



***Institutional Biosafety Committee (IBC)***

***Biosafety Officer***

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October 30, 2018

Dr. Brent Ryckman  
DBS  
University of Montana  
Missoula, MT 59812

Dear Dr. Ryckman,

The University of Montana Institutional Biosafety Committee (IBC) met October 30, 2018 and gave final approval to IBC research application 2018-10, "The role of glycoproteins in human cytomegalovirus replication."

Please be advised of the following items regarding your approved application:

- The principle investigator listed on the application is ultimately responsible for all activities associated with the use of any biological materials associated with this application.
- Approved IBC applications are public information and may be viewed by the public upon request. Therefore, they should not contain sensitive or proprietary information.
- The IBC reserves the right to review (and ask for modifications to) your approved application at any time.
- Your application is good for 3 years from the approval date, October 2, 2018 and will expire on **October 1, 2021.**

The IBC at The University of Montana is at your service and willing to provide assistance as needed. Please see the IBC web page at <http://umt.edu/research/compliance/IBC/default.php> for more information.

Sincerely,

*Kathy Heivilin*

Kathryn Follis Heivilin, CPIA, RBP  
Biosafety Officer  
Senior IACUC Manager

Laboratory Standard Operating Procedure  
(For the use of biohazardous materials or equipment)

**Name of Procedure:** Propagation and experiments involving cultures of human cell lines and human cytomegalovirus (or other herpesviruses) or replication-defective adenovirus vectors.

**Prepared By:** Brent Ryckman

**Revision Date:** 9-1-18

**LOCATION - This procedure may be performed at the following location(s):**

Work with concentrated virus stocks and infected cultures is conducted in ISB206A in the biological safety cabinet. The concentrated viral stocks are stored in the -80 freezer located in room ISB209.

**HAZARDS - The following materials and equipment associated with this procedure presents exposure or physical health hazards. Safety precautions are prudent and mandatory:**

Eyewashes are located at the sinks in ISB206/206A. Safety shower is located immediately inside the main door of ISB 206. Remove contaminated clothing and vigorously wash exposed body area with soap and water for 10 minutes. Obtain immediate medical attention at Curry Health Center. Report the incident to the P.I (Brent Ryckman) and to UM Health and Safety office (ext. 2881/4503).

**ENGINEERING CONTROLS - Prior to performing this procedure, the following safety equipment must be accessible and ready for use: (ex. biological safety cabinet, laminar flow hood, biological disinfectant)**

Biological safety cabinet is located in ISB206A. Perform all work that may create an aerosol or work with open virus stocks in the biological safety cabinet. Infection and post-infection incubations may be performed in CO2 incubators also in ISB206A. Aspiration of primary culture fluid from infected plates must be carried out inside the biological safety cabinet. Virus containing fluids are to be aspirated into the liquid waste trap (containing Wescodyne disinfectant). The concentrated stock of biological disinfectant is located in the cabinet labeled "disinfectant" in ISB206A. After performing any procedure involving live virus, the bench surface of the biosafety hood should be disinfected with a dilute solution (approx 10%) of liqnox or similar lab detergent. Fresh, dilute stock solutions are located in the tissue culture room and elsewhere as needed.

**PROTECTIVE EQUIPMENT - Prior to performing this procedure, the following personal protective equipment must be obtained and ready for use: (ex. Disposable gloves, safety eyewear, lab coat, apron)**

Disposable gloves (latex or nitrile) are required for all procedures involving live virus and/or cell cultures. Lab coats and eye protection are available and encouraged. Gloves are located at every work bench. Replace gloves as soon as possible when visibly soiled, torn or punctured. After work with infectious cultures, remove gloves into biohazard waste bin and wash your hands with soap and water. Wash your hands or any other contaminated skin with soap and water immediately or as soon as possible after removal of gloves and after visible contact with potentially infectious materials. Use facial barrier protection whenever splashes, spray, droplets, or aerosols may be generated (NOTE: Opening containers can create aerosols). Never scrape infected cultures without protective eyewear and face protection. Face protection may include the following: hood sashes, face shields, masks and safety glasses, or chin-length face shields. Always perform work in a biosafety cabinet when generating aerosols or working with primary culture fluids. Remove all PPE immediately upon leaving the work area and as soon as possible if overtly contaminated. Contaminated PPE will be DISPOSED of as biohazardous waste or decontaminated.

**WASTE DISPOSAL - This procedure will result in the following regulated waste, which must be disposed of in compliance with environmental regulations:**

Sharps- Dispose of sharps such as needles, broken glass, scalpels in labeled, hard walled containers. Label with an "infectious substance" marking. Container will be located in ISB206/206A. When 2/3 full, autoclave the sharps container and call EH&RM (x2881) to pick up autoclaved sharps container. Following autoclaving, the biohazard labels will be destroyed to indicate that they are now regular sharp waste.

Glass Pasteur pipettes that contact infectious material must be collected in a hard-walled container in the biosafety cabinet. These will be autoclaved and then dumped into the regular glass waste container.

Solids- Used nonradioactive tissue culture plates, plastic pipettes, pipette tips that may contain infectious material should be collected during the procedure in pails containing double layered orange, autoclave bags marked "biohazard", autoclaved, and then left in the hallway for janitorial pick-up. Following autoclaving, the biohazard labels will be destroyed to indicate that they are now regular waste. Solids such as pipettes and tips that are radioactive AND contain infectious agents should be handled as radioactive waste.

This will be picked up by the standard method (Radiation Safety). Solid waste such as contaminated laboratory coats and clothing will be autoclaved immediately and disposed of.

Liquids- If disinfected with bleach or wescodyne, virus-containing culture fluids can be poured down the drain. If the biohazardous liquid waste contains other chemicals besides bleach or wescodyne, such as radioactive materials, this will be disposed of by RSS by usual procedure once disinfected with bleach.

**ACCIDENTAL SPILL - In the event that a hazardous material spills during this procedure, be prepared to execute the following emergency procedure:**

If a small spill, alert people in the immediate area of the spill. Put on protective equipment. Cover the spill with paper towels or other absorbent materials. Carefully pour a freshly prepared 1 in 10 dilution of household bleach or wescodyne around the edges of the spill and then into the spill. Avoid splashing. Allow a 20-minute contact period. Use paper towels to wipe up the spill, working from the edges into the center. Clean the spill area with fresh towels soaked in disinfectant. Place clean-up materials into a biohazard bag and autoclave. If a large spill, evacuate the lab and call Security.

**CERTIFICATION - I have read and understand the above SOP. I agree to consult with the PI (Brent Ryckman) if I plan to modify this procedure.**

**Signature**

**Name (Print)**

**Date**

## What is CMV?

CMV, or cytomegalovirus, is a common virus that infects people of all ages. Once CMV is in a person's body, it stays there for life. Most infections with CMV are "silent," meaning most people who are infected with CMV have no signs or symptoms. **However, CMV can cause disease in unborn babies and in people with a weakened immune system.**

CMV is a member of the herpesvirus family, which includes the herpes simplex viruses and the viruses that cause chickenpox (varicella-zoster virus) and infectious mononucleosis (Epstein-Barr Virus).

## Who is at risk for CMV disease?

Anyone can become infected with CMV. Most healthy adults and children who have a CMV infection will have few, if any, symptoms. However, certain groups are at higher risk of getting CMV disease. **These groups include**

- unborn babies who are infected during pregnancy
- people with a weakened (immunocompromised) immune system

## How is CMV spread?

- Person to person contact (such as, kissing, sexual contact, and getting saliva or urine on your hands and then touching your eyes, or the inside of your nose or mouth)
- Through the breast milk of an infected woman who is breast feeding
- Infected pregnant women can pass the virus to their unborn babies
- Blood transfusions and organ transplantations

CMV is sometimes found in body fluids, including urine, saliva (spit), breast milk, blood, tears, semen, and vaginal fluids. A person can become infected with CMV when they come in contact with infected body fluids. However, people who are CMV-positive (have been infected with CMV sometime in the past) usually do not have virus in these fluids, so the chance of getting a CMV infection from casual contact is very small.

**Women who are pregnant or planning a pregnancy should follow hygienic practices (e.g., careful handwashing) to avoid CMV infection.** Because young children are more likely to have CMV in their urine or saliva (spit) than are older children or adults, pregnant women who have young children or work with young children should be especially careful.

## What are the signs and symptoms of CMV?

Most healthy children and adults infected with CMV have no symptoms and may not even know that they have been infected. Others may develop a mild illness. Symptoms may include fever, sore throat, fatigue, and swollen glands. These symptoms are similar to those of other illnesses, so most people are not aware that they are infected with CMV.

Most babies born with CMV (in other words, "congenital" CMV) never develop symptoms or disabilities. When babies do have symptoms, some can go away but others can be permanent.

In some children, symptoms do not appear until months or years after birth. The most common of these late-occurring symptoms are hearing loss and vision loss. Children with congenital CMV are more likely to have permanent disabilities and symptoms that get worse if they had symptoms of CMV infection at birth. But, some children who appear healthy at birth can develop hearing or vision loss over time due to congenital CMV. For this reason, if you know your baby was born with CMV, it is important to have her or him hearing and vision tested regularly.

CMV can cause symptoms when the baby is born or later in the baby's life. Most babies born with CMV never develop symptoms or disabilities. In some infants, hearing or vision loss occur months or years after birth.

## How do I know if I have CMV?

Most CMV infections are not diagnosed because the infected person usually has few or no symptoms. However, persons who have been infected with CMV develop antibodies to the virus, which may stay in a person's body for their lifetime. Antibodies are immune proteins that are the body's response to infection.

A blood test can tell a person if they have CMV, but this test is not commonly performed. Laboratory tests can detect the virus in a person's body fluids (blood or urine) or by a tissue biopsy (a small piece of the body's tissue). CMV can also be detected in the body by measuring the antibodies (immune proteins) in the blood targeted against CMV. This is called serologic testing.

Congenital CMV disease is most likely to occur when a woman is infected for the first time during a pregnancy. This is known as a primary CMV infection. Primary infections occur in 1%-4% of seronegative (have no CMV antibodies) pregnant women and lead to fetal infection in one-third of these pregnancies. In women who are already infected before becoming pregnant (CMV seropositive women), CMV reactivation or reinfection leads to fetal infection in less than 1% of pregnancies. Approximately 10% of congenitally infected infants who have symptoms at birth, and of the 90% who have no symptoms, 10%-15% will develop symptoms over months or even years.

### **How do you prevent CMV during pregnancy?**

There is no vaccine to prevent CMV infection; however, there are certain steps pregnant women can take that may reduce their risk of acquiring CMV and other infections that may pose a risk to their unborn children. If you are pregnant,

- Wash your hands often with soap and water for 15-20 seconds, especially after changing diapers or touching saliva or nasal secretions from a young child.
- Reduce contact with saliva and nasal secretions from young children by 1) using soap and water or a disinfectant to clean hard surfaces that have been contaminated by secretions, 2) not sharing food, drinks, or eating utensils with young children, and 3) being careful to limit kissing of young children on the lips.



Wash hands with plenty of soap and water.

### **Is there a treatment for CMV?**

Currently, no treatment is recommended for CMV infection in the healthy individual, including pregnant women. However, antiviral drugs ganciclovir and valganciclovir are being used for patients with weakened immune systems. Antiviral drugs are being tested in infants born with congenital CMV. Because of its strong side effects, ganciclovir should only be considered for infants with severe congenital CMV disease.

Vaccines for preventing CMV infection are still in the research and development stage.

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### **BMBL 5<sup>th</sup> Edition**

Human CMV may pose a special risk during pregnancy because of potential infection of the fetus. All human herpesviruses pose an increased risk to persons who are immunocompromised.

# Adenoviruses

## Key Facts and Q&As about Adenovirus 14

**Clinical features:** Adenoviruses most commonly cause respiratory illness; however, depending on the infecting serotype, they may also cause various other illnesses, such as gastroenteritis, conjunctivitis, cystitis, and rash illness. Symptoms of respiratory illness caused by adenovirus infection range from the common cold syndrome to pneumonia, croup, and bronchitis. **Patients with compromised immune systems are especially susceptible to severe complications of adenovirus infection.** Acute respiratory disease (ARD), first recognized among military recruits during World War II, can be caused by adenovirus infections during conditions of crowding and stress.

**The viruses:** Adenoviruses are medium-sized (90-100 nm), nonenveloped icosohedral viruses containing double-stranded DNA. There are 49 immunologically distinct types (6 subgenera: A through F) that can cause human infections. Adenoviruses are unusually stable to chemical or physical agents and adverse pH conditions, allowing for prolonged survival outside of the body.

**Epidemiologic features:** Although epidemiologic characteristics of the adenoviruses vary by type, all are transmitted by direct contact, fecal-oral transmission, and occasionally waterborne transmission. Some types are capable of establishing persistent asymptomatic infections in tonsils, adenoids, and intestines of infected hosts, and shedding can occur for months or years. Some adenoviruses (e.g., serotypes 1, 2, 5, and 6) have been shown to be endemic in parts of the world where they have been studied, and infection is usually acquired during childhood. Other types cause sporadic infection and occasional outbreaks; for example, epidemic keratoconjunctivitis is associated with adenovirus serotypes 8, 19, and 37. Epidemics of febrile disease with conjunctivitis are associated with waterborne transmission of some adenovirus types, often centering around inadequately chlorinated swimming pools and small lakes. ARD is most often associated with adenovirus types 4 and 7 in the United States. Enteric adenoviruses 40 and 41 cause gastroenteritis, usually in children. For some adenovirus serotypes, the clinical spectrum of disease associated with infection varies depending on the site of infection; for example, infection with adenovirus 7 acquired by inhalation is associated with severe lower respiratory tract disease, whereas oral transmission of the virus typically causes no or mild disease. Outbreaks of adenovirus-associated respiratory disease have been more common in the late winter, spring, and early summer; however, adenovirus infections can occur throughout the year.

**Diagnosis:** Antigen detection, polymerase chain reaction assay, virus isolation, and serology can be used to identify adenovirus infections. Adenovirus typing is usually accomplished by hemagglutination-inhibition and/or neutralization with type-specific antisera. Since adenovirus can be excreted for prolonged periods, the presence of virus does not necessarily mean it is associated with disease.

**Treatment:** Most infections are mild and require no therapy or only symptomatic treatment. **Because there is no virus-specific therapy, serious adenovirus illness can be managed only by treating symptoms and complications of the infection.**

**Prevention:** Vaccines were developed for adenovirus serotypes 4 and 7, but were available only for preventing ARD among military recruits. Strict attention to good infection-control practices is effective for stopping nosocomial outbreaks of adenovirus-associated disease, such as epidemic keratoconjunctivitis. Maintaining adequate levels of chlorination is necessary for preventing swimming pool-associated outbreaks of adenovirus conjunctivitis.



## Lentiviral Guide

[Components](#) | [2nd Generation](#) | [3rd Generation](#) | [Viral Production](#) | [FAQ](#) | [References](#) | [Glossary](#)

### Lentiviral Components

To increase the safety of lentivirus, the components necessary for virus production are split across multiple plasmids (3 for 2nd-generation systems, 4 for 3rd-generation systems). The components of both systems are as follows:

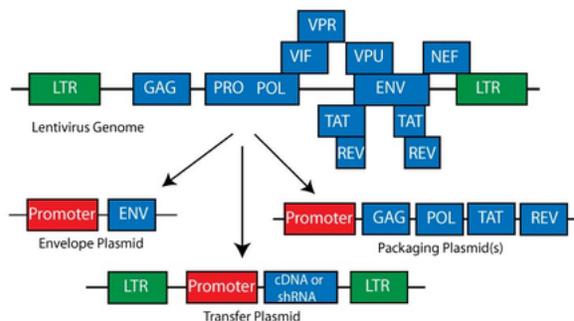
1. Lentiviral transfer plasmid encoding your insert of interest. This sequence is flanked by long terminal repeats (LTRs) that facilitate host genome integration. To improve safety, transfer vectors are all replication incompetent and may additionally contain a deletion in the 3'LTR, rendering the virus "self-inactivating" (SIN) after integration.
2. Packaging plasmid(s)
3. Envelope plasmid

Addgene's packaging and envelope plasmids are generalized and appropriate for varied cell types and systems. When planning your experiment, the important component to consider and optimize is the transfer plasmid. 2nd generation lentiviral plasmids utilize the viral LTR promoter for gene expression, whereas 3rd-generation transfer vectors utilize a hybrid LTR promoter (more information on this below). Additional or specialized promoters may also be included within a transfer plasmid: for example, the U6 promoter is included in the pSico plasmid to drive shRNA expression. Other features that can be included in transfer plasmids include: Tet- or Cre-based regulation and fluorescent fusions or reporters.

[Browse lentivirus plasmids available from Addgene.](#)

### 2nd Generation

The graphic to the right shows how the lentiviral genome is edited down and distributed across the three plasmids comprising the 2nd-generation lentiviral system. This system contains a single packaging plasmid encoding the Gag, Pol, Rev, and Tat genes. The transfer plasmid contains the viral LTRs and psi packaging signal (not pictured). Unless an internal promoter is provided, gene expression is driven by the 5'LTR, which is a weak promoter and requires the presence of Tat to activate expression. The envelope protein Env (usually VSV-G due to its wide infectivity) is encoded on a third, separate, envelope plasmid. **All 2nd generation lentiviral transfer plasmids must be used with a 2nd generation packaging system because transgene expression from the LTR is Tat-dependent.**

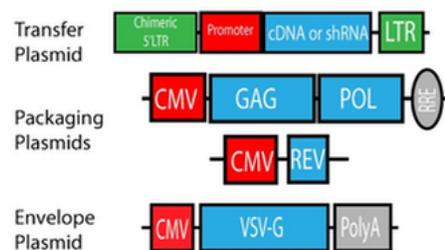


Second Generation Lentiviral Plasmids

### 3rd Generation

The 3rd generation system further improves on the safety of the 2nd generation in a few key ways. First, the packaging system is split into two plasmids: one encoding Rev and one encoding Gag and Pol. Although safer, this system can be more cumbersome to use and result in lower viral titers due to the addition of one additional plasmid.

Second, Tat is eliminated from the 3rd generation system through the addition of a chimeric 5' LTR fused to a heterologous promoter on the transfer plasmid. Expression of the transgene from this promoter is no longer dependent on Tat transactivation. The 3rd generation transfer plasmid can be packaged by either a 2nd generation or 3rd generation packaging system. For a comparison of the key differences between the 2nd and 3rd generation packaging systems, see the table below.



Third Generation Lentiviral Plasmids

### 2nd vs 3rd Generation Lentiviral Systems

| Feature                 | 2nd Generation   | 3rd Generation   |
|-------------------------|--|--|
| <b>Transfer Plasmid</b> | Can be packaged ONLY by a second generation packaging system that includes TAT | Can be packaged by both 2nd and 3rd generation packaging systems |

[Help](#)

| Feature                   | 2nd Generation  | 3rd Generation  |
|---------------------------|---|---|
| <b>Packaging Plasmid</b>  | All on one plasmid: Gag, Pol, Rev, Tat  | Two plasmids: one encoding Gag and Pol and another encoding Rev   |
| <b>Envelope Plasmid</b>   | Interchangeable: usually encodes for VSV-G  | Interchangeable: usually encodes for VSV-G  |
| <b>Safety</b>             | Safe. Replication incompetent: Uses 3 separate plasmids encoding various HIV genes. | Safer. Replication incompetent and always SIN: Uses 4 plasmids instead of 3 and eliminates the requirement for Tat. |
| <b>LTR Viral Promoter</b> | Wild type   | Hybrid: 5'LTR is partially deleted and fused to a heterologous enhancer/promoter such as CMV or RSV                 |

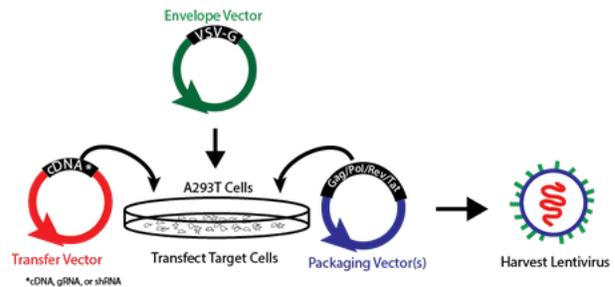
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## Viral Production

The process of producing infectious transgenic lentivirus is outlined in the simple schematic to the right. 3-4 plasmids are transfected into A293T cells:

- one transfer vector
- one or two packaging vector(s)
- one envelope vector

After media change and a brief incubation period, supernatant containing the virus is removed and stored or centrifuged to concentrate virus. Crude or concentrated virus can then be used to transduce the cells of interest. For determination of viral titer and full details, [see the protocol available at the Trono lab website](#).



*Viral Production Schematic*

## Frequently Asked Questions (FAQ) about Lentiviral Plasmids

### What is the difference between 2nd generation and 3rd generation lentiviral systems?

For a full description of 2nd and 3rd generation lentiviruses, [please review 2nd vs 3rd generation above](#). Briefly, 2nd generation lentiviral systems use more HIV proteins (on fewer plasmids) in order to produce functional lentiviral particles than 3rd generation systems.

- 2nd generation packaging systems: express the HIV gag, pol, rev, and tat genes all from a single packaging vector such as [psPAX2](#).
- 3rd generation packaging systems: express gag and pol from one packaging vector and rev from another, such as [pMDLg/pRRRE](#) and [pRSV-Rev](#). 3rd generation packaging systems **DO NOT** express tat. Third generation lentiviral systems are considered safer than second generation systems, but may be more difficult to use because they require transfection with four separate vectors in order to create functional lentiviral particles.

**IMPORTANT:** A 3rd generation transfer vector can be used with a 2nd generation packaging system, but a 2nd generation transfer vector *cannot be used* with a 3rd generation packaging system.

### What is the difference between a lentivirus and a retrovirus?

Lentiviruses are a subtype of retrovirus. From an experimental standpoint the main difference between lentiviruses and standard retroviruses ( $\gamma$ -retroviruses) is that lentiviruses are capable of infecting non-dividing and actively dividing cell types whereas standard retroviruses can only infect mitotically active cell types. This means that lentiviruses can infect a greater variety of cell types than retroviruses.

Both lentiviruses and standard retroviruses use the gag, pol, and env genes for packaging; however, they are different viruses and thus use slightly different isoforms of these packaging components. Therefore, lentiviral vectors may not be efficiently packaged by retroviral packaging systems, and vice versa.

### Which bacterial strain should be used for cloning and producing my lentiviral vector?

Due to the long terminal repeats found in lentiviral vectors, we recommend using a strain that reduces the frequency of homologous recombination of unstable regions, such as Invitrogen Stbl3™ or NEB Stable cells. This will ensure that the repeats will be maintained and often results in a greater yield of DNA. However, if the vector contains a Gateway cassette containing the ccdB gene, a ccdB survival strain is necessary.

### What cell line should be used in order to produce lentivirus?

293T cells are usually used to produce lentivirus. The 293T cell line can be obtained from [GenHunter](#).

### What dictates lentiviral host cell range (tropism)?

Lentiviral tropism is determined by the ability of the viral envelope protein to interact with receptors at the host cell surface. The VSV-G envelope

protein is commonly used in lentiviral particle production because it confers broad tropism over a range of species and cell types. For more information, [see the Cronin, et al. article on different envelopes and their tropism](#).

### How can lentiviral vectors be used to make stable cell lines?

Lentiviral vectors can be used to make stable cell lines in the same manner as standard retroviral vectors. That is, many lentiviral vectors have selectable markers, such as the puromycin resistance gene, conferring resistance to antibiotics. When these antibiotics are added to the growth medium, they kill off any cells that have not incorporated the vector and those cells that survive can be expanded to create stable cell lines which have incorporated the vector and express the insert.

Many lentiviral transfer vectors do not have selectable markers conferring resistance to an antibiotic, but do express some other marker such as GFP. A researcher can use FACS to separate cells expressing fluorescence and later expand these cells into a cell line.

### Where does lentivirus integrate?

Genome-wide studies of viral integration have shown that lentiviruses most often integrate into actively transcribed genes, and that this preference is conserved across target species. Although chromatin availability facilitates integration, it does not explain the lentiviral preference for transcribed genes. Studies comparing the lentivirus HIV and the retrovirus MMLV indicate that the viral integrase plays a role in shaping integration site preferences. A major cellular determinant is LEDGF/p75, a lentiviral tethering protein that recruits the pre-integration complex to transcriptional units and facilitates integration. LEDGF/p75 binding sites are enriched in gene bodies and mostly absent in promoters and intergenic regions, mirroring patterns of lentiviral integration.

### Can lentiviral vectors be used in direct transfections as opposed to making virus?

Some (but not all) lentiviral transfer vectors can be used in transient transfections to achieve expression of the transgene, and those that can are primarily third generation constructs. Lentiviral transfer vectors are not designed specifically for transient transfections; therefore, there may be consequences on transgene expression due to the lentiviral LTRs. While possible, it is not explicitly recommended that you use lentiviral transfer vectors for simple transfections.

### Is it feasible to express cDNA from a lentiviral transfer vector normally used for shRNA expression?

Yes, it is feasible, but first the promoter within the transfer vector must be changed. Most shRNA-expressing lentiviral vectors such as pLKO.1 use a U6 or H1 promoter in order to drive RNA pol III transcription of shRNAs. cDNA expression requires the use of a RNA pol II promoter such as CMV or RSV.

### What techniques can be used to clone an insert into a lentiviral vector containing only one restriction site?

If a lentiviral vector contains only one restriction site, one can use standard cloning techniques to ligate the insert into this site. If it is not immediately feasible to digest and clone the insert from a parent vector, some possible approaches to using this site include subcloning or appending compatible restriction sites onto the insert of interest using PCR. The process of subcloning consists of digesting the insert of interest from its parent vector and ligating into a second vector in such a way that the insert may later be digested from this new vector and cloned into the lentiviral vector. This is basically shuffling restriction sites between vectors until the gene of interest is flanked by sites compatible with those in the vector into which one ultimately wants to ligate the insert. Often times it is less time consuming and easier to simply add restriction sites onto the insert of interest using PCR. This is accomplished by PCR amplifying the insert sequence using primers that contain the restriction sites needed. Functional restriction sites must be a certain number of bases from the ends of the primers used. Alternatively, you could ligate a multiple cloning site (MCS) from a separate vector into the single site in the lentiviral vector and generate more useful restriction sites.

For more information about cloning and changing a MCS, [visit Addgene's Plasmid Reference and Protocol Guide](#).

### How do I clone my insert into a transfer vector with no viable restriction sites but compatible with the Gateway® cloning system?

Gateway® compatible vectors use recombination in order to generate clones containing the insert of interest. In brief, the insert is first cloned into an entry vector at a region flanked by sequences (called attP1 and attP2) that allow the insert to recombine with the destination vector (in this case the destination vector would be the lentiviral transfer vector). The destination vector contains attB sequences with which the attP sequences recombine. [Visit Invitrogen's website for more information on the Gateway® cloning system](#).

### What safety concerns surround the use of lentiviral vectors?

As noted by the NIH, the two main safety concerns surrounding the use of lentiviral are:

1. The potential for generation of replication-competent lentivirus
2. The potential for oncogenesis

The potential for generation of replication-competent lentivirus is addressed by the design of the vectors and by safe laboratory practice. In terms of vector design, 2nd and 3rd generation lentiviral systems provided by Addgene separate transfer, envelope, and packaging components of the virus onto different vectors. The transfer vector encodes the gene of interest and contains the sequences that will incorporate into the host cell genome, but cannot produce functional viral particles without the genes encoded in the envelope and packaging vectors. Unless recombination occurs between the packaging, envelope, and transfer vectors, and the resulting construct is packaged into a viral particle, it is not possible for viruses normally produced from these systems to replicate and produce more virus after the initial infection. In this regard, 3rd generation systems are considered safer than 2nd generation systems because the packaging vector has been divided into two separate plasmids (resulting in a four plasmid system in total). In addition, 3rd generation systems do not use the HIV protein tat in order to produce full length virus from the transfer vector during the viral

production stage.

Many of the lentiviral transfer vectors that have been deposited with Addgene are self-inactivating (SIN) vectors. These vectors have a deletion in the 3'LTR of the viral genome that is transferred into the 5'LTR after one round of reverse transcription. This deletion abolishes transcription of the full-length virus after it has incorporated into a host cell.

The potential for oncogenesis is largely based on the specific insert contained within the lentiviral transfer vector (dependent upon whether or not it is an oncogene) and should be considered on a case by case basis.

Biosafety should always be considered with respect to the precise nature of experiments being performed, and your biosafety office can provide more information on your institution's best practices with regard to lentiviral research. [The NIH has additional information on lentiviral safety considerations.](#)

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## Additional Resources

### Web References

- [Find lentiviral plasmids available from Addgene](#)
- Addgene has put together a webinar ([Lentivirus 101: Plasmids and Viral Production](#)) with Bitesize Bio focused on understanding the components of lentiviruses and how they are produced in the lab. The webinar covers:
  1. Plasmids required to generate lentivirus (both 2nd and 3rd generation systems)
  2. Safety Concerns
  3. Lentiviral-based applications
- [Read our detailed protocol for using the popular cloning vector pLKO.1.](#)
- [Trono Lab Resources:](#)
  - [Lentivectors Toolbox](#)
  - [Standard Lentiviral Production & Titration Protocols](#)
  - [Lentiviral Maps](#)



### Publications

- Retroviral integration site selection. Desfarges S, Ciuffi A. *Viruses*. 2010. Jan;2(1):111-30. [PubMed](#).
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- Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element Enhances Expression of Transgenes Delivered by Retroviral Vectors. Zufferey R, Donello JE, Trono D, and Hope TJ. *J Virol*. 1999. 73(4):2886-92. [PubMed](#).
- A Third Generation Lentivirus Vector with a Conditional Packaging System. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, and Naldini L. *J Virol*. 1998. 72(11):8463-8471. [PubMed](#).
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- In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Naldini L, Blömer U, Gally P, Ory D, Mulligan R, Gage FH, Verma IM, and Trono D. *Science*. 1996. 272(5259): 263-267. [PubMed](#).

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## Glossary

| Plasmid Type     | Element | On Same Plasmid as Transgene? | Purpose   |
|------------------|---------|-------------------------------|---|
| <b>Envelope</b>  | VSVG    | <i>in trans</i>               | Vesicular stomatitis virus G glycoprotein; Broad tropism envelope protein used to pseudotype most lentiviral vectors. |
| <b>Packaging</b> | Gag     | <i>in trans</i>               | Precursor structural protein of the lentiviral particle containing Matrix, Capsid, and Nucleocapsid components.       |

| Plasmid Type    | Element  | On Same Plasmid as Transgene? | Purpose  |
|-----------------|--|-------------------------------|--|
|                 | Pol  | <i>in trans</i>               | Precursor protein containing Reverse Transcriptase and Integrase components.   |
|                 | Rev  | <i>in trans</i>               | On separate plasmid from Gag/Pol in third generation system; Binds to the Rev Response Element (RRE) within unspliced and partially spliced transcripts to facilitate nuclear export.  |
|                 | Tat  | <i>in trans</i>               | Second generation only; Trans-activator; binds TAR to activate transcription from the LTR promoter.  |
| <b>Transfer</b> | cPPT   | <i>in cis</i>                 | Central polypurine tract; recognition site for proviral DNA synthesis. Increases transduction efficiency and transgene expression.   |
|                 | Psi ( $\Psi$ )   | <i>in cis</i>                 | RNA target site for packaging by Nucleocapsid.   |
|                 | RRE  | <i>in cis</i>                 | Rev Response Element; sequence to which the Rev protein binds.   |
|                 | WPPE   | <i>in cis</i>                 | Woodchuck hepatitis virus post-transcriptional regulatory element; sequence that stimulates the expression of transgenes via increased nuclear export.   |
|                 | LTR<br><br><i>Subcomponents:</i><br><ul style="list-style-type: none"> <li>• U3</li> <li>• R</li> </ul> <i>Subelement:</i><br><ul style="list-style-type: none"> <li>◦ TAR</li> </ul> <ul style="list-style-type: none"> <li>• U5</li> </ul> | <i>in cis</i>                 | <p>LTR; Long terminal repeats; U3-R-U5 regions found on either side of a retroviral provirus (see below). Cloning capacity between the LTRs is ~8.5kb, but inserts bigger than ~3kb are packaged less efficiently.</p> <p><b>Subcomponents:</b></p> <p>U3; Unique 3'; region at the 3' end of viral genomic RNA (but found at both the 5' and 3' ends of the provirus). Contains sequences necessary for activation of viral genomic RNA transcription.</p> <p>R; Repeat region found within both the 5' and 3' LTRs of retro/lentiviral vectors. Tat binds to this region.</p> <p><b>Subelement:</b></p> <p>TAR; 2nd generation only; Trans-activating response element; located in the R region of the LTR and acts as a binding site for Tat.</p> <p>U5; Unique 5'; region at the 5' end of the viral genomic RNA (but found at both the 5' and 3' ends of the provirus).</p> |
|                 | 5' LTR   | <i>in cis</i>                 | Acts as an RNA pol II promoter. The transcript begins, by definition, at the beginning of R, is capped, and proceeds through U5 and the rest of the provirus. Third generation vectors use a hybrid 5' LTR with a constitutive promoter such as CMV or RSV.  |
|                 | 3' LTR   | <i>in cis</i>                 | Terminates transcription started by 5' LTR by the addition of a poly A tract just after the R sequence.  |

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