

## Standard Operating Procedure for using lentiviral vectors for cell culture lines.

**Genetic manipulation of cultured primary and immortalized cells *in vitro* using lentiviral vectors.**

### Outline of Basic Workflow

- i. Plasmid production.
- ii. Vector production.
- iii. Vector titer and RCL assays.
- iv. Cell transduction experiments.

#### 1. Plasmids.

The lentiviral vectors will be generated using a modified three-plasmid system. Two of the plasmids produce the envelope coat protein (known as the envelope plasmid) and the structural and packaging proteins (known as the packaging plasmid). The envelope plasmid expresses the rhabdoviral vesicular stomatitis virus G protein (or VSV-G) using a CMV promoter. The packaging plasmid expresses the *gag-pol* polyprotein and its cleaved products, as well as *tat* and *rev*. The other 4 viral accessory proteins (*vif*, *vpr*, *vpu* and *nef*) have been eliminated by nonsense codons or partial deletions of the gene sequence.

The main transfer (or integrating) plasmid contains a multiple cloning site to insert heterologous promoters and cDNAs of interest. The transfer plasmid has a debilitated 3'LTR to make the vector self-inactivating as well as cis-acting DNA elements to produce higher efficiencies of transduction (central polypurine tract sequence) and higher transcript stability (woodchuck post-regulatory element). A 358 bp fragment from the *gag* region of HIV is inserted to help with efficient packaging of the vector particle.

All three plasmids are essential to produce functional vector particles following transfection, and all of the plasmids contain a SV40 ori in the vector backbone to help with propagation in the 293T cells.

The maps for these plasmids are shown in the current amended protocol. At no time during the construction of recombinant plasmids or preparation of plasmid DNA for transfection will more than a 10 liter culture of any host organism that contains recombinant DNA be propagated. *E. coli* will be the host for all recombinant constructions and BSL1 conditions will be followed.

#### **2. Preparation of Lentiviral Vectors by Transient Transfection**

Viral vector particles will be generated by co-transfection of HEK293T cells with the three plasmids explained in the earlier section. Vector will be collected after 48 hours after transfection, and the vector will be either frozen at -80 in aliquots, or if needed, concentrated by centrifugation at 23,000 rpm for 2 hours in a Beckman ultracentrifuge.

**3a. Vector titers.** Viral titers will be determined by infection into HeLa cells and 48 hours later, the genomic DNA will be harvested for real-time PCR assays. Specific PCR primers to the WPRE region have been designed to determine the number of vector genome copies in the vector preparation. Although this vector titering method provides an assessment of infectivity in the absence of promoter activity, it does not discriminate between the proviral versus circular forms of the vector i.e., it can overestimate the number of functional vector particles by a factor of 10-fold. The preparation of the lentiviral vectors will be performed under BSL-2 conditions. Cell culture related to vector preparation will be carried out in a class II biological safety cabinet. Cultures will be grown in a 37°C incubator that is clearly marked with biohazard signs. Incubator space is dedicated to lentiviral vector production and personnel are educated as to which space contains virus-infected cells. Traffic in the cell culture space is restricted to trained personnel during vector preparation.

### **3b. Safety Testing**

Lentivirus preparations are assayed for the presence of Replication Competent Lentivirus (RCL) by serial passage in HeLa cells with quantitative RT-PCR testing of cell culture supernatants for lentiviral RNA. HeLa cells are infected with lentiviral vector stocks and cultured for a minimum of 48 hrs. A sample is then taken for qRT-PCR to a specific DNA segment in the vector genome, and a portion of the supernatant is transferred to fresh HeLa cells. This process is repeated on a weekly basis over a 21 day period.

**4. *In vitro* manipulation of primary and immortalized cells.** Murine and rodent primary and immortalized epithelial cell lines will be obtained from Ellis Avner lab (Department of Pediatrics at MCW). The  $5 \times 10^5$  to  $1 \times 10^6$  cells will be plated into 6-well or 6-cm dishes and topically infected with  $10^6$  to  $10^7$  T.U. for 48 hours and then expanded, frozen down and stored in liquid nitrogen. All vector preparations and cell extracts infected with the vector will be stored in a -80 freezer. These samples will be transported in screw caps in a secured biohazard container to prevent spillage. Future experiments will be performed on these genetically manipulated cells as needed.

All vector production and experimentation, our lab will use 10% bleach to decontaminate all surfaces in the laboratory that have the possibility to contact the vector. The biological safety cabinet is wiped thoroughly with 70% ethanol and the blower will be kept on prior to and after the vector has been handled for a minimum of 30 minutes. Disposable items like plastic ware, pipettes, gloves and

paper towels are collected in biohazard bags that are sealed in leak-proof containers. These materials are de-contaminated and disposed of by autoclaving the bags. Liquids are treated with bleach at a final concentration of 10% for at least 4 hours before being disposed of by pouring down the drain of a sink.

### **Personnel Training and Vaccination**

Dr. Frank Park will educate and show each new individual using his lab culture area on the nuances of vector production and the use of lentiviral vector biology. He will be let his personnel know about the safeguards and how to handle the vector. No vaccination is needed to work with lentiviral vectors. All personnel will use gloves and lab coats while working with any aspect of vector production and experimentation.

If any accidental exposure were to occur, then the person will know to thoroughly and immediately wash the area with a detergent and water for at least 10 minutes. The titers that will be employed in this study are low and from previous experience and literature, the vector will not penetrate the epithelial layer of humans to enter the blood stream.