM_008960 (mRNA) NP_032986 (protein)
Location	Chr 10: 89.61 - 89.72 Mb	Chr 19: 32.82 - 32.89 Mb
Pubmed search	[1]	[2]

The structure of PTEN (solved by X-ray crystallography, see figure to the upper right[1]) reveals that it

consists of a phosphatase domain, and a C2 domain: the phosphatase domain contains the active site which carries out the enzymatic function of the protein, whilst the C2 domain binds the phospholipid membrane. Thus PTEN binds the membrane through its C2 domain bringing the active site to the membrane-bound PIP_3 to de-phosphorylate it.

When the PTEN enzyme is functioning properly, it acts as part of a chemical pathway that signals cells to stop dividing and causes cells to undergo programmed cell death (apoptosis) when necessary. These functions prevent uncontrolled cell growth that can lead to the formation of tumors. There is also evidence that the protein made by the PTEN gene may play a role in cell movement (migration) and sticking (adhesion) of cells to surrounding tissues.

Clinical significance

PTEN is one of the most commonly lost tumour suppressors in human cancer. During tumor development, mutations and deletions of PTEN occur that inactivate its enzymatic activity leading to increased cell proliferation and reduced cell death. Frequent genetic inactivation of PTEN occurs in glioblastoma, endometrial cancer, prostate cancer, and reduced expression is found in many other tumor types such as lung and breast cancer.

PTEN mutation also causes a variety of inherited predispositions to cancer.

Cowden syndrome: Researchers have found more than 70 mutations in the PTEN gene in people with Cowden syndrome. These mutations can be changes in a small number of base pairs or, in some cases, deletions of a large number of base pairs. Most of these mutations cause the PTEN gene to make a protein that does not function properly or does not work at all. The defective protein is unable to stop cell division or signal abnormal cells to die, which can lead to tumor growth, particularly in the breast, thyroid or uterus.

Other disorders: Mutations in the PTEN gene cause several other disorders that, like Cowden syndrome, are characterized by the development of noncancerous tumors called hamartomas. These disorders include Bannayan-Riley-Ruvalcaba syndrome, Proteus syndrome, and Proteus-like syndrome. Together, the disorders caused by PTEN mutations are called PTEN hamartoma tumor syndromes, or PHTS. Mutations responsible for these syndromes cause the resulting protein to be nonfunctional or absent. The defective protein allows the cell to divide in an uncontrolled way and prevents damaged cells from dying, which can lead to the growth of tumors.

See also

- Multiple hamartoma syndrome

References

1. ^ *a b* PDB 1d5r; Lee JO, Yang H, Georgescu MM, Di Cristofano A, Maehama T, Shi Y, Dixon JE, Pandolfi P, Pavletich NP (October 1999). "Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association". *Cell* 99 (3): 323–34. doi:10.1016/S0092-8674(00)81663-3. PMID 10555148.
2. ^ Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH, Tavtigian SV (April 1997). "Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers".

Nat. Genet. **15** (4): 356–62. doi:10.1038/ng0497-356. PMID 9090379.
3. ^ Chu EC, Tarnawski AS (October 2004). "PTEN regulatory functions in tumor suppression and cell biology". *Med. Sci. Monit.* **10** (10): RA235–41. PMID 15448614. http://www.medscimonit.com/fulltxt.php?ICID=11797.
4. ^ "Entrez Gene: PTEN phosphatase and tensin homolog (mutated in multiple advanced cancers 1)".

Further reading

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External links

- The PTEN Protein
- GeneCard
- UMich Orientation of Proteins in Membranes *protein/pdbid-1d5r*
- MeSH *PTEN+Protein*
- Alzgene

This article incorporates text from the United States National Library of Medicine, which is in the public domain.

Retrieved from "http://en.wikipedia.org/wiki/PTEN_(gene)"
Categories: Genes on chromosome 10 | Human proteins | Tumor suppressor genes | Peripheral membrane proteins | EC 3.1.3
Hidden category: Wikipedia articles incorporating text from the United States National Library of Medicine

GeneCopoeia™
Expressway to Discovery

OmicsLink™ Human and Mouse Lentiviral ORF Expression Clones

User's Manual # LV001
Version: 08040902

GeneCopoeia, Inc.
19520 Amaranth Drive
Germantown, MD 20874
USA

www.genecopoeia.com

I. Introduction

Currently GeneCopoeia has multiple sets of 20,000 human and 15,000 mouse lentiviral ORF expression clones in several lentiviral expression vectors. The FIV (feline leukemia virus) based vectors are considered biologically safe, yet have been shown to be as effective as HIV based vectors at transduction of genes into a wide variety of dividing and non-dividing mammalian cells. Once cells are infected with the pseudoviral particles, the expression construct integrates with the genome in target cells and is stably expressed.

Advantages of OmicsLink™ Lentiviral Human and Mouse ORF Expression Clones

1. High Efficiency of Gene Delivery to Virtually All Cell Types and Whole Model Organisms
2. High Expression Levels of Delivered Genes
3. Self-inactivation and No Unwanted Viral Replication

II. OmicsLink™ Human and Mouse Lentiviral ORF Expression Clone Types

OmicsLink™ Lentiviral Human and Mouse ORF Expression Clones are offered in the following vector types

Vector	Promoter	Selection Marker	Tag
pReceiver-Lv01	Lenti-CMV	No	None
pReceiver-Lv41	Lenti-EF1a	Neomycin	None
pReceiver-Lv21	Lenti-CMV	Neomycin	None
pReceiver-Lv26	Lenti-CMV	Neomycin	N-Avi
pReceiver-Lv10	Lenti-CMV	Neomycin	C-Avi
pReceiver-Lv35	Lenti-CMV	No	N-Avi + IRES-Biotin ligase
pReceiver-Lv34	Lenti-CMV	Neomycin	N-eCFP
pReceiver-Lv61	Lenti-CMV	No	C-eCFP
pReceiver-Lv62	Lenti-CMV	Neomycin	C-eCFP
pReceiver-Lv19	Lenti-CMV	Neomycin	N-eGFP
pReceiver-Lv04	Lenti-CMV	No	C-eGFP
pReceiver-Lv08	Lenti-CMV	Neomycin	C-eGFP
pReceiver-Lv20	Lenti-CMV	Neomycin	N-eYFP
pReceiver-Lv05	Lenti-CMV	No	C-eYFP
pReceiver-Lv09	Lenti-CMV	Neomycin	C-eYFP
pReceiver-Lv23	Lenti-CMV	Neomycin	N-Flag
pReceiver-Lv03	Lenti-CMV	No	C-Flag
pReceiver-Lv07	Lenti-CMV	Neomycin	C-Flag
pReceiver-Lv33	Lenti-CMV	No	C-Flag + IRES-eGFP
pReceiver-Lv02	Lenti-CMV	No	C-HA
pReceiver-Lv06	Lenti-CMV	Neomycin	C-HA
pReceiver-Lv32	Lenti-CMV	No	C-HA + IRES-eGFP
pReceiver-Lv64	Lenti-CMV	Neomycin	N-HaloTag
pReceiver-Lv65	Lenti-CMV	Neomycin	C-HaloTag
pReceiver-Lv31	Lenti-CMV	No	IRES-eGFP
pReceiver-Lv36	Lenti-CMV	No	IRES-luciferase
pReceiver-Lv40	Lenti-CMV	Neomycin	IRES-Neomycin
pReceiver-Lv25	Lenti-CMV	Neomycin	N-Myc
pReceiver-Lv17	Lenti-CMV	Neomycin	C-Myc
pReceiver-Lv43	Lenti-CMV	No	C-Myc+ IRES-eGFP
pReceiver-Lv44	Lenti-CMV	No	C-Myc+ IRES-eYFP
pReceiver-Lv47	Lenti-CMV	Neomycin	C-Myc+IRES-Neomycin
pReceiver-Lv18	Lenti-CMV	Neomycin	C-Myc-His

III. Getting Started.

We recommend on receipt of a new expression clone that the plasmid is transformed into DH5α or equivalent cells (the expression plasmid contains the ampicillin resistance gene) and that a midi-prep of the plasmid is made. As the efficiency of transfection is dependent on the quality of plasmid DNA, we recommend the use of the QIAGEN Endotoxin-free Plasmid Kit or purification by CsCl gradient. Normal transfections use 2ug of plasmid for each 10-cm plate.

IV. Pseudovirus Production

The lentiviral expression vector contains the elements required for packaging, transduction, and stable integration of the viral expression construct into genomic DNA, leading to expression of the open reading frame. However, it lacks the elements essential for transcription and packaging of an RNA copy of the expression construct into recombinant pseudoviral particles. These factors are supplied by a packaging kit (Cat. # pLV-PK-01) that comes with a mixture of two packaging plasmids, pLV-PK-FIV and pLV-PK-VSG. pLV-PK-FIV contains the structural, regulatory, and replication genes required to produce lentivirus. pLV-PK-VSG expresses the envelope protein of vesicular stomatitis virus, which defines the range of infectible cells. The packaging kit, pLV-PK-01, also has a positive lentiviral expression clone that expresses eGFP protein.

To generate pseudoviral particles, producer cells need to be transiently co-transfected with the expression clone and packaging vectors. After transfection, the producer cells secrete pseudoviral particles into the culture media. The pseudoviral particles can be used to directly infect target cells. The expression construct is reverse transcribed and integrated into the genome of target cells, providing high levels of expression of the open reading frame of choice. See figure 1 for schematics of this process.

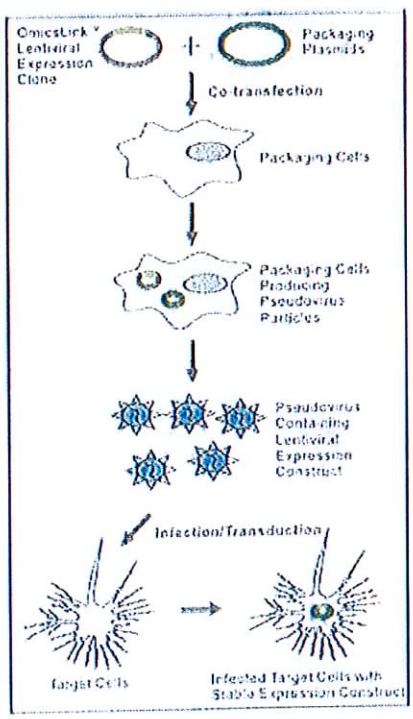

Figure 1. Schematics of pseudovirus production and infection of target cells

The following protocol describes the generation of pseudoviral particles containing the lentiviral expression construct. The yield of recombinant lentiviral particles typically produced under these optimized conditions is 10 ml of $1\text{-}3\times10^{6}$ (fu/ml) for the FIV system per 10-cm culture plate when measured by transduction of 111299 cells. This amount of pseudoviral particles is generally sufficient to infect $5\text{-}10\times10^{5}$ target cells at a MOI (multiplicity of infection) equal to 1.

Caution: Following this protocol results in the production of infectious pseudoviral particles. The recommended guidelines for working with BSL-2 safety class must be adhered to.

1. We recommend the use of 293Ta as producer cells. These should be grown in D-MEM medium supplemented with heat-inactivated serum and antibiotics for 2 to 3 days (50-70% confluency) prior

to transfection.

2. Mix 10 µg of the packaging plasmid mix with 2µg of the lentiviral expression construct (eGFP control plasmid at a 1:100 ratio may be included at this point if desired). Add the mixture to 400 µl D-MEM medium without serum and antibiotics. Add 20 µl of Plus™ Reagent (Invitrogen, Cat. # 11514-015), mix, and incubate at room temperature for 15 min.
3. Dilute 30 µl of Lipofectamine™ Reagent (Invitrogen, Cat. # 18324-111) into 400 µl of D-MEM medium without serum and antibiotics. Mix gently.
4. Add diluted Lipofectamine™ Reagent (from step 3) dropwise to DNA/Plus™ Reagent complex (from step 2). Mix gently by inversion and incubate at room temperature for 15min.
5. While incubating the mixture in step 4 to form the transfection complexes, wash the 293Ta cells with 10 ml of D-MEM without serum and antibiotics, and then add 9 ml of D-MEM with 2% serum and without antibiotics.
6. Add the DNA/ Plus™ Reagent/ Lipofectamine™ Reagent complex (from step 4) to the plate from step 5, and mix complexes with medium gently by inversion and incubate at 37°C in a CO_2 incubator overnight.
7. Replace the medium containing complexes with fresh D-MEM medium supplemented with 2% serum and antibiotics and continue incubation in the CO_2 incubator at 37°C . Peak virus production is normally achieved 48 hours post infection. We recommend collecting supernatants at 24, 36, 48, and 60 hours. Supernatants should be replaced with fresh D-MEM medium supplemented with 2% serum without antibiotics.
8. Collect all 10 ml of the pseudovirus-containing medium in a 15-ml sterile capped conical tube, and centrifuge at 3,000 rpm at room temperature for 5 minutes to pellet cell debris.
9. Following centrifugation, filter the supernatant through Millex-HV 0.45 µm PVDF filters (a low protein binding filter from Millipore, Cat. #SLHVR25LS).

The supernatant containing the pseudoviral particles can be used directly to determine the pseudoviral titer and directly used to infect target cells *in vitro* as long as the target cells can survive in conditioned medium. The supernatant can be stored at –80°C without cryoprotectant. Expect a 20-30% loss of viral titer with each freeze/thaw cycle.

V. Pseudoviral Titer Estimation

At this point we recommend that the pseudoviral stock is titered to ensure it is viable and to test what fraction of target calls can be transduced. This enables the number of copies of viral construct per target cell to be controlled.

Pseudoviral titer is normally achieved by mixing the eGFP control plasmid at a 1:100 ratio with the expression construct as an internal control. Alternatively the eGFP control plasmid can be expressed in parallel as an external control. To determine the relative pseudoviral titer, the packaged lentiviral expression clone should be transduced into H1299 cells along with cell lines appropriate to the user's experimental system. Relative titers may vary up to 20 fold.

Day 1.

1. For each viral stock, plate H1299 cells in a 24-well plate at a density of $0.6\text{-}1x10^5$ cells per well 24 hours prior to viral infection. Add 1 ml of complete D-MEM medium (with serum and antibiotics) and incubate cells at 37°C with 5% CO_2 overnight.

Day 2.

2. Prepare complete D-MEM medium plus 10% FBS with Polybrene at a final concentration of 5µg/ml. (The optimal concentration of Polybrene depends on cell type and may need to be empirically determined, but is usually in the range of 2-10 µg/ml) Prepare enough for an extra well as a negative control.

3. Remove culture medium and replace with 0.5 ml of complete D-MEM medium with 10% serum and Polybrene (from Step 2). For each pseudoviral stock, use three wells. Infect H1299 cells by adding 1 µl of viral stock into the first well (dilution factor of 500), 10 µl of viral stock into the second well (dilution factor of 50), and 100 µl of viral stock into the third well (dilution factor of 5). For one additional well (mock well control), add 0.5ml of D-MEM medium with Polybrene (from Step 2). Incubate cells at 37°C with 5% CO_2

overnight. (Note: Excessive exposure to Polybrene (>12 hr) can be toxic to some cells.)

Day 3.

4. Remove culture medium and replace with 1 ml of complete D-MEM medium (without Polybrene). Incubate the cells at 37°C with 5% CO_2 overnight.

Day 4.

5. Split the cells 1:3 to 1:5, depending on the type of cells, and incubate in complete D-MEM for an additional 24 hours.

Day 6.

6. The fraction of eGFP fluorescent cells can be counted by FACS (fluorescent activated cell sorting). Alternatively the eGFP fluorescence may be visualized. Normally 10 random fields of view are used to estimate the overall fraction of fluorescing cells on a plate. The average of this number is multiplied by $1.5x10^5$ (expected # of cells on plate at the time of infection) and by the corresponding dilution factor, then divide by 0.5ml to determine the relative titer of the pseudov rus in the supernatant.

VI. Transduction of the Packaged Lentiviral Expression Clones

The transduction efficiency depends upon the target cells and experimental procedure. It is recommended that the titered pseudoviral stock containing the positive control eGFP construct is used to determine the concentration of pseudoviral particles required for the desired MOI of target cells. After these test transductions are performed, it should be possible to determine the optimum concentration of pseudoviral particles for transduction based on eGFP fluorescence.

Day 1.

1. Plate target cells in a 24-well plate at a density of $0.6\text{-}1x10^5$ cells per well 24 hours prior to viral infection. Add 1 ml of complete optimal medium (with serum and antibiotics) and incubate cells at 37°C with 5% CO_2 overnight.

Day 2.

2. For each well, prepare 0.5 ml of virus suspension diluted in complete medium with Polybrene at a final concentration of 5-8 μg/ml.

Note:

a. We recommend to start with 0.5 ml of non-diluted viral supernatant (if the virus was not concentrated), if possible. The higher the concentration of virus in solution, the higher is the transduction efficiency.
b. Use several dilutions of pseudoviral stock if necessary. In addition, we recommend including a transduction with the eGFP control construct and other appropriate positive and negative control constructs.
c. Mix the virus with the medium gently by rotation or inversion. Do not vortex.

3. Infect target cells by removing the culture medium from cells and replacing it with 0.5 ml of prepared virus dilution. For one well (mock well control), add 0.5 ml of D-MEM medium with Polybrene. Incubate cells at 37°C with 5% CO_2 overnight. For extremely fast-growing and metabolizing cell lines such as 293Ta, use 3% FBS in the medium.

Day 3.

4. Remove the culture medium and replace with 1 ml of complete medium (without Polybrene). Incubate the cells at 37°C with 5% CO_2 overnight.

Day 4.

5. By day 4, the culture will be confluent (depending on cell type). Split it 1:3 to 1:5, depending on the type of cells, and continue incubating for 48 hours in complete D-MEM.

Day 6.

6. The infected target cells can be analyzed for transient expression of the expression construct using an appropriate biological assay. If you have used an internal eGFP control, determine the percentage of infected cells by counting fluorescing cells by flow cytometry. Alternatively, the infected cells can be identified by selecting and counting based on selection marker genes contained in the expression clone constructs (*e.g.*, neomycin), but the titer determined by neomycin selection is usually less than the titer determined by eGFP selection.

VII. Limited Use License and Warranty

Limited Use License

Following terms and conditions apply to use of all OmicsLink™ ORF Expression Clones in all lentiviral vectors and Packaging Kit (the Product). If the terms and conditions are not acceptable, the Product in its entirety must be returned to GeneCopoeia within 5 calendar days. A limited End-User license is granted to the purchaser of the Product. The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use. The Product must not be resold, repackaged or modified for resale, or used to manufacture commercial products without prior written consent from GeneCopoeia. This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research. Use of any part of the Product constitutes acceptance of the above terms.

Limited Warranty

GeneCopoeia warrants that the Product meets the specifications described in the accompanying Product Datasheet. If it is proven to the satisfaction of GeneCopoeia that the Product fails to meet these specifications, GeneCopoeia will replace the Product. In the event a replacement cannot be provided, GeneCopoeia will provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to GeneCopoeia within 30 days of receipt of the Product. GeneCopoeia's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. GeneCopoeia's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. GeneCopoeia does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose. GeneCopoeia is committed to providing our customers with high-quality products. If you should have any questions or concerns about any GeneCopoeia products, please contact us at 301-515-6982.

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PHYSIOLOGY 473B/573B: Cell Signaling in Tissue, Injury & Repair
[old name: Advanced Cellular Physiology]

Course Manager: Dr. R. Wang

Wednesday, 1:30-3:30, Jan. 7 -- April 8, 2009
Medical Sciences Building, Room 282

INSTRUCTORS:

Instructors:
Dr. R. Wang, VRL, Rm A5-140, 685-8500 x55098 [on Sabbatical Leave]
Dr. A. Leask, *DSB 0067, 661-2111 x81102* [Course Manager]
Dr. S. Cregan, Robarts Research Institute, Rm 308, 663-5777 x34134

LECTURE SCHEDULE:

Date	Lecturer	Topic
January 7	Cregan	Molecular mechanisms of proliferation and differentiation in neurodevelopment
January 14	Cregan	Molecular mechanisms of apoptosis
January 21	Cregan	Non-apoptotic mechanisms of cell death [In Class Term Exam 1hr]
January 28	Cregan	Cell death in neurodevelopment and neurodegenerative conditions
February 4	Leask	Cell adhesion, contraction and matrix deposition in normal tissue repair
February 11	Leask	Signaling in fibrotic disease (I)
February 18	Leask	Signaling in fibrotic disease (II) [In Class Midterm - 1hr]
Feb. 23-March 1	CLASS CANCELLED - STUDY WEEK	
March 4	Leask	Function and regulation of the CCN family of proteins
March 11		Student Presentations
March 18		Student Presentations
March 25		Student Presentations
April 1		Student Presentations
April 8		Student Presentations
Last day of class: April 10, 2009 Final Examination Period: Apr. 10 - 30, 2009 (2hr)		

General Information About Mammalian Virus Vectors

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All use of **mammalian** recombinant virus vectors at UR must be approved by the IBC through the registration process. *Non-mammalian vectors (such as baculovirus) are exempt from this requirement.*

Please note that the NIH Guidelines stipulate that Institutional Biosafety Committee approval be obtained prior to use of recombinant mammalian virus vector systems. See NIH Guidelines Section III-D.

Overview of selected vector systems

General comment on containment: Suggested biosafety containment levels are provided for each vector system. Note, however, that use of a higher-level containment facility may be required in some cases, depending on the specific properties of the vector and/or insert. **Special care** should be given to the design, risk assessment (Section 2 of NIH Guidelines), and handling of virus vectors containing genes that make growth-regulating products, products released into the circulation, products that may have a general effect on the host-immune system.

Adenovirus: Adenoviruses are infectious human viruses, which often cause mild respiratory illness. Rare cases of severe disease can occur, and its use as a genetic vector therefore requires the use of adequate containment equipment and practices. Biosafety Level 2 (BL2) is appropriate for many constructs (see above). **Particular care** should be given to vectors containing genes that make products that may be similar to products made by the deleted adenovirus genes.

See Adenovirus Vector Safety Guidelines for additional information.

Adeno-associated virus: These are infectious human viruses with no known disease association. Some AAV types are common in the general population, and these viruses have the ability to integrate into the host chromosome. The NIH Guidelines (Appendix B) state that "adeno-associated virus (AAV) types 1 through 4, and recombinant AAV constructs, in which the transgene does not encode either a potentially tumorigenic gene product or a toxin molecule and are produced in the absence of a helper virus" can in most cases be handled at biosafety level 1 (BL1). This level of containment made is modified by other considerations (see above).

Herpesvirus: Herpesviruses include infectious human viruses such as herpes simplex virus type 1 (HSV-1), which is the most commonly used vector system. HSV-1 is

common in the general population, but can cause encephalitis in rare cases; its utility as a vector system stems from its broad host cell range, ability to transduce neurons, and its large insert capacity. Biosafety Level 2 (BL2) is appropriate for many constructs (see above).

Lentivirus: Lentiviruses are a subset of retroviruses, with the ability to integrate into host chromosomes, and to infect non-dividing cells. These viruses can cause severe immunologic and neurologic disease in their natural hosts. Lentivirus vector systems can include viruses of non-human origin (feline immunodeficiency virus, equine infectious anemia virus) as well as simian viruses (simian immunodeficiency virus) and human viruses (HIV). Typical lentivirus vectors take the form of virus pseudotypes bearing envelope proteins from vesicular stomatitis virus (VSV). It is usually recommended that work with non-human lentiviruses that are incapable of establishing productive infections in humans (FIV, EIAV) be conducted at biosafety level 2 (BL2). Work with simian or human lentiviruses (SIV, HIV) is typically conducted at a higher containment level (please consult the IBC).

> **See FIV Vector Safety Guidelines for additional information.**
>
> **See NIH OBA's Biosafety considerations for Research with Lentiviral vectors for additional information.**
>
> **See Assessment for Recombinant, Pseudotyped Lentiviral Vectors (HIV and SIV vectors) for additional information.**
>
> **See Assessment for Recombinant Amphotropic Retroviral Vectors Containing Oncogenes and Lentiviral Vectors Containing Oncogenes for additional information.**

Poxvirus: Poxvirus vectors include avian viruses (avipox vectors) such as NYVAC and ALVAC, which cannot establish productive infections in humans, as well as mammalian poxviruses, which can productively infect humans -- such as vaccinia virus and modified vaccinia viruses (MVA). Poxviruses are highly stable, and vaccinia virus can (rarely) cause severe infections in immunocompromised persons, persons with certain underlying skin conditions, or pregnant women. Such individuals should not work with vaccinia virus (see IBC guidelines). The use of biosafety Level 2 (BL2) is appropriate for many poxvirus and constructs (see above).

Retrovirus: These are infectious viruses which can integrate into transduced cells with high frequency, and which may have oncogenic potential in their natural hosts. Retrovirus vector systems are typically based on murine viruses -- most commonly, these systems include ecotropic viruses (which can infect only murine cells), amphotropic viruses (which can infect human cells) or pseudotyped viruses (which can also infect human cells). Containment for vectors with the ability to infect human cells will usually be recommended at biosafety level 2 (BL2), as per the NIH Guidelines (Appendix B) *("For agents that are infectious to human cells, e.g., amphotropic and xenotropic strains*

of murine leukemia virus, a containment level appropriate for RG2 human agents is recommended."), whereas for ecotropic vectors with no ability to infect human cells, BL1 containment may be appropriate *("[ecotropic] murine retroviral vectors...that contain less than 50% of their respective parental viral genome and that have been demonstrated to be free of detectable replication competent retrovirus can be maintained, handled, and administered, under BL1 containment").*

Baculovirus: Non-mammalian virus vectors are exempt from these registration requirements.

See **Suggested Biocontainment Levels for Common Viral Vectors** for additional information.

PDF version: **Mammalian Virus Vector Registration**
Word version: **Mammalian Virus Vector Registration**

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