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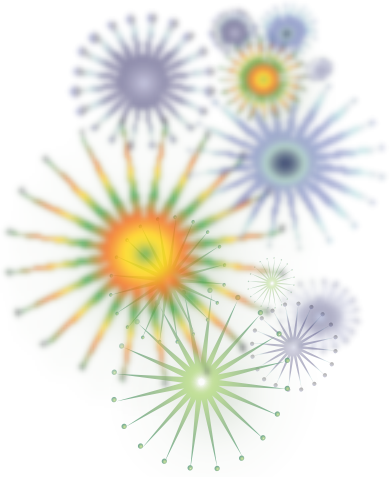
Lentivirus Biosafety Information and Handling Guidelines



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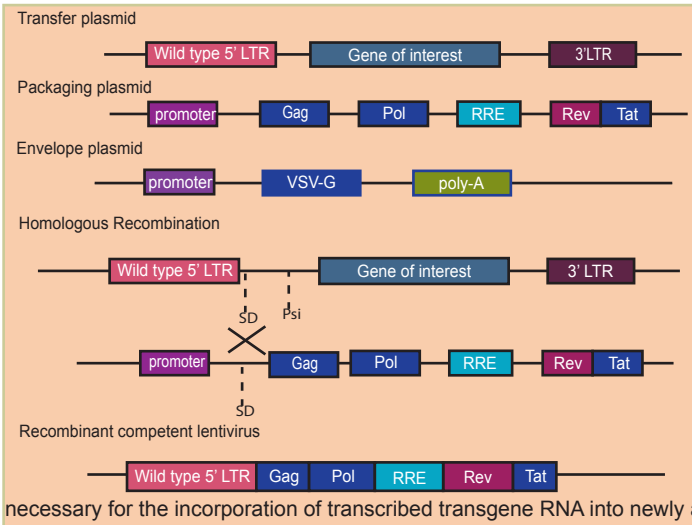
Introduction

Lentiviruses, a genus of the Retroviridae family, are enveloped retroviruses that replicate in a host cell through reverse transcription. Lentiviruses are classified as Biosafety Level 2 (BSL-2) organisms due to their ability to infect primary human cells. Pathogenic lentiviruses cause severe immunologic or neurologic diseases in humans and other mammals. As a gene delivery system, lentivirus-derived vectors can efficienctly deliver transgenes into a host cell genome and infect both dividing and non-dividing cells [2], making them powerful molecular biology research tools.

In recent years, lentiviruses or lentivirus-derived vectors have been increasingly used in research; therefore, it is important that principal investigators and researchers understand the risks associated with lentiviral exposure. This document is meant to provide general guidelines on biosafety issues that must be taken into consideration while working with lentivirus. This manual should provide a basic foundation for academic laboratories to establish their own standard working practices or protocols, and institute a proper risk management program to minimize lentivirus-related occupational hazards.

An Overview of Development of Lentivirus Packaging Systems

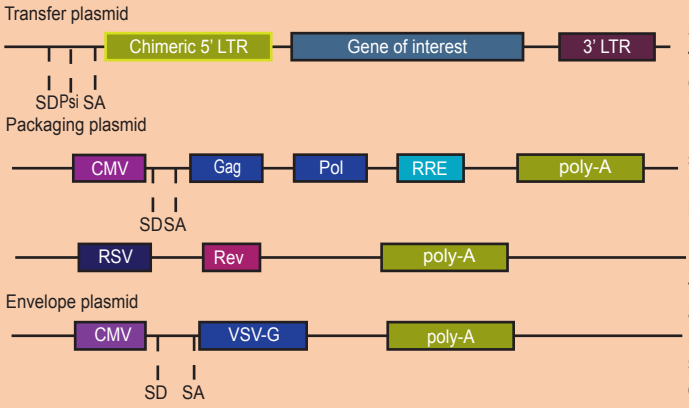
In the first-generation of engineered lentivirus packaging vectors, live viral particles were produced by expressing HIV-1 core proteins, enzymes and accessory factors on one plasmid and the broad host-tropic envelope gene from Vesicular Stomatitis Virus (VSV-G) on a separate plasmid [2]. The second-generation packaging system, still commonly used, improved biosafety by reducing the total number of HIV-1-derived components to *gag, pol, rev* and *tat* [2, 4]. However, safety concerns related to the production of replication-competent virus (RCL) still exist, which led to further improvements to this system. The third-generation lentivirus system further reduced risks of handling by inactivating additional viral components to limit the potential for generating replication-competent virus particles. Below is a detailed comparison on the biosafety features of 2nd versus 3rd generation packaging systems.



A schematic drawing of the second-generation lentivirus packaging system. 3 plasmids are used: a transfer plasmid, a packaging plasmid and an envelope plasmid. Lentiviral genes key to packaging *gag, pol, rev* and *tat* are all expressed on the same construct. The transfer plasmid carries the transgene under control of a wildtype 5' LTR. SD denotes splicer donor site and psi represents the packaging sequence necessary for the incorporation of transcribed transgene RNA into newly assembled lentiviruses. As shown in the figure above, one single step of homologous recombination between transfer plasmid and packaging plasmid could lead to the generation of RCLs.

Features of the second-generation packaging system:

- Contains 3 lentivirus structural genes *gag, pol* and *env,* and 2 lentivirus regulatory genes, *tat* and *rev*
- The vector itself carries HIV-derived cis-acting sequences including an HIV-derived wildtype 5' LTR, 5' splice donor site, *env* gene containing RRE and a splice acceptor site, and a truncated 3' LTR
- Transcription from the vector LTR occurs only in the presence of *tat*



Schematic representation of the third-generation lentivirus packaging system. Essential viral genes are split into 2 separate plasmids and the lentiviral components have been further inactivated [1]. The transfer plasmid carries a chimeric 5' LTR, which is generated by substituting an enhancer or constitutively active promoter for the U3 region (RRL) of wildtype 5' LTR [1]. This feature makes *tat* dispensable and allows the gene to be completely removed from the packaging system. SD: splicer donor site; SA: splicer acceptor site; Psi: packaging sequence.

Features of the third-generation packaging system:

- Contains 3 lentivirus structural genes *gag, pol* and *env*, and only 1 lentivirus regulatory gene *rev*
- *Tat*, a viral gene essential for viral self-replication, is eliminated and transactivation of the vector transcription is achieved by placing a strong constitutive promoter upstream of the vector transcript
- *Rev* gene is expressed *in trans* on a packaging construct separate from *gag* and *pol* genes
- In the packaging construct carrying *gag* and *pol* genes, transcription will be activated only if *rev* binds to RRE
- Transfer plasmid carries a chimeric 5' LTR with the U3 region replaced by a constitutively active promoter/enhancer. The transgene downstream of this promoter will be the only portion transferred to the target cell genomes and does not contain a wildtype HIV-1 LTR
- 3' LTR has a deletion at the U3 region, which removes the template to generate LTRs in the integrated proviruses and leads to self-inactivation

Comparison of Biosafety Features of 2nd and 3rd Generation System:

2nd-generation packaging system	3rd-generation packaging system
• Utilizes a wildtype 5' LTR; • Contains 4 lentiviral genes (*gag, pol, rev* and *tat*);	• The 5' LTR is inactivated; • The number of lentiviral genes is reduced to 3 (*gag, pol* and *rev*) from 4;

2nd-generation packaging system	3rd-generation packaging system
• 3 plasmids total in the packaging system, with *gag, pol, rev* and *tat* genes all being expressed on the same packaging construct; • A single-step double crossover could cause the wildtype 5' LTR and packaging sequence psi to be placed in front of lentiviral genes, thus generating replication-competent lentiviruses.	• Lentiviral genes required for packaging and replication have been split into 2 separate packaging plasmids. *Rev* gene is expressed on a construct separate from *gag and pol*. This separates the key elements required for virus production into 4 different plasmids and makes lentivirus packaging contingent on complementation *in trans* available only in packaging cells; • 3' LTR is truncated, reducing the possibility of producing replication-competent viruses; • The number of recombination events needed for RCL greatly increases, which should lead to increased biosafety.

Risk Management:

LENTIVIRUS HANDLING mainly includes the following risks [3]:

• The potential for generating replication-competent lentiviruses. The likelihood of a RCL event depends on:

►The number of essential genes present in the packaging system

►The number of recombination steps required to reassemble a replication-competent virus

• The potential for oncogenesis:

►Risk of oncogenesis as a result of packaging known oncogenes

►Risk of oncogenesis as a result of packaging genes with oncogenic potential

Warning: Do not use this system to package known oncogenes or genes with oncogenic potential into the lentiviral vector!

Note: A roadmap to control risks of virus-associated oncogenesis is provided by the National Institutes of Health in "NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules". The guide is available at: http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines_prn.pdf

• Potential for vector recombination in cells of HIV-positive, virus-negative patients

• General risks associated with the laboratory use of infectious lentivirus of high titer and volume

In order to control and reduce the risks associated with lentivirus handling, the following are **recommended:**

• Use of later generation lentivirus-packaging systems to package your virus. The third-generation lentivirus system is designed to reduce the risk of generating replication-competent virus. To date, there have been no reports of RCL production as a result of 3rd-generation lentivirus packaging;

• Use proper containment when working with lentiviruses as per BSL-2 and institutional guidelines;

• Refer to your institutional guidelines on animal containment requirements and husbandry or housing guidelines if you are using live lentivirus in animal studies;

• Conduct a comprehensive of risk assessment prior to any lentiviral operations:

► Your institution's Environmental Health and Safety department may wish to perform a RCL assessment. Note that such assessment requires live HIV-1 control viruses and may increase biosafety risks of operators performing these tests. According to one RCL testing on 60 liters of lentiviruses packaged via different vector systems by National Gene Laboratory (NGVL), no RCL was detected from lentivirus produced using 3rd-generation system. This strongly suggests that the frequency of RCL generation using the latest lentivirus packaging system is extremely low;

► Determine the oncogenic potential and other potential toxicity of the transgene to be incorporated into lentiviral vector;

► Examine the titer and total volume of lentivirus to be used. Use the minimum virus titer and total volume required to achieve desired experimental outcome.

Containment Guidelines

Containment requirements for research involving lentiviruses are addressed by a Recombinant DNA Advisory Committee (RAC)-issued document entitled "Biosafety Considerations for Research with Lentiviral Vectors." This document is available for download at:
http://oba.od.nih.gov/oba/rac/Guidance/LentiVirus_Containment/pdf/Lenti_Containment_Guidance.pdf

It is important that all work on lentivirus must be conducted under BSL-2 containment conditions. Live lentiviral vectors that incorporate known oncogenes or genes with oncogenic potential and lentivirus produced at large scale (greater than 100ml volume) requires higher levels of protection such as BSL-2 enhanced containment (including BSL-3 practices and Personal Protective Equipment to reduce potential mucosa exposure to the vector). 3-plasmid lentivirus system should also be used and generated at BSL-2 enhanced (laboratory research) or at Animal Biosafety Level-2 (ABSL-2) enhanced (animal research) containment. Reduced biosafety level may be used for

3-plasmid lentivirus system following demonstration that no detectable RCL is present in the virus preparations. Researchers must strictly abide by all published BSL-2 guidelines for lentiviral waste disposal.

Each institution may also draft their own guidelines for safe lentivirus handling. Research with BSL-2 organisms such as lentiviruses or lentiviral vectors may require review and approval from the Institutional Biosafety Committee (IBC).

Voices on Lentiviral Safety Considerations

Center of Disease Control (CDC):

"The risk associated with retroviral vector systems can vary significantly, especially lentiviral vectors. Because the risk associated with each gene transfer system can vary, no specific guideline can be offered other than to have all gene transfer protocols reviewed by an IBC."

- "Biosafety in Microbiological and Biomedical Laboratories", 5th edition (Available at: www.cdc.gov/biosafety/publications/index.htm)

National Institute of Health (NIH):

"A comprehensive risk assessment and determination of containment for research with lentiviral vectors should consider the nature of the vector system, transgene insert, and type of manipulations involved. For many experiments, either BL-2 or enhanced BL-2 will be appropriate. Examples of biosafety considerations may include vector generation considerations, animal research considerations and practices, containment and training considerations."

---------"Biosafety Considerations for Research with Lentiviral Vectors"

References

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2. Naldini L, Blömer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. 1996, "In Vivo Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector", Science, vol. 272, no. 5259, pp. 263-267

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4. Romain Zufferey, Dea Nagy, Ron J. Mandel, Luigi Naldini , Didier Trono 1997, "Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo", Nature Biotechnology, vol. 15, pp. 871-875.