Policy Date of Issue: 5.3.11

Purpose:

To provide guidelines and procedures for safely working with lentiviral vectors at the University of British Columbia. Note that these are guidelines and each protocol requires a risk assessment review by the Biosafety Committee through RISe.

Background:

Lentiviruses are a subclass of retroviruses which are able to infect both proliferating and non-proliferating cells. In research these are based on immunodeficiency viruses including, Human Immunodeficiency Virus (HIV), Feline Immunodeficiency Virus (FIV), and Simian Immunodeficiency Virus (SIV). The focus of this document is on HIV – based lentiviral vectors. The vectors have been modified to provide a safer version of the HIV virus in which the viral replication genes have been removed and the envelope genes replaced with those of Vesicular Stomatitis Virus (VSV). There have been several generations of lentiviral vectors, with each subsequent generation having more safety features in place, thus they need to be treated differently.

The use of lentiviral vectors has been increasing as they allow highly efficient gene delivery in a wide variety of cell types. The two inherent risks with Lentiviral vectors are: the potential generation of Replication Competent Lentiviruses (RCL), and the potential for oncogenesis. This policy provides guidelines for mitigating the risk of the vector system and/or the transgene insert.

Applicable Legislation, Standards, and Guidelines:

Public Health Agency of Canada: Laboratory Biosafety Guidelines, 3rd edition, 2004
Canadian Food Inspection Agency: Veterinary Standards for Animal Facilities
National Institutes of Health (NIH), Recombinant DNA Advisory Committee (RAC), Biosafety Considerations for Research with Lentiviral Vectors, March 2006

Lentiviral Vector Features:

Please see Appendix A.

Containment Level Guidelines:

Table 1: The following are the **suggested** containment level for all *in vitro* work with lentiviral vectors. **Note: all protocols still require the approval of the Biosafety Committee.**

Lentiviral Vector System	Containment Level
Non-HIV based lentiviral vector (known genes and no oncogenes)	**CL1**
Non-HIV based lentiviral vector (unknown genes, oncogenes, and suppressor genes)	**CL2**
3rd generation or higher unaltered from a commercial source (all genes)	**CL2 with CL3 procedures.** [1]
Modified 3rd generation or higher from a commercial source or personally developed backbones (all genes)	**CL2 with CL3 procedures.** [2]
1st and 2nd generation HIV based lentiviral vector (all genes)	**CL2 with CL3 procedures**

[1] Based on a risk assessment of the gene, work may be performed in CL2 without RCL testing.
[2] May perform RCL testing to downgrade to CL2.

Table 2: The following are the **suggested** containment levels for all *in vivo* work with lentiviral vectors. **Note: all protocols still require the approval of the Biosafety and Animal Care Committees.**

Lentiviral Vector System	**Animals**	**Containment Level**
Non-HIV based lentiviral vector (known genes and no oncogenes)	All animals	**CL1**
Non-HIV based lentiviral vector (unknown genes, oncogenes, and suppressor genes)	All animals	**CL2** [1]
3rd generation or higher unaltered from a commercial source (all genes)	All animals without human cells	**CL2** [1,2]
	All animals with human cells added	**CL2**
Modified 3rd generation or higher from a commercial source or personally developed backbones (all genes)	All animals without human cells	**CL2** [2]
	All animals with human cells added	**CL2**
1st and 2nd generation HIV based lentiviral vector (all genes)	All animals	**CL2 with CL3 procedures**

[1] Based on a risk assessment of the gene, procedures with the concentrated virus are performed at this level. All animals may be moved from CL2 to CL1 spaces after 72 hours or the first cage change, whichever is longer.
[2] Bases on the risk assessment of the gene and/or backbone, RCL testing is required before animal procedures begin. If results are negative then all animals may be moved from CL2 to CL1 spaces after 72 hours or the first cage change, whichever is longer.

Containment Requirements:

- For Containment Level 1 and 2 Physical and Operational requirements please see Laboratory Biosafety Guidelines Chapters 3 and 4.
- For Containment Level 2 with Level 3 Procedures please see Appendix B.

Replication Competent Lentivirus Testing:

- Fill out the Lentivirus Production form.
- Once complete submit in RISe under section 5.1.1.

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Approved: *(a signed copy exists on file)*

Originally produced: November 21, 2011

Initiator and Biosafety Advisor: Stephanie Thomson, March 5, 2012

Committee Chair: Keith Humphries, March 5, 2012

Revision: March 5, 2012

Appendix A:

To understand lentivirus, there needs to be an understanding of basic retroviruses. These RNA viruses use a DNA intermediate in order to replicate. The basic structure of a retrovirus is a viral protein core containing two identical single-stranded RNA molecules and replication enzymes[1]. A viral envelop consisting of host cell membrane and viral encoded envelop glycoproteins surrounds the viral protein core. Figure 1[1] describes the basic replication cycle of a generic retrovirus.

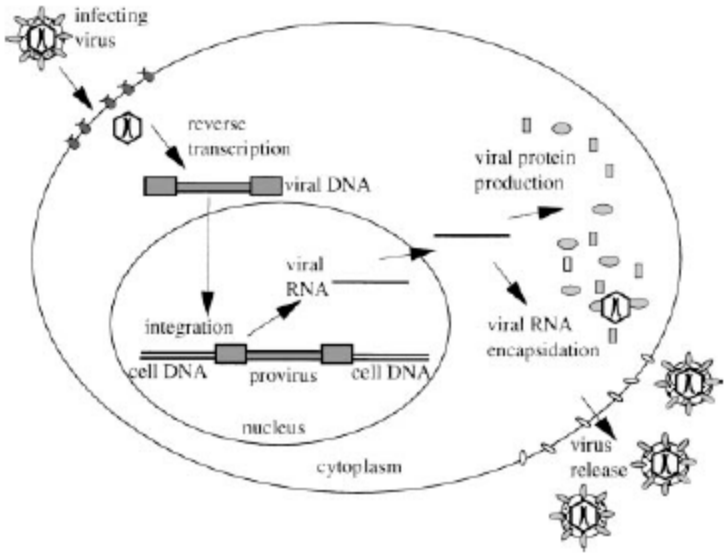


Figure 1.
The envelope glycoproteins recognize specific receptors on the cell's surface. The envelope and membrane then fuse releasing the viral core into the cytoplasm. In the cytoplasm the RNA undergoes reverse transcription, thus forming double stranded DNA. Though the timing is not actually known for this process, it is believed that the DNA is then transported to the nucleus where it is randomly integrated into the genome. The viral genome is now stable and replicated during DNA synthesis. The produced viral RNA is transported out of the nucleus when it is translated into viral proteins and enzymes. These proteins are then assembled forming new core particles, which are encapsulated in glycoproteins and membrane. These mature progeny virions are now released from the cell and are capable of infecting other cells.

Retroviruses contain the sequences for three genes: *gag*, *pol* and *env*.[1, 2] The exact make up of these genes is different depending on the virus, but the overall function is the same. The viral core proteins, structural protein matrix, capsid and nucleocapsid, are encoded within the *gag* gene. While the viral replication enzymes, reverse transcriptase and protease, are encoded in the *pol* gene. Finally, the viral envelope glycoproteins and transmembrane proteins are encoded within the *env* gene.[1, 2] In the case of HIV-1 there are additional genes present making the process more complicated, including *tat*, *rev* and *vpr*. These genes will be discussed below.

The term "Lentivirus" actually means a slow acting virus that is characterized by a long interval between infection and the onset of symptoms.[3] This means that there are many retroviruses that fall under this category, including HIV-1 and SIV. The scientific community, however, has come to use the term "Lentivirus" for a viral vector adapted from HIV-1. This vector was developed as a means to integrate a

gene of interest into the genome of a cell. HIV-1 was used as the model because it is able to infect both dividing a non-dividing cells. And though this process is not completely understood, it is believed that the matrix protein from Gag, the integrase protein, and the accessory protein Vpr are involved.[2, 4] Little is known of the functions of the various accessory proteins, excluding Tat and Rev. This combined with the fact that it was difficult to generate a stable packaging cell line made it so that the early forms of lentivirus had little difference to HIV-1.

***First Generation*:** Early studies using the HIV-1 for gene delivery found that the wild-type Env proteins gave low titers and limited the cells that could be infected. The *env* gene was then removed from the backbone and the envelope from a different virus used. In most cases the virus envelope used is from vesicular stomatitis virus (VSV) or the amphotropic Moloney leukemia virus (MLV).[8] These two envelopes alter the wild-type entry pathway from fusion at the plasma membrane to entry through the endocytic pathway. This increases the types of cells that maybe infected by the virus. In this system three plasmid constructs are used to generate a single vector. First plasmid (Envelope Plasmid) contains the VSV *env* gene, the second (Packaging Plasmid) contains the *gag* and *pol* genes of HIV, and the third contains (Transfer Plasmid) the gene of interest under control of a heterologous promoter.[2, 4] It should be noted that the third plasmid also contains: cis-acting signals for encapsidation, reverse transcription and integration sites at the end of the flanking long terminal repeats (LTRs), and a small sequence overlap with the *gag* sequence.[8] These three plasmid constructs are co-transfected in 293T cells (HEK cells transformed with E1α and SV40 T-antigen). The media is collected and the viral vectors concentrated using ultracentrifugation.

The constructs developed through the above method are only able to transfer the vector RNA, or gene of interest. The absence of the viral gene inhibits the vector to one round of infection. There is a possibility during the recombination of the three different constructs that replication-competent retroviruses (RCRs) are generated.[4] RCRs present a hazard to the recipient since they are now able to replicate within the cells. This means that whatever gene of interest is being studied will be replicated at will to many cells. The use of VSV envelope helps to reduce the risk of RCRs however, there is still a chance, and the VSV envelope allows the virus to infect most types of cells.

***Second Generation*:** Later studies found that deletion of the accessory genes *vif*, *vpr*, *vpu* and *nef* from the packaging plasmid resulted in little change to the transduction efficiency.[4] Studies have shown that in wild type HIV the deletion of any of these genes renders the virus incapacitated. In other words the HIV loses its pathogenicity.[4] It is believed that these genes are dispensable in the lentivirus because the function of the genes are no longer needed in the altered vector. The first example of this is Vpr. The function of Vpr is to aid in the nuclear import of HIV in macrophages. This function is not needed in many types of cells, including neurons. Also, with the use of VSV envelope the entry pathway of the virus has been altered thus changing the proteins and enzymes needed for importation.[4] The function of Nef is also affected by entry through the endocytic pathway. Nef is responsible for optimal fusion of the virus to the cell membrane.[4] Infectivity of wild-

type HIV is almost completely prevented with the deletion of the *nef* gene. It is also believed that because of the artificial environment in which the lentiviral vectors are produced, there is a reduced time-constraint on the virus to reproduce. In wild-type HIV in an *in vivo* situation the virus needs to efficiently uncoat, reverse transcribe, undergo nuclear import and integrate its genome before there is an immune response by the body. The transfected plasmids can undergo a more "relaxed" state of infection and production, thus eliminating the accessory proteins does not affect the efficiency.[4]

The same safety features are present in the second generation, as with the first generation. But there is an even greater reduction in the risk of producing RCRs. As mentioned above, deleting the accessory genes inactivates the wild-type virus and the belief is that the same is true for RCRs. However, there is still a small risk that the virus recombines to form a replicating version of the virus.

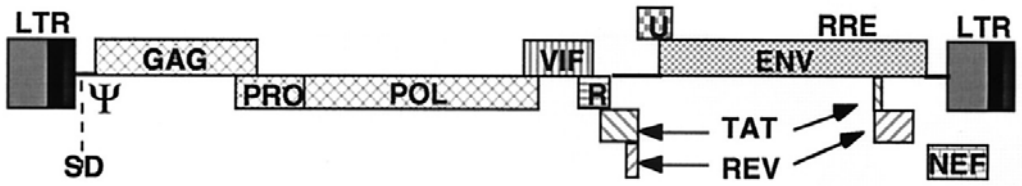
It should be noted that some new additions were also introduced into the second generation vectors. Woodchuck hepatitis posttranscriptional regulatory element (WPRE) was added downstream of the transgene thus increasing the expression levels by several folds.[5] The HIV genome contains a central polypurine tract (cPPT) that creates a DNA flap due to the initiation and termination of the second strand synthesis by the reverse transcriptase. This flap appears to aid in the import of the viral genome to the nucleus.

***Third Generation*:** It was always believed that the accessory proteins Tat and Rev were essential for the replication of lentiviral and HIV vectors. Tat proteins are responsible for regulating HIV gene expression at the transcriptional level, while Rev regulates expression at the posttranscriptional level. It was found that the HIV structural genes were only expressed in the presence of Tat and Rev. [6] In 1998 is was found that by using a constitutively active promoter (like CMV) in place of part of the upstream LTR in the transfer plasmid, the *trans*-acting function of Tat is dispensable.[6] So the third generation vectors contain a different *env* gene, and lack the accessory genes *vpr*, *nef*, *vif*, *vpu* and *tat*. The packaging plasmids were further altered using this system as well. A fourth plasmid was introduced to supply the *rev* gene. So the plasmids to be co-transfected to produce the lentiviral vector are: Envelope plasmid, Transfer plasmid, Packaging plasmid (lacking all the accessory genes), and Expression plasmid (containing the *rev* gene).[6]

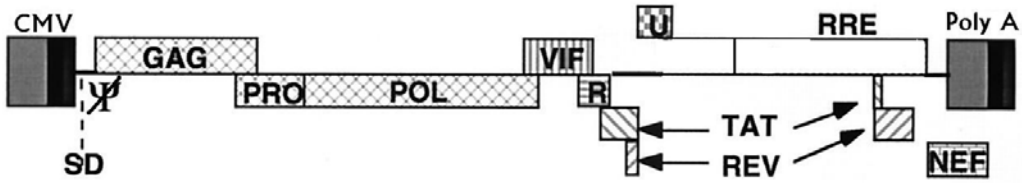
Again, all the same safety features mentioned above are incorporated into the third generation. And the risk of RCRs is again greatly reduced by the deletion of the *tat* gene.

***Self-Inactivation*:** Most of the third generation systems and some second generations also have this feature. A deletion in the U3 region of the downstream LTR in the transfer plasmid results in the inactivation of promoter activity with could lead to transcriptional activation of any genes downstream of the integration site.[7] This works because the reverse transcriptase in the transduced cell transcribes the inactive U3 region into both the LTRs rendering them inactive as well.[7]

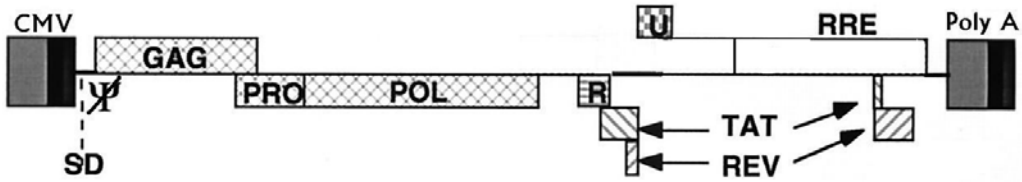
HIV Provirus



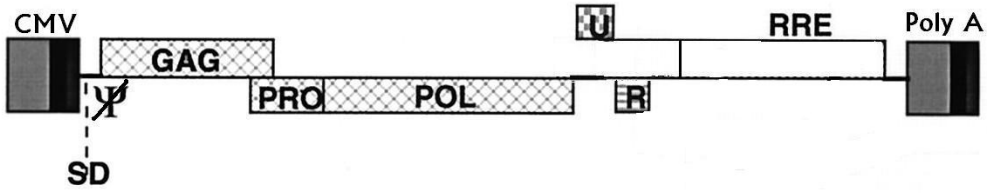
First Generation Packaging Plasmid



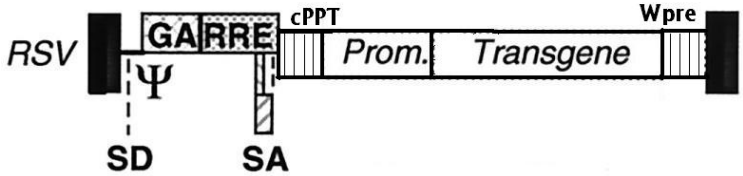
Second Generation Packaging Plasmid



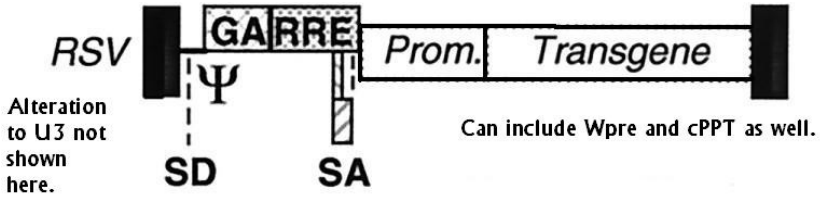
Third Generation Packaging Plasmid



Second Generation Transfer Plasmid

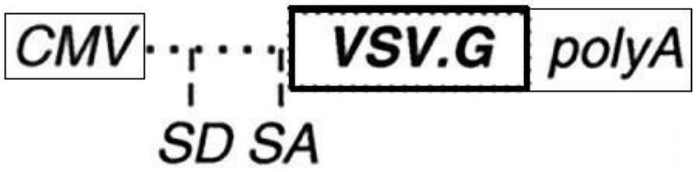


Third Generation Transfer Plasmid



Third Generation Expression Plasmid

Envelope Plasmid (For all generations)



All diagrams are adapted from #2, 6 and 8.

Appendix B

Containment Level 2 with Level 3 Procedures:

In addition to the operational practices for all laboratories handling infectious substances and those minimum requirements for containment level 2, the following describe the minimum operational practices required at containment level 3. The Office of Biosafety requires that the following procedures be incorporated into your existing experimental procedures and protocols.

1. There must be a program for the management of biological safety issues in place with appropriate authority to oversee safety and containment practices.
2. Everyone entering the containment laboratory must have completed a training course in procedures specific to the containment laboratory and must show evidence of having understood the training; training must be documented and signed by the employee and supervisor.
3. A protocol specific to the operation of the laboratory must be developed and read by personnel; employees must certify in writing that they have understood the material in the protocol. This should include entry and exit protocols for people, animals, equipment, samples and waste. General protocols must be supplemented with protocols specific to each project in progress.
4. Personnel must have demonstrated proficiency in microbiological practices and techniques.
5. People entering a containment facility must be well prepared and bring all materials they will need with them; if something has been forgotten, established traffic patterns must still be adhered to (i.e., do not go back to get it; either phone for someone to bring it or exit using proper protocols).
6. Routine laboratory cleaning must be done by personnel using the containment facility or by specific personnel dedicated and trained for this task.
7. The containment laboratory must be kept locked.
8. Infectious agents should be stored inside the containment laboratory; agents stored outside of the zone must be kept locked, in leakproof containers; emergency response procedures are to take into account the existence of such infectious agents outside of the containment laboratory.
9. Personal items such as purses and outdoor clothing must not be brought into the containment laboratory.
10. Drainage traps must be filled with liquid (i.e., through regular sink usage, automatic primers or by filling traps in areas that are not frequently used).
11. Laboratory samples and supplies may be carried into the containment laboratory or passed in through a pass-box.

12. Personnel entering the containment laboratory must wear an additional layer of protective clothing (i.e., solid-front gowns with tight-fitting wrists, double gloves) over dedicated laboratory clothing and shoe covers over dedicated laboratory shoes; this additional layer of protective clothing and shoe covers must be removed before leaving the containment laboratory in a manner that minimizes any contamination of the skin with the potentially contaminated dedicated laboratory clothing laboratory clothing when infectious materials are directly handled and should be removed after completion of work (e.g., dedicated for use at the BSC).
13. The use of full coverage protective clothing (i.e., completely covering all street clothing) is acceptable. When a known or suspected exposure may have occurred, all clothing, including street clothing, requires appropriate decontamination. Laboratories manipulating organisms, such as HIV, that are not infectious via inhalation, are not required to remove street clothing.
14. Centrifugation of infectious materials must be carried out in closed containers placed in sealed safety cups or rotors that are unloaded in a BSC.
15. Animals or arthropods that have been experimentally infected must remain in the laboratory or appropriate animal containment facility.
16. All activities with infectious materials are conducted in a BSC; if this is not possible, other primary containment devices in combination with personal protective clothing and equipment must be used; no work with open vessels containing infectious materials is conducted on the open bench.

Research activities not specific to the protocols involving the above mentioned agents, must not be performed when research with these agents is occurring.

Appendix C: Emergency Procedures:

Hazardous Spill Cleanup

A **Minor Biological Spill** is one that the laboratory staff is capable of handling safely without the assistance of safety and emergency personnel. The decontaminant used is dependant on the facility, but 10% bleach solution is common.

- Alert people in the area of the spill and evacuate the area.
- Remove and decontaminate any material that has been splashed on you and remove and decontaminate grossly contaminated clothing. Use shower station if necessary.
- Secure the affected area and post biohazard warning signs. Wait 30 minutes for aerosols to fall.
- Assess the situation and don the appropriate PPE and decontaminant for the cleanup operation. (Double gloves, lab coat, goggles)
- Spill Cleanup Procedure
    - Cover the spill with paper towels or other absorbent material moist or wet with decontaminant to absorb the spill and to prevent further aerosolization.
    - Pour the appropriate decontaminant around the covered spill and working from the outside inwards push the decontaminant to the middle with the absorbent material.
    - Wait at least 15 minutes for the bleach to penetrate through the contained spill.
    - Remove the absorbent material to an orange biohazard plastic bag for disposal*.
    - Repeat Spill Cleanup Procedure over the original spill area 3x to ensure decontamination and cleanup.
    - Notify your safety officer and the Department of Health, Safety and Environment at 604-822-2029 of the incident.

*Please follow the disposal procedures for your building. Remember if bleach is used these materials cannot be autoclaved as they could cause an explosion.

A **Major Biological Spill** requires the assistance of safety and emergency personnel. This procedure should be used when high concentrations and/or volumes of lentivirus are spilt.

- Alert people in the area of the spill and evacuate the area.
- Remove and decontaminate any material that has been splashed on you and remove and decontaminate grossly contaminated clothing. Use shower station if necessary.
- Secure the affected area and post biohazard warning signs.
- Call for Hazardous Materials Response: xxxx.
- Have a person knowledgeable of the incident and the laboratory assist emergency personnel.
- Notify your safety officer, PI and HSE of the incident.

Loss of Electrical Power

- The Biosafety Cabinet (BSC) is connected to Emergency Power. In the event of a power outage in the lab when lentiviral work is in progress, work will be contained and stopped as soon as possible, the cabinet sash will be closed, and a sign will be posted on the cabinet to keep the sash closed until power is restored. Note: Remove outer gloves and leave in the BSC and then close the sash. Then leave the room following proper exiting procedures.
- If there is a power failure during a centrifuge run, wait until the centrifuge slows down and stops and then remove samples to the BSC and exit the room as above.

Medical Emergencies

- Provide immediate first-aid. Stop the bleeding of wounds and wash the affected area with disinfectant/soap.
- Call for first aid: xxxx
- In the event of an exposure incident, report the incident to First Aid attendants and your safety officer, who will record the details of the exposure incident including the route of exposure, the infectious agent and an estimate of the dosage.
- In the event of a major medical emergency, dial 911 and then your security officer.

Building Emergencies

- In the event of a fire in the lab, dial xxxx.
- Suspend work in the BSC as soon as possible, lower the sash, and attach a sign to the cabinet to keep the sash closed. Evacuate the building and proceed to your labs designated meeting area outside of the building.

In the event of a fire drill or other building evacuation notice, suspend work in the Biosafety Cabinet as soon as possible, lower the sash, attach a sign to the cabinet to keep the sash closed, and evacuate the building, as above.

References

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