Lentiviral Vectors

**LENTIVIRUS**

**Virus Characteristics**: Lentiviruses are medium-sized (120 nm), enveloped viruses composed of a nucleocapsid containing two copies of single-stranded positive-sense RNA.

Lentivirus is a genus of slow viruses (lente-, Latin for "slow") of the Retroviridae family, characterized by a long incubation period.

The viruses are species-specific in host range and several have been recognized as pathogens of domestic animals, non-human primates and humans.

Although viral vector systems based on Feline Immunodeficiency Virus (FIV) and Equine Infectious Anemia Virus (EIAV) are available, lentiviral vectors are typically based on the best characterized of all lentiviruses: Human Immunodeficiency Virus serotype 1 (HIV-1).

Lentiviruses can deliver a significant amount of genetic information into the DNA of the host cell and have the unique ability among retroviruses of integrating into the genome of non-dividing cells. The viral genome remains in the host genome and is passed on to the progeny of the cell when it divides. For this reason, they are one of the most efficient methods of a gene delivery vector.

Advantage of lentiviruses over gamma-retroviruses: They do not require mitosis for productive infection.
The genomes of lentiviruses are complex, encoding a number of regulatory and accessory proteins not found in other retroviruses. The information provided in this section will be focused on the HIV-1.

In addition to the \textit{gag}, \textit{pol} and \textit{env} genes common to all retroviruses, HIV-1 contains two regulatory genes, \textit{tat} and \textit{rev}, essential for virus replication and four accessory genes, \textit{vif}, \textit{vpr}, \textit{vpu} and \textit{nef} that, while dispensable for virus growth \textit{in vitro}, they are critical for \textit{in vivo} replication and pathogenesis.

The ~10 kb HIV genome is flanked by long terminal repeats (LTRs) which are noncoding sequences that play an important role in virus replication and gene transcription. LTRs include R, U5 and U3 sequences.
HIV is primarily spread through direct contact of the virus with mucous membranes and broken skin (e.g. scratches, cuts, abrasions, dermatitis, or other lesions). Percutaneous (e.g. needle stick) exposure is also an important route of transmission.

Infection occurring via the respiratory tract has not been documented, and is unknown. However, since many of lentiviral vectors are pseudotyped with the glycoprotein of the Vesicular Stomatitis Virus, which can be transmitted by aerosol route, this route should be considered as well.

The clinical manifestations of HIV infection is divided into three different stages: initial, chronic and final.

The symptoms of the initial stage are very unspecific. Individuals usually present with flu-like syndrome after two weeks of infection followed by the chronic stage which no symptoms are present. The final stage of HIV infection corresponds to AIDS. AIDS symptoms will vary according to the acquired opportunistic infection and/or malignancy developed.
HIV can infect a variety of human immune cells such as CD4+ T cells, macrophages, and microglial cells.

Viral entry to target cells is mediated through interaction of the virion envelope glycoproteins (gp120) with the CD4 molecule and chemokine co-receptors of the host cells (CCR5 or CXCR4).

**Altering Cell Tropism - Pseudotyping**

CD4 is the major receptor for the native HIV envelope glycoprotein and, therefore, the tropism for lentiviral vectors with this envelope protein is highly restricted. In order to infect cells without CD4 expression, several heterologous envelope proteins have been used for pseudotyping.

The Vesicular Stomatitis Virus glycoprotein G (VSV-G) is preferentially used to allow gene transfer to a broad range of cell types and species (from insects to humans). A further advantage of VSV-G pseudotyping is the increased stability of the viral particles, which enables concentration of the viral particles by ultracentrifugation.

Other envelopes used for pseudotyping lentiviral vectors may include envelope proteins of other retroviruses, such as human foamy virus, gibbon ape leukemia virus (GALV) and the feline endogenous virus (RD114). All still maintain the tropism of the vector for human cells.

For transfection of murine cells, lentiviral vectors can be pseudotyped with a murine ecotropic envelope. This would remove exposure risks to the workers, since they cannot infect human cells.

*Although pseudotyping a lentiviral vector with VSV-G provides advantages, this increases the risks of infection following an accidental exposure where not only CD4+ cells are going to be the target, but any cells in the site of inoculation.*
Although viral genomic integration is essential to obtain stable expression of the gene of interest, it may potentially contribute to insertional mutagenesis.

The integration of the provirus can disturb the function of cellular genes and lead to activation of oncogenes or inactivation of tumor suppressors promoting the development of cancer.

Even a non-replicative lentiviral vector, carrying a “non-harmful” gene (e.g. GFP) or gene inhibitor, in theory, can cause harm.

Integrase-Deficient Lentivirus: Circumventing oncogenic potential

Lentiviral vectors can be produced through the use of mutations in the integrase protein that minimize proviral integration. The resulting integrase-deficient lentivirus (IDLV) generates circular vector episomes in transduced target cells that are gradually lost by dilution in dividing cells (transient expression), but are stable in quiescent cells. Inherently, IDLVs have a greatly reduced risk of causing insertional mutagenesis compared to integrating lentiviruses.
New viral RNA and proteins move to cell surface and a new, immature, HIV virus forms and leaves the cell through “budding.”

The risk of insertional mutagenesis due to chromosomal integration is a safety concern. The provirus can disturb the function of cellular genes and lead to activation of oncogenes or inactivation of tumor suppressors promoting the development of cancer.

Lentiviruses are unique among the Retroviridae family in the fact that they can infect non-dividing cells by actively entering the nucleus of a cell through the nuclear pore.

Even a non-replicative lentivirus, carrying a “non-harmful” gene (e.g. GFP) or gene inhibitor, in theory, can cause harm.

Transcription of the provirus DNA into RNA to generate genomic RNA and to make viral proteins.

New viral RNA and proteins move to cell surface and a new, immature, HIV virus forms and leaves the cell through “budding”.

Viral particles mature by the protease enzyme which modifies viral protein chains to make the particle ready to infect other cells.
Lentiviral Vector Production

**Biosafety Concern**

Lentiviruses are known as having high mutation and recombination rates and the possibility that the HIV could self-replicate and could be produced during vector manufacture by recombination process is a real concern.

To reduce the reversion probability, viral accessory genes (*i.e.* *vif*, *vpr*, *vpu* and *nef*) are deleted and essential genes have been separated in different plasmids, so that multiple recombination events would be required in order to form a replicative competent lentivirus (RCL).

**Vector Packaging:**

Generation of recombinant lentiviral vector particles involves subcloning the gene of interest (transgene) into an HIV-1 Transfer Vector backbone, which is co-transfected with Helper Plasmids into a recipient cell line (Packaging Cell).
Transfer Vector (Viral Construct)

The genetic information contained in the Transfer Vector genome is the only one transferred to the target cells. In this construct, the gene of interest (or gene inhibitor) is cloned into a vector sequence that contains the Packaging Signal (Psi-sequence - \( \Psi \)) and is flanked by the viral LTRs.

The LTRs are necessary since they are the control center for gene expression. Packaging signal (\( \Psi \)) is the element required for encapsidation of the viral genome, in other words, it indicates in which genome structural proteins should be assembled.

Helper Plasmids

Helper plasmids lack the packaging signal, therefore, their genes will not be enclosed by the viral capsid.

Genes for structural viral proteins (\( gag \)) and enzymes (\( pol \)), as well as regulatory genes (\( tat \) and \( rev \)), are provided in plasmid(s) separated from the one with the transgene or gene inhibitor (Transfer Vector). The number of the Packaging Plasmids varies according to the vector system.

Envelope Plasmid

In lentiviral packaging systems, the native envelope (HIV env gene) is typically replaced with a helper plasmid expressing heterologous envelope glycoproteins (e.g. VSV-G).

Although vector is rendered replication-incompetent, it remains infective!

The final viral vector particle will not contain any of the HIV genes provided by the helper plasmids, only their proteins. Without the essential genes, the viral vector will not be able to replicate, but still has the ability to infect cells and to have its transgene expressed. Depending on the nature of the transgene, a vector may be harmful following an accidental exposure.
Lentiviral vector production systems have been refined over time to improve their performance and safety. Depending on their features, vector systems are divided into different generations.

**First-generation** - The first-generation lentiviral vectors were manufactured using a packaging system that comprised all HIV genes, except the *env* gene (usually heterologous), which is separated in one plasmid.

- **Transgene**
- **gag, pol, tat, rev and accessory genes**
- **env** (het)

**Second-generation** - It was shown subsequently that none of the four HIV-1 accessory genes *vif, vpr, vpu, or nef* were required for HIV-1 replication in immortalized cell lines. This led to the development of a “second generation” of HIV-1 vector systems.

In this system, the accessory genes were eliminated leaving the *gag* and *pol* reading frames, which encode for the structural and enzymatic components of the virion, respectively, and the *tat* and *rev* genes, fulfilling transcriptional and post-transcriptional functions.

**Third-generation / Self-Inactivating (SIN)** In a third-generation system, only *gag, pol,* and *rev* genes remain present (*tat* is eliminated). The *rev* gene is provided in a separated plasmid. Since the HIV promoter in 5'LTR depends on *tat,* a vector which lacks *tat* needs to have its wild type promoter replaced with a heterologous enhancer/promoter such as CMV or RSV to ensure transcription.

* The Transfer Vector may also carry oligonucleotides (e.g. micro RNA, shRNA) that will inhibit target gene(s) of the host.
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<tr>
<th></th>
<th>First generation</th>
<th>Second generation</th>
<th>SIN Third generation</th>
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<tr>
<td><strong>Number of Plasmids</strong></td>
<td>3</td>
<td>3</td>
<td>4</td>
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<tr>
<td><strong>Deletion in the 3′LTR - SIN</strong></td>
<td>NO</td>
<td>NO</td>
<td>YES</td>
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<td>(&quot;self-inactivation&quot;)</td>
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<tr>
<td><strong>Number of Packaging</strong></td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Plasmids containing HIV genes</td>
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<td></td>
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<tr>
<td><strong>Accessory genes</strong></td>
<td>All present</td>
<td>All absent</td>
<td>All absent</td>
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<td><em>vif, vpr, vpu, nef</em></td>
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<tr>
<td><em>tat</em> and <em>rev</em> genes*</td>
<td><em>tat</em> and <em>rev</em> are present on a single packaging plasmid</td>
<td><em>tat</em> and <em>rev</em> are present on a single packaging plasmid</td>
<td><em>tat</em> is absent, <em>rev</em> protein is expressed from a separate plasmid</td>
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<tr>
<td><em>gag</em> and <em>pol</em> genes*</td>
<td>On the same plasmid</td>
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<td><strong>Recombination events to</strong></td>
<td>2 recombinations</td>
<td>3 recombinations</td>
<td>4 recombinations, between plasmids without homology and pick up of a promoter to complement ‘SIN’ deletion</td>
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<td>generate Recombinant Competent</td>
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<td>Lentiviruses (RCL)</td>
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Table adapted from Pauwels et al - Current Gene Therapy, 2009, Vol.9, No.6.459-474
Self-Inactivating (SIN) Vectors: Increasing Safety

Although the recombination event possible between three independent plasmids is extremely small, ways to decrease this probability even further when dealing with HIV-1 based vectors have been undertaken. This is the basis of the development of self-inactivating vectors or SIN vectors.

SIN vectors have modifications in their 3’ Long terminal repeat (LTR).

What are LTRs?

LTRs are noncoding sequences that are located on each end of the HIV genome.

The Viral RNA LTR contains two parts: the unique sequences (U5 at the 5’-end and U3 at the 3’-end); and the repetitive sequence (R - located at both ends of the RNA genome).

The U3 region is in fact the functional HIV-1 promoter, and contains the transcriptional enhancers. The R region marks the starting point of transcription, while the U5 region is involved in reverse transcription.

Provirus (DNA): When viral RNA is reverse transcribed to DNA, a U3 region is added to the 5’-LTR, and a U5 is added to the 3’-LTR, resulting in two identical terminal structures.

Transcript (RNA): Transcription of the provirus generates a RNA with the same LTR structure as the original Viral RNA.

In a SIN vector, the promoter sequences in the U3 region of the 3’LTR is deleted (Δ3’-LTR). When a vector infects a target cell, during the process of reverse transcription (RNA to DNA), the 3’LTR is copied to the 5’LTR and the 5’-LTR of the provirus will lack an active viral promoter.

By design, SIN vectors require an internal heterologous promoter for transgene expression. This can be either a viral (e.g. CMV, Rous sarcoma virus) or a cellular promoter (e.g. EF1-α ).
Advantages

Stable integration into the host genome with stable expression of the transgene

Ability to carry transgenes of reasonable sizes (up to 8Kb)

Infect dividing and non-dividing cells (different from gamma-retroviruses)

Efficient gene transfer

No immunogenic proteins generated

Stable integration into the host genome with stable expression of the transgene

Disadvantages

Potential for generation of replication competent lentivirus (RCL)

Oncogenesis potential

The risk of RCLs formation exists not only during viral vector production, but also during experiments involving materials infected with wild type HIV.

The wild type HIV may recombine with the vector potentially leading the creation of novel viruses with unpredicted potential for diffusion and pathogenesis.

Experiments involving human materials unscreened for HIV must be performed with extreme precaution.
Due to the presence of a lipid envelope in their structure, lentiviral vectors are rapidly inactivated when exposed to drying environmental conditions. They are susceptible to most of common use disinfectants, such as 70% ethanol.

A mixture of 70% ethanol or isopropanol diluted in water is effective against a wide spectrum of organisms, including enveloped viruses.

Alcohol based disinfectants are not recommended, however, for spill clean-up due to evaporation, which results in brief contact times. Also, they have a limited activity in the presence of organic material.
Although the parent virus for most of lentiviral vectors belongs to the Risk Group Classification 3 (i.e. HIV), in general, guidelines recommend BSL2 practices for working with these agents.

Non-human lentiviruses such as FIV and EIAV are classified as Risk Group 1 organisms. Although BSL1 facilities and practices are normally appropriate for this Risk Group class, it is important to note that those vectors usually are pseudotyped with a human tropic envelope, such as the VSV-G envelope. In this case, BSL2 containment must be implemented since these viruses now have the capability of transducing human cells.

The implementation of BSL2 measures is adequate for the production or handling of most replication-defective lentiviral vectors, unless large volumes are exceeded (according to NIH this is >10L production volumes).

The handling of lentiviral vector suspensions, in particular high titer stocks and/or volume, increases the possible accidental exposure of the lab worker and therefore is considered to be an activity associated with higher risks. In such case, BSL2 facilities with BSL3 practices should be considered (i.e. BSL2+).

Handling first generation lentiviral vectors may also require the implementation of additional measures because these vectors are considered to be less safe and the probability of RCL is not negligible.
Containment: Animal Experiments

The recombinant DNA advisory Committee (RAC) of the National Institutes of Health (NIH) recommends ABSL2 containment for animals that are not permissive for lentiviral infection (e.g. mice, rats).

Animals that do not support replication of HIV-1 that are injected with safer lentiviral vectors systems (e.g. SIN third generation vectors), can be housed in an ABSL1 lab after a period of time that ranges between 1 to 7 days.

At the University of Cincinnati, animals receiving lentiviral vectors must be housed in ABSL2 conditions for a period of 72 hours.
Exposure Risks

**Percutaneous Exposure** - The major potential hazard is infection of the researcher by parenteral inoculation (e.g. needle stick accidents). The use of sharps including needles, blades and glassware should be strictly limited. Strongly consider the use of engineered sharps systems. If feasible, use plastic disposable transfer pipettes rather than glass Pasteur pipettes.

Open wounds, cuts, scratches, and grazes should be covered with waterproof dressings in addition to PPE.

**Mucosal Exposure** - Laboratory personnel may also be exposed by direct contact of vector suspension (e.g. splash) with oral, ocular or nasal mucosa. Extreme care must be taken to avoid spilling and/or splashing infected materials, especially if working with high volumes (>10L).

Proper restraint technique (physical or chemical) is critical to minimize accidental exposure during *in vivo* experiments involving sharps.

Lentiviral vector manipulations should be conducted in a biological safety cabinet which not only provides respiratory protection, but also protects the worker against splash.

For activities conducted outside of a biosafety cabinet (e.g. stereotactic injection), the use of mucous membrane protection devices is of extreme importance.

The use of face shields provides protection against ocular, nasal and oral mucosa exposure. If eye protection is used (e.g. goggles), nasal and ocular protection can be achieved by the use of surgical masks.
Post Exposure Prophylaxis : PEP

Following an accidental exposure, a lentiviral vector can potentially infect the lab worker cells. This could not only result in permanent transgene expression with its associated harmful effects, but might also result in activation (or inhibition) of neighbouring genes due to insertional mutagenesis.

The current guidelines for managing occupational exposure to HIV-based viral vectors rely on the use of antivirals. In this type of exposure, antivirals should target the pre-integration steps of the viral cycle to prevent insertional risks.

Ideally, it is recommended that post-exposure prophylaxis (PEP) be administered immediately, but certainly no later than 72h following exposure.

Antivirals that inhibit the viral Reverse Transcriptase (RT) are used as PEP drugs. When RT is blocked, DNA copy cannot be formed from the viral RNA. Also, inhibitors of the viral Integrase may be added to the regimen (see replication cycle).

PEP treatment needs to be prescribed by an occupational health physician who must determine whether treatment is necessary based on the biological hazards associated with the expressed transgene.

Each PI should develop an emergency response plan in case of exposure to lentiviral vectors. It is the responsibility of the PI to provide risk assessment information if emergency department providers have any questions regarding health hazards.