



# Viral Vectors 101

A Desktop Resource

Created and Compiled by Addgene  
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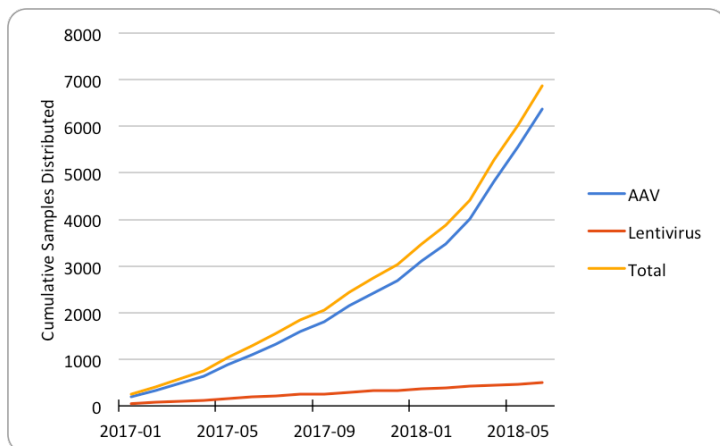
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# Viral Vectors 101: A desktop resource

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# Viral Vectors 101: Introduction to this desktop resource

By Tyler J. Ford | July 16, 2018



Cummulative ready-to-use virus distribution through June 2018.

adeno-associated viral vectors.

To further enable researchers, we started our viral service in 2017. Through this service, we distribute ready-to-use, quality-controlled AAV and lentivirus for direct use in experiments. As you can see in the chart to the left, this service is already very popular and its use has grown exponentially.

With this Viral Vectors 101 eBook, we are proud to further expand our viral vector offerings. Within it, you'll find nearly all of our viral vector educational content in a single downloadable resource. We hope you can use this eBook to expand your molecular biology tool kit, to introduce new members of your lab to the many uses of viral vectors, to brush up on viral vector background, to gain insights into new viral vector technologies, and to find troubleshooting tips for your viral vector experiments.

If you have any comments, questions or suggestions for this eBook, please send an email to [blog@addgene.org](mailto:blog@addgene.org) and we'll be happy to help in any way we can.

Happy reading!

~The Addgene Team



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# CHAPTER 1

## Introduction to viral vectors

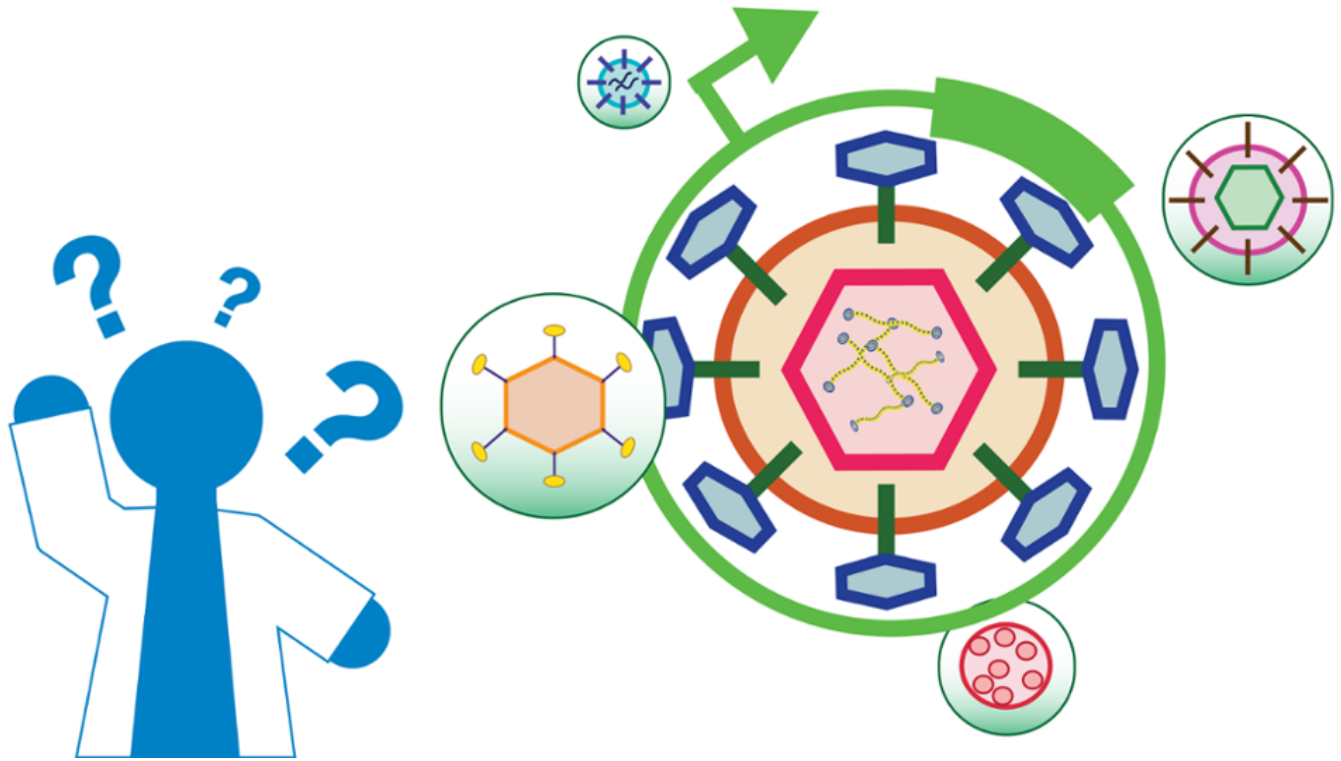


# Introduction to viral vectors

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# Using virus in your research - A primer for beginners

By Leila Haery | June 7, 2016



With new technologies being developed and shared more easily than ever, it may be difficult to keep up with how much there is to know. One well-established and widely popular technology (that scientists love to discuss) is virus-- specifically, using viruses as research tools. Viruses are appealing because they are widely available, customizable, and allow manipulation of genetic material within a living cell. Thanks to major advances in DNA sequencing, synthesis, and cloning over the past decade, more researchers rely on viruses to perform complex [genome engineering](#) tasks. The wide array of viral tools and applications available can make it difficult for the uninitiated researcher to determine the best way to design an experiment using virus. In this post, I will give a brief introduction to viruses and discuss some of their important characteristics to help get any virus novice started.

In general, viruses allow us to deliver genetic material to living cells. Researchers typically use viruses for two types of studies—(1) knock-in; introducing a protein-coding gene into cells to study its function, or (2) knockout/knockdown; studying gene function through deletion (potentially using [CRISPR lentivirus knockout libraries](#)) or reduction of gene expression, respectively. If you are a researcher who will be using virus for the first time, here are a few considerations to get you started.

## Viruses come in different flavors

Addgene provides vectors that can be used to produce four main types of virus: [gamma-retrovirus](#), [lentivirus](#) (a sub-type of retrovirus), [adenovirus](#), and [adeno-associated virus \(AAV\)](#). Each type of virus can be used for specific research applications. Retroviruses, a family of viruses that stably integrates into the host genome, are one of

# Using virus in your research - A primer for beginners (CONT'D)

the most popular types of virus. While retroviruses generally only infect dividing cells (because their access to the host genome is thought to rely on the breakdown of the nuclear envelope that occurs in mitosis [[Roe et al., 1993](#)]), lentiviruses are a genus of the retroviral family that can infect non-dividing cells (possibly through the use of nuclear localization signals by the viral components [[Bukrinsky et al., 1992](#)]), and thus, offer an advantage when transducing certain cell types that exhibit limited cell division (e.g., neurons). Adenoviruses, which are part of a separate family, can also infect non-dividing cells and are generally used to achieve transient high gene expression. Because adenoviruses do not integrate into the host genome and instead remain episomal, they do not disrupt the host genome and thus avoid the insertional mutagenesis associated with the random insertion of some retroviruses [[Coffin et al., 1997](#)]. Adeno-associated viruses (AAVs) also remain episomal, have the ability to infect non-dividing cells, and (because of their low immunogenicity) are attractive tools for gene therapy. Details about the pros and cons of each virus are listed in the table below.

| Type of Virus     | Pros   | Cons  |
|-------------------|--|---|
| <b>Retrovirus</b> | <ul style="list-style-type: none"> <li>• <b>Stable gene expression</b></li> <li>• <b>Broad tropism</b></li> </ul>  | <ul style="list-style-type: none"> <li>• <b>Only infects dividing cells</b></li> <li>• <b>Moderate immunogenicity</b></li> <li>• <b>Insertional mutagenesis possible</b></li> </ul>                           |
| <b>Lentivirus</b> | <ul style="list-style-type: none"> <li>• <b>Infects non-dividing cells</b></li> <li>• <b>Broad tropism</b></li> </ul>  | <ul style="list-style-type: none"> <li>• <b>Insertional mutagenesis possible</b></li> </ul>   |
| <b>Adenovirus</b> | <ul style="list-style-type: none"> <li>• <b>Remains episomal (little risk of insertional mutagenesis)</b></li> <li>• <b>High gene expression</b></li> <li>• <b>Infects non-dividing cells</b></li> <li>• <b>Large packaging capacity</b></li> <li>• <b>High transduction efficiency</b></li> <li>• <b>Broad tropism</b></li> </ul> | <ul style="list-style-type: none"> <li>• <b>Transient gene expression</b></li> <li>• <b>High immunogenicity</b></li> </ul>  |
| <b>AAV</b>        | <ul style="list-style-type: none"> <li>• <b>Remains episomal (little risk of insertional mutagenesis)</b></li> <li>• <b>Very low immunogenicity</b></li> <li>• <b>Infects non-dividing cells</b></li> </ul>  | <ul style="list-style-type: none"> <li>• <b>Transient gene expression</b></li> <li>• <b>Low tropism for mouse cells and hematopoietic cells</b></li> <li>• <b>Low packaging capacity (~4.5 kb)</b></li> </ul> |

## The buck stops at the transduced cell

For safety reasons (see below), viral vectors are engineered such that once the virus particle is produced it cannot reproduce (this feature is termed replication incompetence or replication deficiency). In other words, virus particles can transduce host cells (just like natural viruses), but (unlike natural viruses) the transduced host cell is not able to produce new virus particles.

# Using virus in your research - A primer for beginners (CONT'D)

Given that they cannot use a host cell, production of replication incompetent virus particles is achieved by particular cells that are able to replicate the viral genome (called packaging cells) and necessitates the presence of other plasmids (called helper plasmids or packaging plasmids) besides the original viral vector that provide replication machinery and virus structural components. Once a sufficient number of virus particles have been generated by the packaging cells, they can then be harvested and used to transduce target cells of interest.

A commonly used packaging cell type is the 293T (or HEK293T) cell line. These cells are so called because they stably express the SV40 large T antigen, which is required for efficient replication of plasmids containing the SV40 [origin of replication](#). Given that many viral vectors contain the SV40 origin of replication, 293T cells are able to produce high viral titers. In addition to the viral vector itself, each virus particle comprises a viral envelope that fuses with the target cell's membrane and determines target cell specificity or tropism (see [Viruses of a feather infect together](#), below), and a protein capsid that encloses the genetic material. These components are delivered through packaging plasmids encoding the gag, pol, and env genes, which contribute to the virion structure. Some packaging cells already stably express these genes (e.g., 293GP), which eliminates the requirement for packaging plasmids during viral particle production

## Viruses of a feather infect together

Depending on the composition of the viral envelope, viruses exhibit particular patterns of cell tropism, and thus are suited for infecting specific cell types. [Pseudotyping](#) is the process of producing viruses with foreign envelope proteins and is frequently used to generate viruses with altered cell tropism [[Cronin et al., 2006](#)]. For example, VSV-G pseudotyped viruses can be used to transduce cells of almost any species (termed pantropic). Alternatively, ecotropic viruses have tropism that is generally limited to a small group of species or cell types (like mice and rats), while amphotropic refers to a virus that can transduce a wide range of hosts.

## Not all vector sequences are created equal

Viruses are often used to study the function of a particular gene, either through overexpression, knockdown, or knockout. Designing viral vectors for gene overexpression can be relatively straightforward in that the gene coding sequence can simply be subcloned into a viral vector (though codon optimization of the sequence could increase protein expression within different species). Designing vectors to knockdown (with [RNAi](#)) or knockout (with [CRISPR](#)) gene expression, however, can be more complicated because the same gene can be targeted by a variety of vector sequences, each of which can exhibit a different targeting efficiency and exhibit unintended off target effects. Fortunately, there are resources available to help researchers design sequences to minimize off target effects and other potential complications. Some of these resources are listed below. However, whether using RNAi or CRISPR, it is of utmost importance to test multiple targeting sequences. By comparing the effects of multiple sequences, you can determine the extent of off target effects and infer the true biological effect of a gene product.

For designing an RNAi sequence or a guide RNA sequence:

<http://www.broadinstitute.org/rnai/public/>

# Using virus in your research - A primer for beginners (CONT'D)

For designing RNAi sequences or finding predesigned sequences:

<http://www.operon.com/tools/oligo-design-tools.aspx>

An Addgene blog post on how to design guide RNAs, including links to bioinformatics tools for minimizing off target effects:

<http://blog.addgene.org/how-to-design-your-grna-for-crispr-genome-editing>

## The safety dance!

When using virus for the first time or bringing virus into a new lab space, there are safety protocols that should be established. Two major safety concerns of working with viruses are the potential for generation of replication competent virus, and the potential for oncogenesis (in a host cell) via insertional mutagenesis. Separating the virus into multiple plasmids (as described above) can minimize the replication potential of the virus. In addition, careful containment of reagents and tools that have come in contact with the viral particles is essential for preventing exposure to the virus. Before working with virus, researchers should consult their institutions to receive safety training and learn important safety protocols.

Overall, viruses are an exciting research tool that can be used to investigate many types of research questions. This post hopefully served as an introduction to some of the important features of viruses and provided some useful terms and articles to further expand your understanding of these complex molecules.

## References

1. Roe T, et al. "Integration of murine leukemia virus DNA depends on mitosis." EMBO J. 12 (1993) 2099-2108. PubMed [PMID: 8491198](#). PubMed Central [PMCID: PMC413431](#).
2. Bukrinsky MI, et al. "Active nuclear import of human immunodeficiency virus type 1 preintegration complexes." Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 6580–6584. PubMed [PMID: 1631159](#). PubMed Central [PMCID: PMC49545](#).
3. Coffin J, Hughes S, Varmus H. Retroviruses. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY, USA: 1997. PubMed [PMID: 21433340](#).
4. Cronin J, et al. "Altering the tropism of lentiviral vectors through pseudotyping." Curr. Gene. Ther. 5 (2005) 387-398. PubMed [PMID: 16101513](#). PubMed Central [PMCID: PMC1368960](#).

# Beginner's guide to viral vectors (infographic)

By Leila Haery | June 2, 2017

You can use viral vectors for many experimental purposes. To help you make sense of all the viral vector information that's out there, Addgene Leila Haery has summed up some of the most important characteristics of retroviruses, lentiviruses, AAVs, and adenoviruses in this easy-to-use guide. Print out the guide and use it for quick reference when you're designing your next virus experiment.

## Beginner's Guide to Viral Vectors

### Immunogenicity How will cells react to my virus?

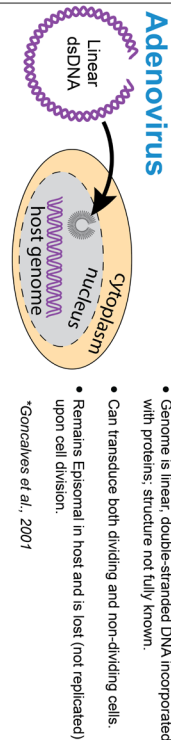


### Packaging Capacity How much genomic material can be delivered?

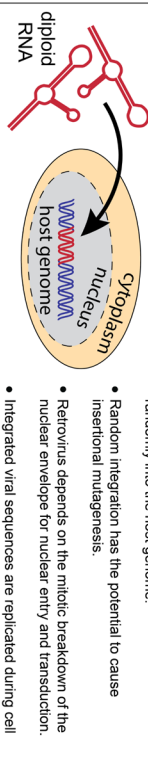
Recombinant viruses used for research have been derived from wild-type viruses and modified for safety and efficiency. These modifications enable the virus to direct expression of specific transgenes, whose sequences are packaged within the virus. Due to physical restrictions based on the size of the virion, the length of the transgenes delivered by viral vectors is limited by the packaging capacities below.



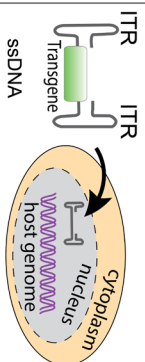
### Genome Composition What is the structure of the viral genome and where does it integrate in the host cell?



### Retrovirus/Lentivirus



### Adeno Associated Virus



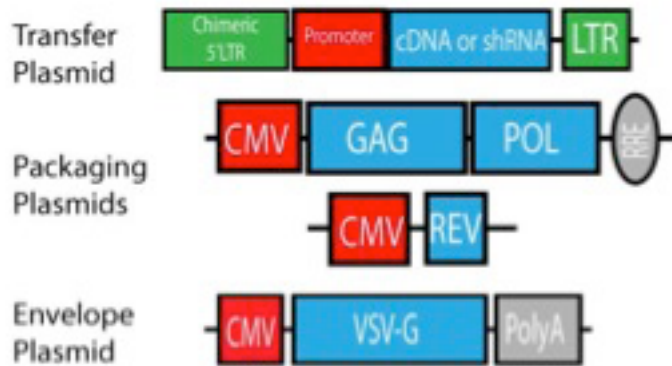
**Need More Info on Virus?**  
[addgene.org/viral-vectors](http://addgene.org/viral-vectors)

 **addgene**  
The nonprofit plasmid repository



# Viral vector elements

By Marcy Patrick | July 17, 2014



The use of viral vectors in research is beneficial for a number of reasons, including but not limited to: helping to get difficult-to-deliver DNA into mammalian cells, increasing the efficiency of gene transduction, allowing for control over which cells are infected through viral pseudotyping, and ease of vector cloning and modification. At the most basic level, viral vectors consist of a viral genome that has been adapted into a plasmid-based technology and modified for safety through the removal of many essential genes and the separation of the viral components. Read on for a brief description of the viruses used to make these vectors as well as a table defining the major elements found within the plasmids comprising the viral vector systems.

## Viral vectors

Of the many viruses out there, only a subset are commonly used in the lab and we will discuss the three most prevalent: gamma-retrovirus, lentivirus, and adeno-associated virus.

### Gamma-retrovirus and Lentivirus

A retrovirus is an RNA virus that uses reverse transcriptase to make a DNA provirus which can be incorporated into the host's genome. There are two common genera of retrovirus used by scientists: Gamma-retrovirus (many times shortened to just "retrovirus") and lentivirus. From these two genera, the most studied types are Murine Leukemia virus (MLV) and human immunodeficiency virus-1 (HIV-1), respectively. Since these viruses are closely related, their life cycle and the required components are basically the same, although differences do exist at the sequence level. This means that plasmids containing virus-specific elements such as the LTRs or structural proteins are not interchangeable; however, other, more general, viral components such as a heterologous envelope or a post-transcriptional regulatory element can be used across either system. The most notable difference between gamma-retrovirus and lentivirus is the fact that lentivirus can infect both dividing and non-dividing cells, whereas gamma-retrovirus is restricted to dividing cells only.

### Adeno-associated Virus

Adeno-associated virus or AAV is a small parvovirus that infects humans and some other primate species, but is not currently known to cause disease. If AAV is exclusively used to infect human cells, it stably integrates at a specific chromosomal site as part of its lysogenic life cycle; however, the addition of a helper virus such as adenovirus or a helper plasmid containing the specific viral proteins E1A, E1B, E2A, E4, and VA will cause AAV to enter a lytic cycle and replicate. There are at least eleven different serotypes of AAV, with likely more to be discovered. The most studied and experimentally used serotype is AAV2; however, many factors including species and cell type may make other serotypes more desirable.

# Viral vector elements (CONT'D)

## Common viral vector elements

When developing viral vectors, scientists strive for a number of features: low risk, high expression, large payload capacity, ability to infect target cells, no immune response from the host, and easy to develop/use in the lab. In virtually all viral expression systems employed by scientists, non-essential components are stripped away and the remaining native genes are spread over multiple plasmids to ensure safety. The choice for how the elements are divided up is dependent on whether the component needs to be provided *in cis* (on the same plasmid) or *in trans* (on a separate plasmid) as your insert. The tables below list some components most commonly found in viral vector systems.

### Gamma-retroviral and lentiviral elements:

| Element | Plasmid type                   | Provided?     | Purpose  |
|---------|--------------------------------|---------------|--|
| LTR     | Transfer                       | <i>in cis</i> | 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.   |
| U3      | Transfer                       | <i>in cis</i> | 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.   |
| R       | Transfer                       | <i>in cis</i> | Repeat region found within both the 5' and 3' LTRs of retro/lentiviral vectors. Tat binds to this region.  |
| U5      | Transfer                       | <i>in cis</i> | Unique 5'; region at the 5' end of the viral genomic RNA (but found at both the 5' and 3' ends of the provirus).   |
| 5' LTR  | Transfer                       | <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. |
| TAR     | Transfer (2nd generation only) | <i>in cis</i> | Trans-activating response element; located in the R region of the LTR and acts as a binding site for Tat.  |

# Viral vector elements (CONT'D)

## Gamma-retroviral and lentiviral elements (CONT'D):

| Element        | Plasmid type | Provided?       | Purpose  |
|----------------|--------------|-----------------|--|
| 3' LTR         | Transfer     | <i>in cis</i>   | Terminates trascription started by 5' LTR by the addition of a poly A tract just after the R sequence.   |
| cPPT           | Transfer     | <i>in cis</i>   | Central polypurine tract; recognition site for proviral DNA synthesis. Increases transduction efficiency and transgene expression.                     |
| 3' LTR         | Transfer     | <i>in cis</i>   | Terminates trascription started by 5' LTR by the addition of a poly A tract just after the R sequence.   |
| cPPT           | Transfer     | <i>in cis</i>   | Central polypurine tract; recognition site for proviral DNA synthesis. Increases transduction efficiency and transgene expression.                     |
| Psi ( $\Psi$ ) | Transfer     | <i>in cis</i>   | RNA target site for packaging by Nucleocapsid.   |
| RRE            | Transfer     | <i>in cis</i>   | Rev Response Element; sequence to which the Rev protein binds.   |
| WPRE           | Transfer     | <i>in cis</i>   | Woodchuck hepatitis virus post-transcriptional regulatory element; sequence that stimulates the expression of transgenes via increased nuclear export. |
| Gag            | Packaging    | <i>in trans</i> | Precursor structural protein of the lentiviral particle containing Matrix, Capsid, and Nucleocapsid components.  |
| Pol            | Packaging    | <i>in trans</i> | Precursor protein containing Reverse Transcriptase and Integrase components.   |

# Viral vector elements (CONT'D)

## Gamma-retroviral and lentiviral elements (CONT'D):

| Element | Plasmid type   | Provided?       | Purpose  |
|---------|--|-----------------|--|
| Rev     | Packaging (on separate plasmid from Gag/Pol in third generation systems) | <i>in trans</i> | Binds to the Rev Response Element (RRE) within unspliced and partially spliced transcripts to facilitate nuclear export. |
| Tat     | Packaging (second generation only)                                       | <i>in trans</i> | Trans-activator; binds TAR to activate transcription from the LTR promoter.  |
| VSVG    | Envelope   | <i>in trans</i> | Vesicular stomatitis virus G glycoprotein; Broad tropism envelope protein used to pseudotype most lentiviral vectors.    |

Please see “Your Lentiviral Plasmid FAQs Answered” or our [Lentiviral FAQ webpage](#) for more detailed information on lentiviral vectors including Biosafety FAQs.

## AAV elements (CONT'D):

| Element | Plasmid type | Provided?     | Purpose  |
|---------|--------------|---------------|--|
| ITR     | Transfer     | <i>in cis</i> | Inverted terminal repeat; 145 bases each. Symmetry of ITRs is required for efficient multiplication of the AAV genome. Forms a T-shaped hairpin that serves as the origin of viral DNA replication. Contains D region required for packaging. Cloning capacity between the ITRs is ~4kb. |

# Viral vector elements (CONT'D)

## AAV elements (CONT'D):

| Element | Plasmid type | Provided?       | Purpose  |
|---------|--------------|-----------------|--|
| Rep     | Packaging    | <i>in trans</i> | Packaging proteins with four possible variants: Rep78, Rep68, Rep52, and Rep40; Required for genome replication and necessary for integration. Rep proteins from most serotypes can be interchangeably used with any ITR serotype.*  |
| Cap     | Packaging    | <i>in trans</i> | Structural capsid proteins with three variants: VP1, VP2, and VP3; VP1 possesses phospholipase A2 activity, which is likely necessary to release the AAV particles from late endosomes. VP2 and VP3 are crucial for correct virion assembly. Determines the serotype/ viral tropism. |

\*Exception is AAV5, which requires AAV5 Rep and AAV5 ITRs for packaging.

## Find Viral Vectors at Addgene

- [Addgene's most popular lentiviral plasmids](#)
- [Addgene's most popular retroviral plasmids](#)
- [Addgene's collection of AAV plasmids](#)

# Viral vector elements (CONT'D)

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# CHAPTER 2

## Adeno-associated virus (AAV)



# AAV: A versatile tool for gene expression in mammalian cells

By Didem Goz Ayturk | July 26, 2016

[Adeno-associated virus \(AAV\)](#) has emerged as a favorite viral tool for both research and clinical applications. AAV can be used to transiently express a gene of interest in a variety of cell types. It was first described about 50 years ago as a contaminant of adenoviral preparations, hence the name ([Atchison et al., 1965](#)). AAV is a single stranded, DNA virus belonging to the family Parvoviridae. It has a “simple” genome packaged in an icosahedral capsid. It does not have a lipid coat, also called an envelope, and thus cannot support the addition of a glycoprotein, such as VSV-G, to its surface. In research applications, the genome is typically gutted so that precious cargo space is opened for gene delivery, and for safety. You can easily complement the virus in a tissue culture setting, in other words “*in trans*”, by supplying the genes that encode the replicase functions and capsid proteins. This gives researchers the ability to produce more virus in a controlled setting. Even though AAV is isolated from a wide range of organisms, it has not been associated with disease, and it is considered a biosafety level 1 (BSL1) viral agent.

## Four considerations for the perfect recombinant AAV (rAAV) tool

AAV can be used in a wide variety of applications including transient gene expression in a particular cell type, [CRISPR genome editing](#), [optogenetics](#), and [chemogenetics](#) experiments. If you are new to utilizing rAAVs as gene delivery tools, here are some things you should consider before getting started:

- 1. Cargo capacity:** Even the gutted version of rAAV has a cargo capacity of < 4.8 kb, which is one of the key limitations of the virus for gene delivery. However, if your gene(s) of interest is small enough, you can design single rAAV vectors with two genes packaged in them, and use elements such as [IRES or 2A](#), to co-express them from one promoter. Co-infection rates for different rAAVs are also quite high if the titers are high, so if you cannot fit both your genes in one vector, you can coinfect (although this may not always work efficiently *in vivo*).
- 2. Specificity:** There are a few different rAAV components that can drive gene expression in specific cells/tissues; these include the [promoter](#), Flp or [Cre](#) dependent gene switches, and the serotype (serotype will be discussed more below). If you are aiming for broad expression, a broadly active promoter, such as CAG (chicken beta actin promoter with CMV immediate early enhancer), is a good choice. If you have a specific target cell type, you may want to try a different promoter, e.g. Camk2a for a neuron-specific one. rAAV vectors with Cre- or Flp-dependent gene expression, on the other hand, can be injected into animals with Cre or Flp expressed in specific cell types resulting in gene expression only in these cells.
- 3. Serotype:** Capsid proteins are very important rAAV vector components and drive the biology of these vectors. Although multiple studies have shown that different serotypes differ in their ability to infect different cell types, a recent study showed that most (or all) serotypes use the same receptor (AAVR) ([Pillay et al., 2016](#)). The observed tropisms may be due to other factors, such as the attachment of the virions to the cell surface, or perhaps steps subsequent to entry, such as uncoating.

AAV nomenclature can be confusing. You will likely see designations such as AAV2/2 or AAV2/8, but what do these numbers actually mean? The first number stands for the inverted terminal repeat (ITR) type. ITRs are short DNA sequences that flank the AAV genome and allow it to form concatemers in host cells. They, along with the Rep protein, facilitate integration into the human genome at the AAVS1 site on chromosome 19, something that is only observed with wild type AAV – not the vector forms. Almost all vectors contain type 2 ITRs. Type 2 ITRs can be packaged with a variety of capsid types. The type of capsid used to package an rAAV vector, i.e the serotype



# AAV: A versatile tool for gene expression in mammalian cells (CONT'D)

- is denoted by the second number. For example, if an rAAV vector has an ITR of type 2 and a capsid of type 8, it will be denoted AAV 2/8.

**4. Genome:** Wild type AAV are single-stranded DNA viruses. After uncoating of the DNA, the virus relies on the host cell replication machinery to synthesize the complementary DNA strand. This step is considered to be a limiting factor in the transduction efficiency of rAAV. To overcome this, ([McCarty et al., 2001](#)) engineered a dimeric, or self-complementary, AAV (termed an scAAV) by mutating one of the ITRs. scAAV vectors display rapid onset (days) and higher level transgene expression than standard single stranded (ss) AAV. However their packaging capacity (<2.5Kb) is half that of ssAAV (<4.8Kb), restricting the number of genes and regulatory elements that can successfully be packaged.

scAAV may be valuable if you require rapid transgene expression. A lower titer may be able to achieve desired transgene expression levels, minimizing the chance of seeing toxicity or immunogenicity due to high virion concentrations.

Quick side note: You can use [retrograde AAVs](#) to map neuronal connections.

## Producing your rAAV

There are several facilities nation-wide where you can obtain excellent quality, high titer rAAV ([including Addgene!](#)), but you can also generate rAAVs in your own lab with standard molecular biology tools and tissue culture experience.

Briefly, you start by transfecting HEK293T cells with an rAAV vector carrying your gene of interest, an adenoviral helper plasmid, and a plasmid containing Rep and Cap (Figure 2, commonly referred to as “triple plasmid transfection”). After 2-3 days, you collect the supernatant, (or in some cases make a cell extract as some rAAV serotypes are released into the culture media while others are not) ([Vandenberghe et al., 2010](#)) from which you precipitate the virions using PEG. You then purify the virions further by density gradient centrifugation using a high-speed ultracentrifuge. The virions will form a band due to their high density, and you can collect this band from the gradient. You then remove the density gradient material by dialysis/buffer exchange and can further concentrate the virions if needed.

The virion prep can be titrated by qPCR using primers targeting the viral genome, and/or protein gels ([Veldwijk et al., 2002](#)). These titers are for physical particles, many of which are not infectious. The ratio of physical particles to infectious particles can vary greatly, from 1 to >100.

AAV virions are quite stable. They will withstand freeze-thaw cycles and dehydration, which makes contamination from previous preps that might be on your bench, in your centrifuge, or in your incubator, possible. To minimize this, you should treat all disposable items and surfaces that come into contact with the AAV with a disinfectant, such as Coverage Plus NPD. Also very important is to apply common [cell culture practices](#) diligently.

# AAV: A versatile tool for gene expression in mammalian cells (CONT'D)

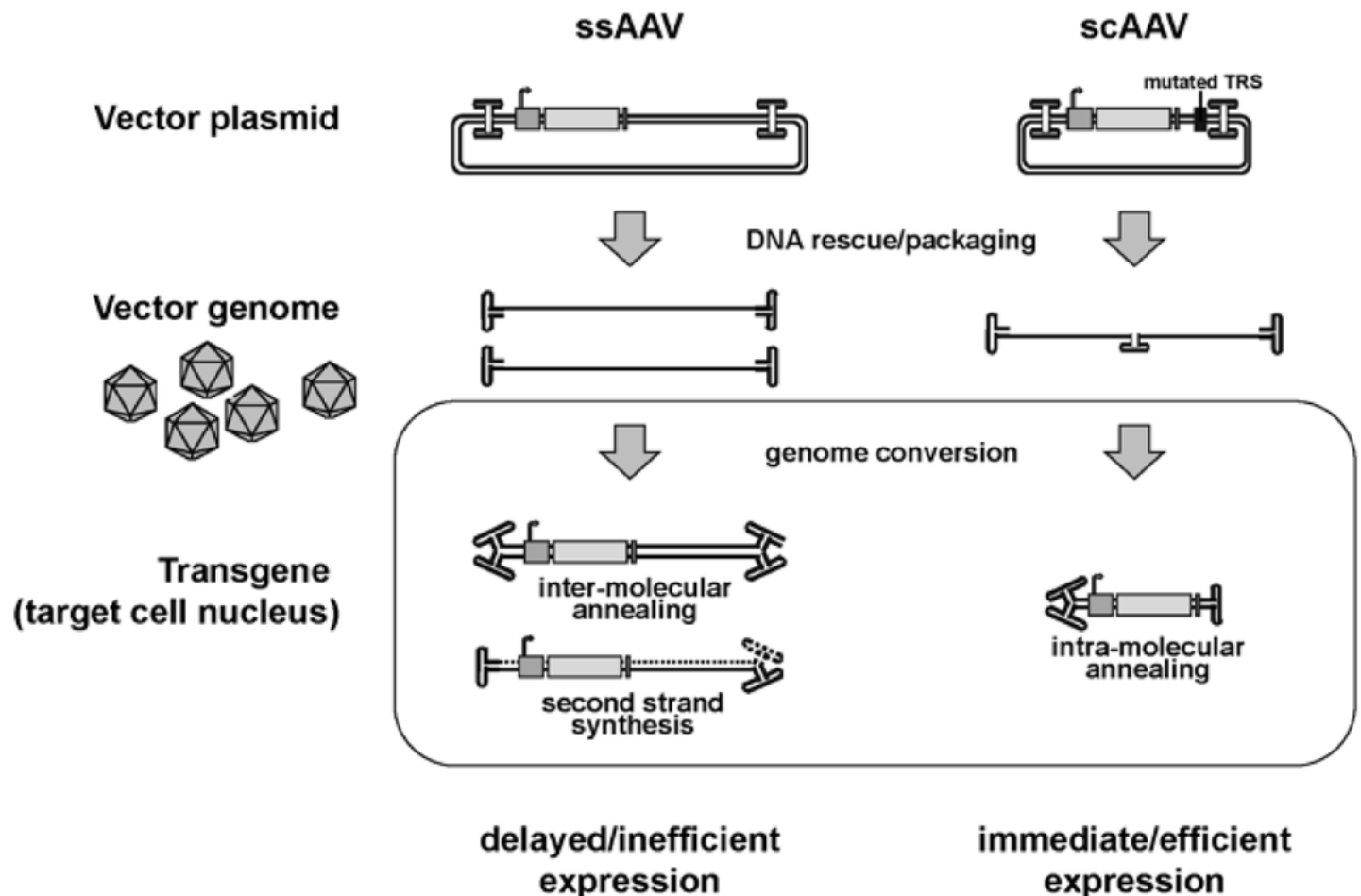


Figure 1: Comparison of ssAAV and scAAV genomes, packaging, and transduction. From Takashi Okada (2013). Efficient AAV Vector Production System: Towards Gene Therapy for Duchenne Muscular Dystrophy, Gene Therapy - Tools and Potential Applications, Dr. Francisco Martin (Ed.), InTech, DOI: 10.5772/53023.

## In vivo rAAV delivery

There are several factors concerning AAV delivery *in vivo* that vary, depending upon your biological system. These factors greatly influence the level of success of your infection. Whatever your application might be, listed below are a few parameters you may want to consider before proceeding with your injections:

**Injection titer:** The number of viral particles you can deliver to the tissue will be determined by the titer of your viral prep and the maximum volume that can be delivered, and is of great importance. Unfortunately, as mentioned above, the titer of the physical particles cannot be directly translated to what you will observe *in vivo*, although it is a starting point. You can start by scouring the literature for advice on particular tissues, but, often, the optimal dose will need to be determined empirically *in vivo* by trying a series of dilutions.

**Age of the animal:** First of all, it is important to remember that terminally differentiated cells (i.e. postmitotic cells) will have long term AAV expression, but, in dividing cells, expression will be lost. The age of the injected animal

# AAV: A versatile tool for gene expression in mammalian cells (CONT'D)

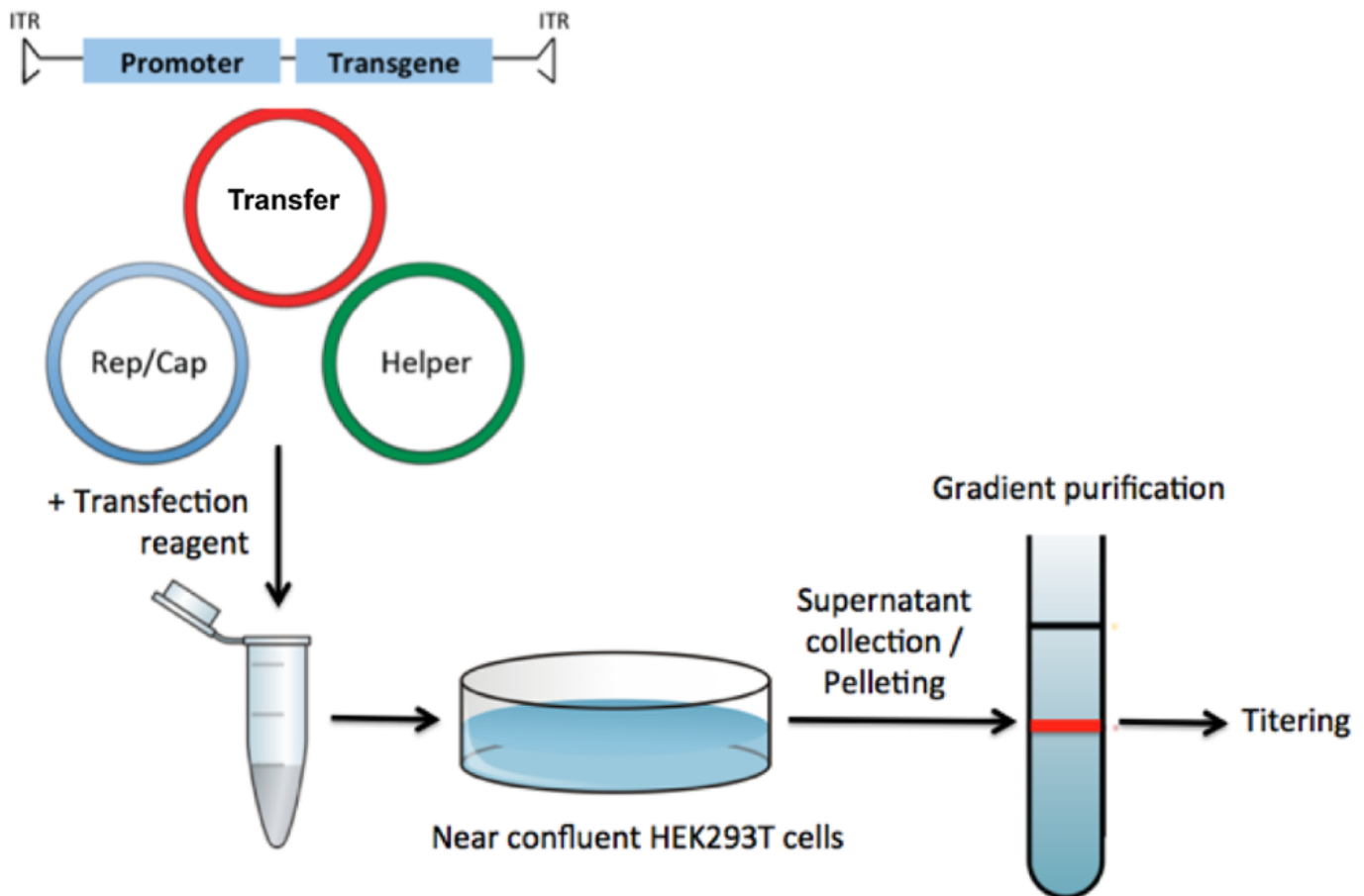


Figure 2: Recombinant AAV (rAAV) transfer vectors typically carry a transgene driven by a promoter of choice between ITRs, but do not encode Rep/Cap. Rep/Cap ORFs encode proteins essential for viral genome replication, virion assembly, and the 60-mer viral capsid. In this case, Rep/Cap are encoded by plasmids that are co-transfected with the transgene plasmid in tissue culture. Also needed for replication is an adenoviral helper plasmid that induces the lytic life cycle for AAV, and its release.

will determine what types of cell you can infect successfully. (i.e. at an early developmental time point, cells that are not yet born, even if their mitotic progenitor cells are present, cannot be infected by AAV) ([Xiong and Cepko, 2016](#))

**Visualization of the infection:** You should also think about how you will visualize your rAAV infection before you start the delivery process. Have you included a reporter gene in your rAAV construct, such as [GFP or mCherry](#)? These will make it easy to directly observe where expression is occurring via fluorescence measurements. If not, you can consider co-injecting an AAV that carries a reporter gene under the same promoter driving your gene of interest and measuring reporter gene expression as a proxy for your gene of interest, which may not be easy to detect. If co-expression or co-infection are just not possible, detecting your protein by immunohistochemistry or *in-situ* hybridization might be your best bet.

**Time after infection:** An adequate amount of time needs to pass between injection and tissue processing to detect AAV-mediated gene expression. Timing will highly depend on the capsid type and on the tissue you're

# AAV: A versatile tool for gene expression in mammalian cells (CONT'D)

infecting. Waiting ~2 weeks is a good starting point for many tissues. AAV-mediated gene expression has been reported to be quite stable, lasting for several years in human clinical trials and in dogs ([Wonjo et al., 2013](#)).

## Considerations when preparing AAV:

| Before design and growth       | At and after delivery          |
|--------------------------------|--------------------------------|
| Cargo capacity                 | Administration titer/volume    |
| Promoter (specific vs. broad)  | Age of the animal              |
| Serotype - tissue/cell tropism | Visualization of the infection |
| Production (DIY or purchase?)  | Time after infection           |

AAV is an extremely versatile tool for viral gene delivery. The demands of your particular research application will determine the appropriate parameters of AAV design and delivery. Although your final serotype/titer/age determination will heavily rely on pilot experiments, you can look to the ever-growing literature for design recommendations appropriate to many different biological systems. With more than a hundred clinical trials involving AAV started in the last two decades, it seems that this tool will remain popular in both therapeutic and basic research settings in the years to come.

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# AAV: A versatile tool for gene expression in mammalian cells (CONT'D)

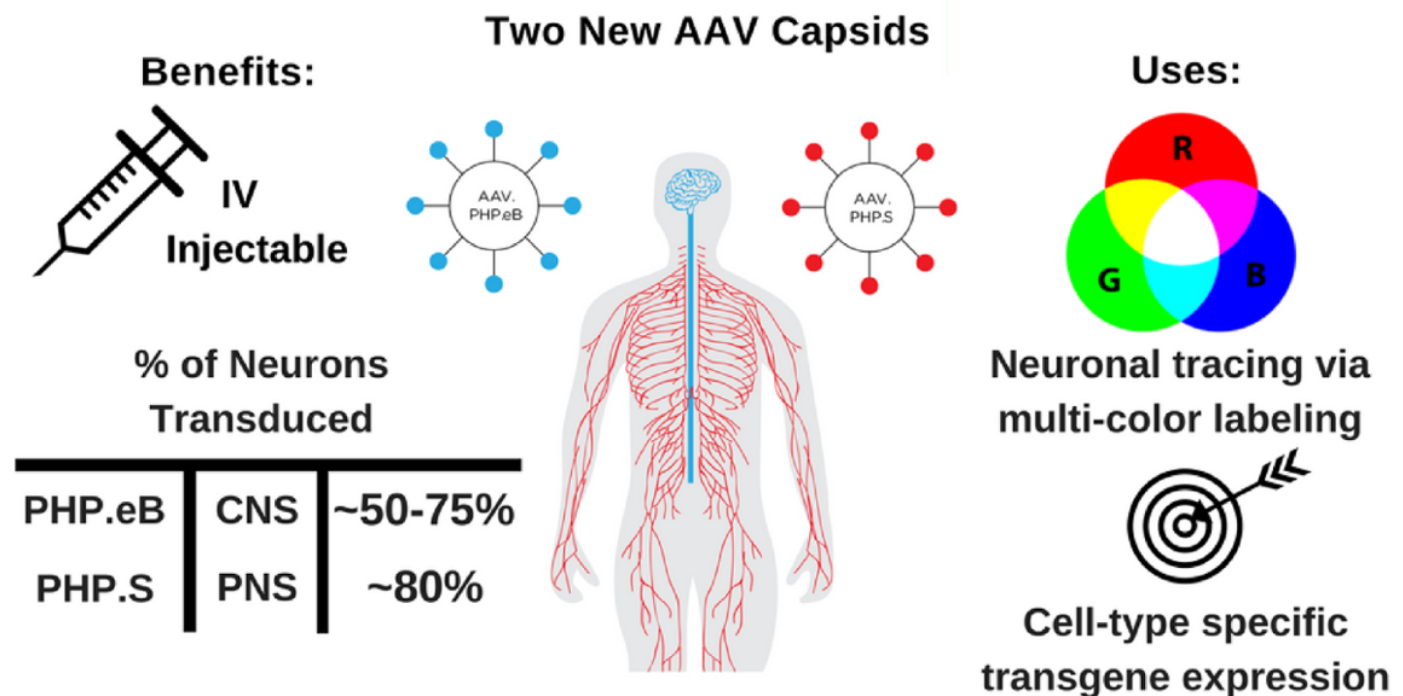
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# AAVs CREATED for gene delivery to the CNS & PNS

By Beth Kenkel | Sep 28, 2017

Adeno-associated viral (AAV) vectors are the most frequently used gene-transfer tools in the study of the brain and spinal cord, which together are known as the central nervous system (CNS). AAVs are popular tools because: 1) their genomes are easy to manipulate, 2) they have long-term expression; and 3) they have limited toxicity. However, a key challenge of using AAVs for neuroscience research is the lack of a method for genetically manipulating neurons throughout the whole brain. Neurons of the peripheral nervous system (PNS), which connect the heart, lung, gut, and other organs to the CNS, are also an important target for gene delivery, especially for the study of pain. While many new capsids (i.e. the part of the virus that determines tropism) have been developed that increase transduction efficiency, none allow for simple and efficient transduction of both the CNS and PNS. That is until the Gradinaru Lab at Caltech stepped up to the challenge.

## IV Delivery of AAVs to the Central and Peripheral Nervous Systems



Chan et al. Nature Neuroscience. June 2017



Their Solution: Use the CREATE method to engineer two new IV injectable AAV capsids, AAV.PHP.eB and AAV.PHP.S, that target the CNS or the PNS, respectively. Read on to learn more about these two new AAV capsids and how they can be useful tools for studying the CNS and PNS!

# AAVs CREATED for gene delivery to the CNS & PNS (CONT'D)

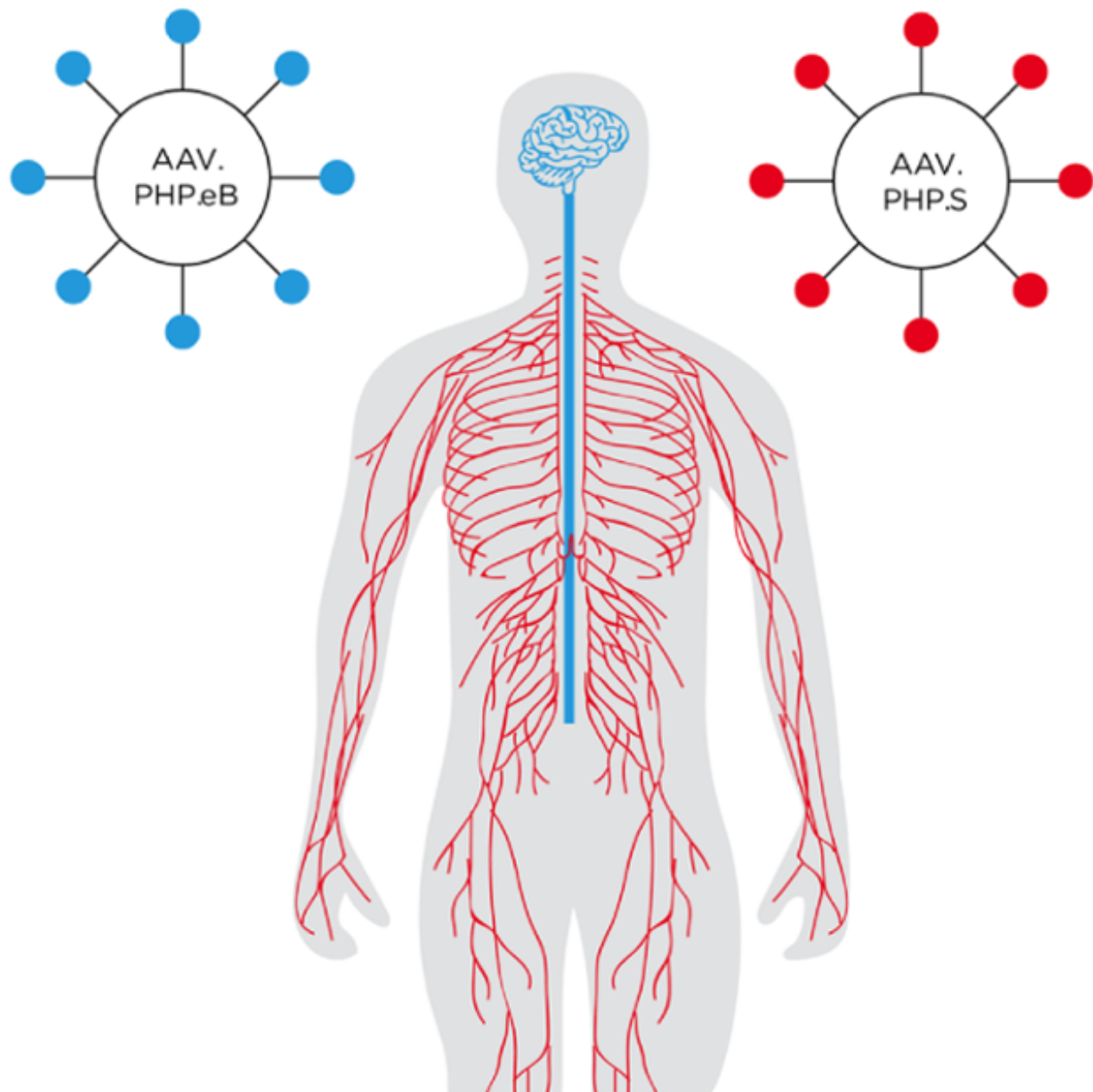


Figure 1: AAV Capsids for targeting the CNS and the PNS. AAV.PHP.eB capsid targets the central nervous system (blue). AAV.PHP.S capsid targets the peripheral nervous system (red).



# AAVs CREATED for gene delivery to the CNS & PNS (CONT'D)

## What is the CREATE method?

CREATE stands for **Cre Recombinase-based AAV Targeted Evolution**. In this technique, a pool of mutant AAV capsid (cap) expressing plasmids is generated. Diversity is introduced into the cap plasmid library by inserting short DNA sequences that contain all possible combinations into a region of the cap gene. In this special CREATE cap plasmid, loxP sites flank the downstream polyA sequence. When viruses produced from this plasmid pool are injected into an animal with cell-type-specific Cre, the polyA sequence is inverted only in the Cre-expressing tissues. This inversion creates a template that's suitable for PCR amplification using pre-designed primers.

By using this library to infect an animal expressing Cre in a cell type of interest, a scientist can isolate DNA from the infected animal and use the pre-designed primers to determine which version(s) of the capsid gene successfully infected the cell type of interest. Repeat rounds of infection and screening by PCR enrich for capsid variants with increased ability to infect the cell type of interest, which, in the case of Chan et al, was neurons and astrocytes.

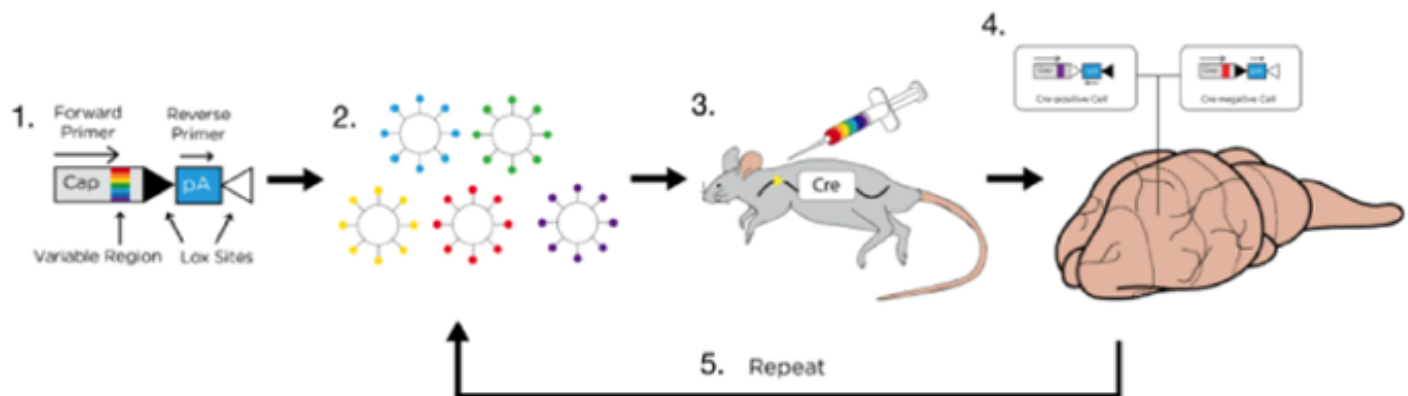


Figure 2. The CREATE method has 5 main steps:

- 1) Construct the CREATE capsid library. Random DNA sequences encoding a heptamer peptide are inserted into an existing AAV capsid gene (cap). The polyA (pA) of cap is flanked by loxP sites. When Cre is present, the pA become inverted allowing for PCR amplification of the heptamer sequence by primers that bind to the pA and the constant region of the cap gene.
- 2) Generate the AAV vector library. Next the CREATE genome library is packaged to create the AAV vector library.
- 3) In vivo screening in a Cre mouse. The AAV library is IV injected into mice that express Cre in a cell type of interest. Chan et al used mice that express Cre in neurons and astrocytes.
- 4) Recover AAV capsids. Several days post-injection, the brain and spinal cord are isolated and PCR-amplified for capsid sequences.
- 5) Repeat screen. Capsid sequences identified in step 4 are cloned back into the CREATE vector and a second round of in vivo screening is completed to identify a winning capsid.

## Why screen for IV deliverable AAVs?

Typically AAVs are directly injected into the brain, but this tends to limit expression to near the site of the injection. IV injection into the bloodstream allows for dispersion of the virus but it has to be able to cross the blood-brain barrier to reach the brain. Delivering the CREATE AAV library via IV screens for capsids that are able to cross this barrier - only those that cross the barrier can be identified by PCR later. The PNS presents a different problem: it's spread throughout the body and is often difficult to surgically access. Therefore an IV injection is an ideal way to deliver AAV to the PNS. It's a happy accident that AAV.PHP.S targets the PNS since it was actually engineered to target the CNS.



# AAVs CREATED for gene delivery to the CNS & PNS (CONT'D)

## How well do these AAVs transduce the CNS and PNS?

Both plasmids infect the PNS or the CNS much better than the AAV vectors they were derived from. Additionally, AAV.PHP.eB was used at a lower dose than its parent AAV.PHP.B:  $1 \times 10^{11}$  vs  $1 \times 10^{12}$  viral genomes (vg) per mouse. Figure 3 shows a gross comparison of AAV.PHP.eB and AAV.PHP.S to their parental vectors. To quantify this comparison, capsids were packed with a GFP transgene and GFP intensity and transduction efficiency of neurons were measured. On average, AAV.PHP.eB and AAV.PHP.S had 1.5- to 2-fold higher GFP intensity compared to the strains they were derived from. They also transduced a higher number of neurons overall (GFP+ cells that also stained for a neuronal marker). Check out tables 1 and 2 below for a summary of the quantified transduction efficiency of AAV-PHP.eB and AAV-PHP.S versus their parental AAV vectors.

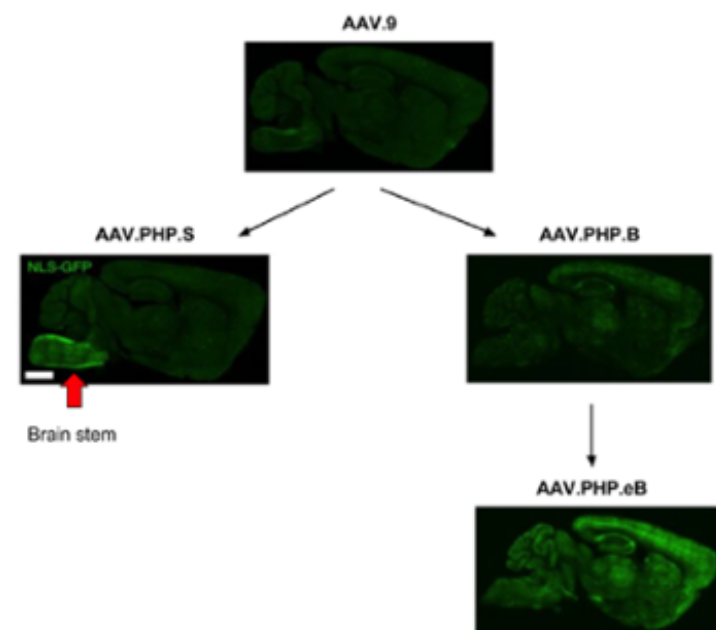


Figure 3: Gross comparison of AAV.PHP.eB and AAV.PHP.S to their parental vectors. A GFP construct was packaged in the indicated capsid, viral particles were IV injected into mice, and 3 weeks later brains were collected. Shown here are representative brain sections showing neuron transduction via GFP expression. Arrows indicate how capsids were derived. Figure adapted from Figure 1 of Chan et al.

## What can these AAV be used for?

### 1. Multi-color labeling of neurons

Multi-color labeling of neurons is for more than making pretty pictures (see figure 4 on p31). It also enables single-cell anatomical study and neuronal tracing, aka determining where a neuron's axons and dendrites extend to. The keys to this method are 1) high levels of color diversity so that cells are labeled with their own unique color; and 2) low levels of labeling so that a cell's projections are easy to trace and not lost in a jumble of fluorescent neurons. A simple but flawed approach to multi-color labeling is to inject a mix of AAV vectors that constitutively express a red, green, or blue (RGB) fluorescent protein (4a-c). When high doses of this viral cocktail are injected, by chance lots of individual cells will be transduced with multiple viral particles and labeled a variety of colors (figure 4b). But notice in figure 4b how difficult it is to trace individual cell's axons, particularly the two green neurons near the arrows. When lower doses of virus are used in order to decrease the number of neurons that get labeled, neurons are more likely to be transduced by only one RGB AAV and red, green, or blue are the predominate colors expressed (figure 4c).

To achieve the full diversity of colors AND sparse labeling of neurons, Chan et al separated color labeling and color expression into a two part system (figure 4d):

**Part 1:** A cocktail of three AAV vectors expressing RGB proteins under the control of an inducer, i.e. [tet-off transactivator \(tTA\)](#), is injected at a high dose to maximize the number of cells that are labeled.

**Part 2:** An AAV expressing the inducer (i.e. tet-off transactivator) is co-injected. The dose of this AAV can be adjusted to achieve sparse levels of labeling, without loss of color diversity.

# AAVs CREATED for gene delivery to the CNS & PNS (CONT'D)

With this two part system, color diversity is maintained at both high (figure 4e) and low doses (figure 4f) of the tTA inducer virus, but the desired sparsity of labeling is achieved with low doses of inducer virus. Additionally, this system is less cumbersome than [brainbow](#), another method for multi-color labeling of neurons, because it doesn't require the generation of a transgenic mouse.

## 2. Cell-Type-Specific Restricted Transgene Expression in the CNS and PNS

Previously, in order to broadly introduce a transgene into a specific cell type in the brain, you needed to make a transgenic mouse. Combining AAV.PHP.eB's or AAV.PHP.S's great neuronal transduction efficiency, as shown in tables 1 and 2, with a cell-type specific promoter or enhancer now allows for cell-type-restricted expression in both the CNS (figure 5 in Chan et al) and the PNS (supplemental figure 8 of Chan et al). These new AAVs can serve as tools for basic science research (neuronal tracing and morphologic studies), as well as translational applications (mechanistic studies and gene therapy treatments for neurological diseases).

| AAV Vector           | Percent of Neurons Transduced |           |            |
|----------------------|-------------------------------|-----------|------------|
|                      | Cortex                        | Striatum  | Cerebellum |
| AAV.PHP.B (parental) | 49 +/- 7%                     | 36 +/- 8% | 37 +/- 10% |
| AAV.PHP.eB           | 69 +/- 4%                     | 55 +/- 7% | 76 +/- 5%  |

Table 1: Transduction efficiency of AAV-PHP.B versus AAV-PHP.eB in the CNS. A GFP transgene was packaged into either an AAV-PHP.B or an AAV.PHP.eB capsid. Both viruses were IV injected at a dose of  $1 \times 10^{11}$  vg per mouse and the level of transduction, measured via GFP expression, was determined 3 weeks later. Table was derived from figure 2e in Chan et al.

| AAV Vector       | Percent of Neurons Transduced in Dorsal Root Ganglion |
|------------------|---|
| AAV.9 (parental) | 46 +/- 0.7%   |
| AAV.PHP.S        | 82 +/- 2%   |

Table 2: Transduction efficiency of AAV.9 versus AAV.PHP.S in the PNS. A GFP transgene was packaged into either an AAV.9 or an AAV.PHP.S capsid. Both viruses were IV injected at a dose of  $1 \times 10^{12}$  vg per mouse and the level of transduction, measured via GFP expression, was determined 3 weeks later. Table was derived from figure 3b in Chan et al.

## Key takeaways from Chan et al

- Two new AAV capsids (figure 1) were engineered with the CREATE method (figure 2) to allow for efficient IV delivery of virus to the CNS or PNS (figure 3 & 4, tables 1 & 2).
- These capsids are useful tools for neuronal tracing and morphology studies when used as part of a two part fluorescent tag-then-express system that allows for controlled induction of multi-color labeling of neurons (figure 5).

# AAVs CREATED for gene delivery to the CNS & PNS (CONT'D)

- 4).
3. These capsids can serve as powerful cell-type-restricted gene vectors for neurons, no transgenic mouse required (see figure 5 and supplemental figure 8 in Chan et al).

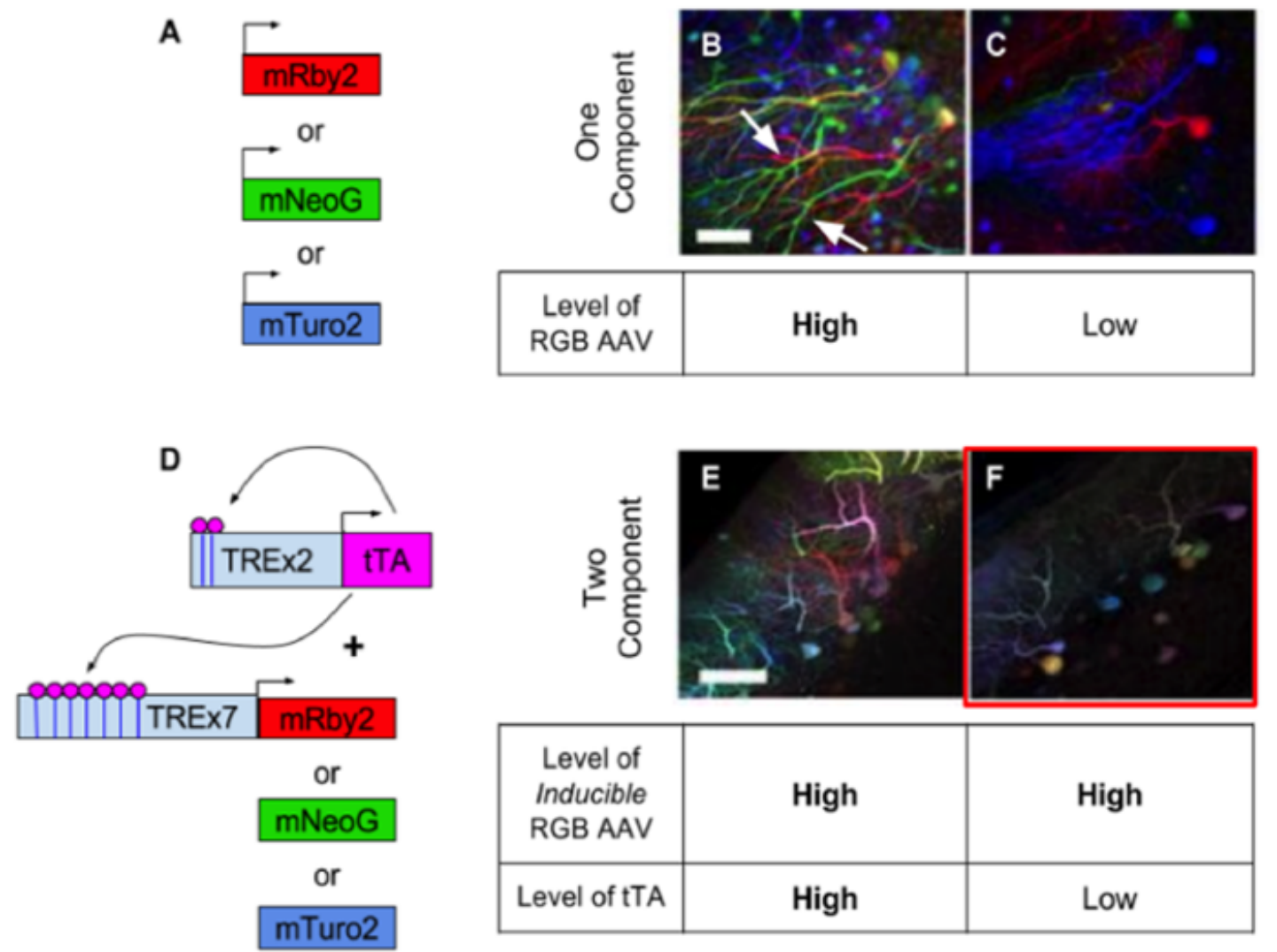


Figure 4: Multi-color labeling of neurons in the cerebellum. Co-injection of a cocktail of three AAV vectors (a) that each express one of three fluorescent proteins (red, green, blue) from a neuron-specific promoter. At high doses of virus (b), a range of hues are expressed due to higher rates of multiple viral particles transducing each cell. At lower doses of virus (c), color diversity drops as cells are more likely to receive only one viral particle. Separating color tagging and color expression into a two part system (d) eliminates this issue. Color expression is driven by a tet-response element (TRE) and required co-injection of an inducer virus which expresses the tet-off transactivator (tTa). The dose of the inducer virus can be varied to tune the desired level of labeling while maintaining color diversity (high levels of inducer = e, low levels of inducer = f). Scale bar = 50 um. Figure adapted from Figure 4 of Chan et al.

# AAVs CREATEd for gene delivery to the CNS & PNS (CONT'D)

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# Using AAV for neuronal tracing

By Klaus Wanisch | July 5, 2018

A key aspect to understanding the brain's function is knowing its architecture, in particular the connections between different brain regions. For example, communication between the hippocampus and the prefrontal cortex brain regions is involved in the formation of episodic memory, a special type of memory which includes autobiographical events (see Jin & Maren, 2015). Directional flow of information between different parts of the brain is mediated via individual neurons. Neurons are composed of a cell body, with dendrites receiving incoming information, and a projecting axon sending information onwards to other neuronal cells. Synapses at the terminals of axons form connections to dendrites of proximal neuronal cells. In the specific example of episodic memory, a subset of hippocampal neurons projects axons directly to the prefrontal cortex, but also indirectly via synapses to neurons in other brain regions. Further, the connections between regions are often reciprocal, forming a neuronal loop which is activated and strengthened during memory formation and memory retrieval.

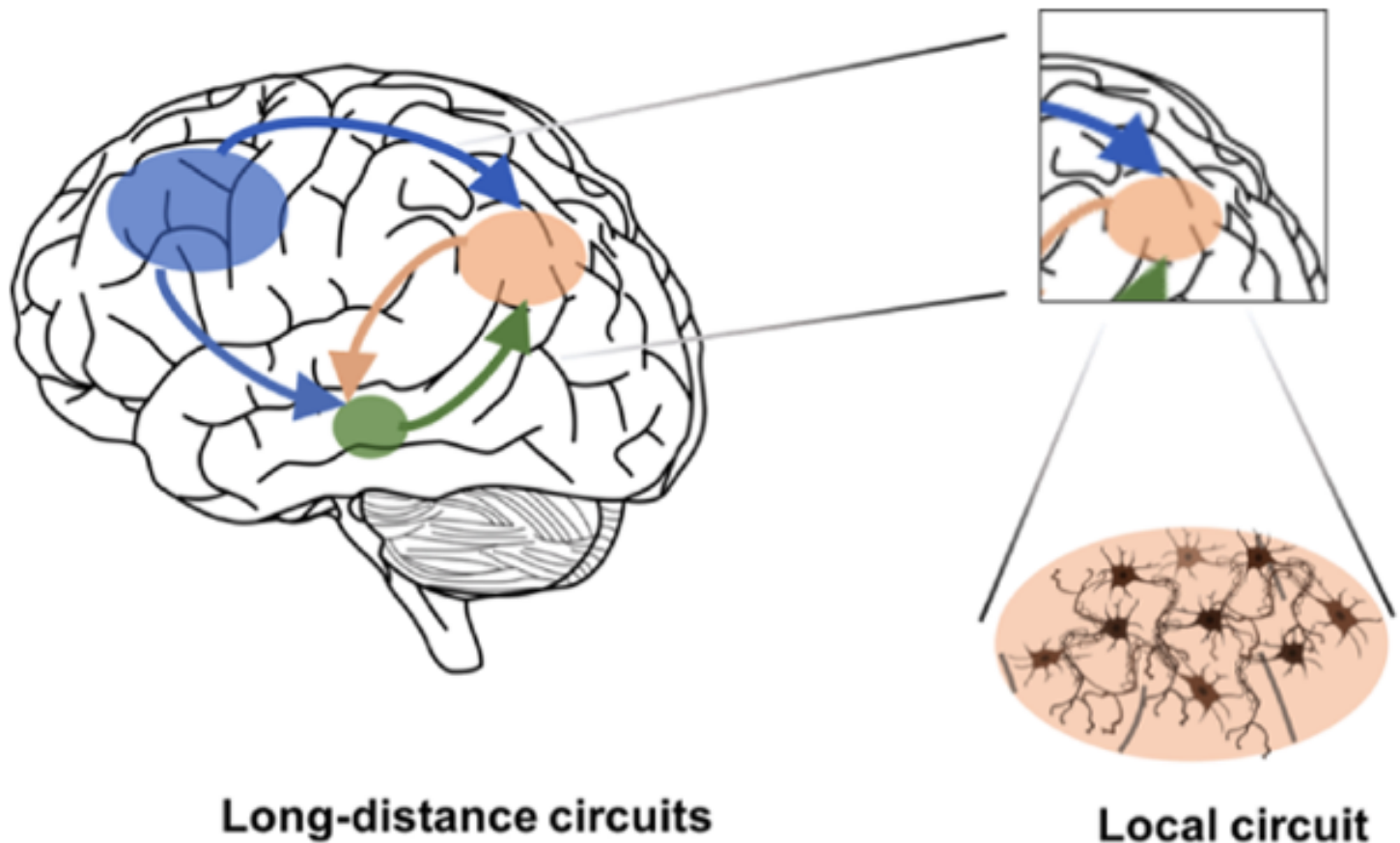


Figure 1: Examples of neuronal circuits.

In neuronal tracing experiments, brain tissue is injected with compounds that intracellularly spread away from the site of application and aid in visualization of the morphology of individual neurons, including dendritic and axonal extensions and, most importantly, any connections the neurons make to other distant brain areas. It is desirable for tracing compounds to outline neurons in the direction of information flow in order to understand which brain regions communicate with each other and how they do it (i.e. where the signals come from and what implications their signaling is likely to have). Anterograde tracing outlines neurons from their cell bodies to the

# Using AAV for neuronal tracing (CONT'D)

terminals of their axons; while retrograde tracing outlines neurons in the opposite direction, from the terminals of their axons to their cell bodies.

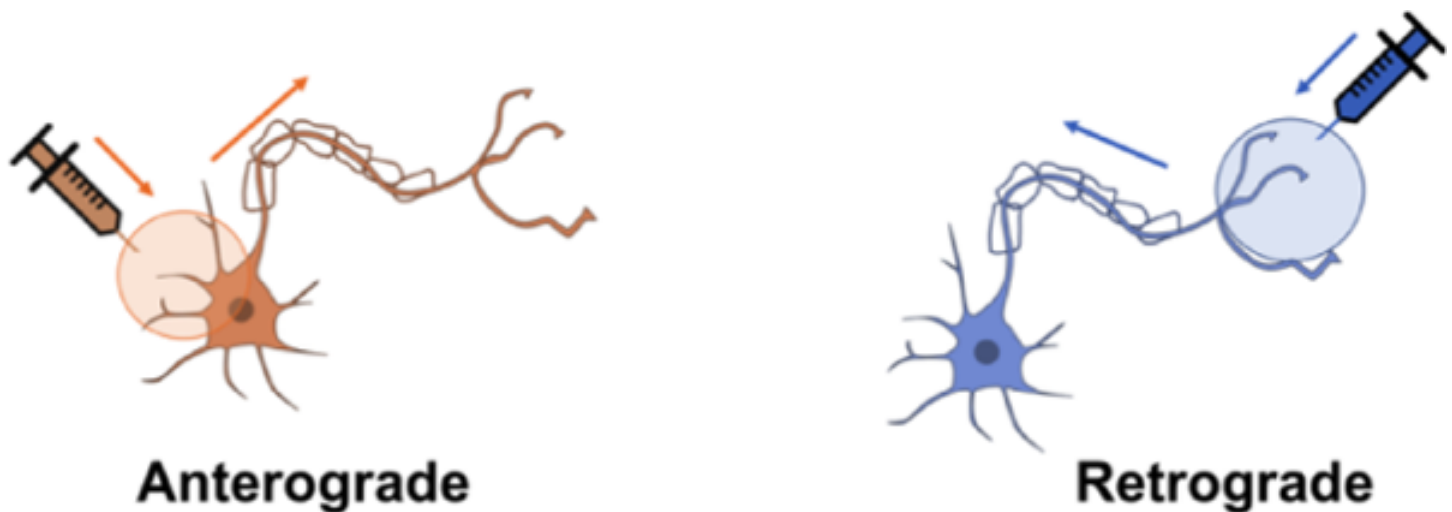


Figure 2: Directions of neuronal tracing - the tracer spreads as shown by the arrows, either away from the cell body (anterograde) or towards it (retrograde).

Anterograde and retrograde tracing take advantage of existing transport pathways in neurons. Anterograde transport is typically used for the trafficking of organelles, such as mitochondria, as well as macromolecules like actin and myosin, and enzymes for transmitter synthesis. Retrograde transport is used for transport of endocytosed material or molecules targeted for degradation. These two pathways also use different cytoskeletal machineries to facilitate transport: retrograde transport relies on dynein, while anterograde transport relies on kinesin. Different rates of speed for various forms of retrograde and anterograde transport suggest that there are several parallel mechanisms in place (for review see Maday et al., 2014). It is currently unclear which proteins facilitate transport of neuronal tracing compounds and this is an area that requires further study.

## Traditional neuronal tracing methods

Traditional tracing compounds either produce a colorimetric product or are fluorescent. While dyes usually spread unidirectionally, there are some reports of bi-directionality. For predominately anterograde tracing, biotinylated dextran amines (Veenman et al., 1992) or fluorescent molecules like rhodamine-isothiocyanate (RITC; Thanos et al., 1987) are used. For predominantly retrograde tracing, albumin protein labelled with horseradish peroxidase (HRP; Kristensson and Olsson, 1971), plant lectins (wheat germ agglutinin WGA, Schwab et al., 1978) or fluorescent molecules such as Fluoro-Gold (Schmued and Fallon, 1986) are used.

To facilitate cellular uptake of these dyes *in vivo*, microinjection, pressure injection or iontophoresis (applying small electric currents via inserted electrodes) may be applied, but these methods can have a negative impact on cellular health. Another limitation of traditional tracing compounds is that their diffusion across the synaptic cleft is usually severely impaired, and there are few reports of dye molecules that are transported from one neuron to another at synapses resulting in faint signals (Schwab et al., 1979; Buttry & Goshgarian, 2015). Nevertheless, the use of these tracers over the past few decades was essential to build up our current understanding of neuroanatomy



# Using AAV for neuronal tracing (CONT'D)

and neural connectivity, providing valuable insights for neuronal communication.

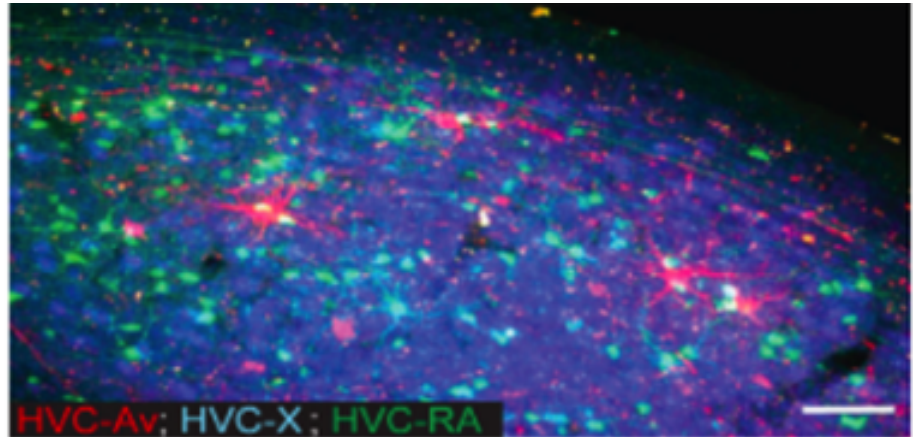
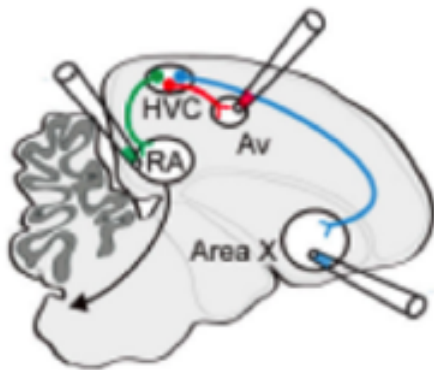


Figure 3: Retrograde labeling of the zebra finch brain. Left: Various dyes were injected into the following regions of the zebra finch brain - Dextra-Alexa Fluor 594 (red) into Avalanche area (Av); Dextran-Alexa Fluor 488 (green) into song motor nucleus (RA); Fast Blue (Blue) into Area X (basal ganglia region). Right: Dyes were retrograde transported to the telencephalic nucleus (HVC) and labeled neuronal cell bodies found there. Scale bar, 100  $\mu$ m. Adapted from Roberts et al., 2017 with permission.

## Viral vectors and the nervous system

With the advent of [viral vector technology](#), new methods have been developed to study neuronal connectivity. These methods are less neurotoxic and better compatible with other neuroscience methods such as electrophysiological recordings. The origins of these methods are naturally occurring viruses that infect, persist, and migrate within neurons, and that are also capable of spreading across synaptic connections. Most famous is the [Rabies Virus \(RABV\)](#) which moves retrogradely from a peripheral site of infection to the central nervous system where it replicates, spreads, causes neurotoxicity, and is lethal if left untreated (for review see Dietzschold et al., 2008). Herpes Simplex Virus (HSV) also migrates through neurons where it can replicate and by doing so spread across several synaptic connections (Ugolini et al., 1989). When using these viruses to mark neuronal connectivity a large majority of neuronal cells are labeled overtime due to the continued replication of the virus. While this can be useful, if too many neurons are labeled, it can be difficult to accurately map network connections (Lo & Anderson, 2011).

In the past two decades, several virus species have been modified and used as tools for tracing. With some engineering, RABV and HSV, but also others including pseudorabies virus type 1, vesicular stomatitis virus (VSV; Beier et al., 2011), [adenovirus](#), and [adeno-associated virus \(AAV\)](#), have been used for tracing and visualizing motor, visual, and auditory neuronal circuitry *in vivo*. A variant of RABV has been developed to only label a neuronal connection via a single synaptic step, thus preventing further widespread expansion of RABV. This improvement resulted in a clearer picture of neuronal connectivity in the mammalian brain (Wickersham et al., 2007; [plasmids at Addgene](#)).

## Viral vectors and the nervous system

AAV vectors have been widely used by neuroscientists for some time due to their advantageous characteristics for brain research and their ability to introduce genetic material into neuronal cells. The AAV genome is a single-

# Using AAV for neuronal tracing (CONT'D)

stranded DNA molecule and AAV vectors are replication deficient (i.e. they cannot easily spread between cells like natural RABV or HSV can). AAV does not have a membrane envelope like lentivirus, but instead is contained by a capsid which is comprised of the viral proteins VP1, VP2 and VP3. To date several hundred variants, or serotypes, have been found naturally or have been genetically engineered to have differences in their capsid proteins. For a brief overview, see [Addgene's AAV guide](#).

AAV particles are relatively small (20 nm) in size which makes them capable of diffusing in the extracellular space; they also have low immunogenicity and cellular toxicity. AAV vectors can be equipped with strong promoters for high and neuron-specific transgene expression. [Fluorescent proteins](#) are widely used for tracing and their expression is often made [Cre-dependent](#). If Cre-dependent vectors are used in transgenic animals that express Cre in only specific cell types, for example only dopaminergic neurons, neuronal tracing has the added benefit of only labelling the subset of infected cells that also express Cre. Another advantage of AAV vectors is that there are well-established protocols for their production and purification that yield high titers (see also AAV protocols for [production](#), [purification](#) and [titration](#) on the Addgene website).

| Tissue                           | Optimal serotype                   |
|----------------------------------|------------------------------------|
| CNS                              | AAV1, AAV2, AAV4, AAV5, AAV8, AAV9 |
| Heart                            | AAV1, AAV8, AAV9                   |
| Liver                            | AAV7, AAV8, AAV9                   |
| Lung                             | AAV4, AAV5, AAV6, AAV9             |
| RPE (Retinal Pigment Epithelium) | AAV1, AAV2, AAV4, AAV5, AAV8       |
| Skeletal muscle                  | AAV1, AAV6, AAV7, AAV8, AAV9       |

Table 1: Common AAV Serotypes and their target tissues (adapted from [www.addgene.org/viral-vectors/aav/aav-guide/](http://www.addgene.org/viral-vectors/aav/aav-guide/))

Commonly used serotypes include AAV1–9, with most of these serotypes capable of entering neurons (see table 1) ([Choi et al., 2005](#); [Taymans et al., 2007](#); [Howard et al., 2008](#); [Watakabe et al., 2015](#)). There are several reports of both retrograde and anterograde intracellular transport of some AAV serotypes, but overall with rather low efficiency. Not all measures of transport efficiency are consistent which may be caused by the diversity of techniques, species, and brain regions used. Evidence for retrograde transport has been found for AAV1, -2, -5, -7, -8 (Taymans et al., 2007), AAV1 ([Hollis et al., 2008](#)) AAV6 ([Towne et al., 2010](#)), AAV8, -9 ([Masamizu et al., 2011](#)); AAV5 ([Aschauer et al., 2013](#)). Anterograde transport has been observed for AAV1, -5, -8 (McFarland et al., 2009), and bidirectional transport for AAV1, -8, -9 ([Castle et al., 2014](#)). Differences between AAV serotypes may be also linked to differences in viral uptake by cells and to the uncoating step in the viral life cycle.

## Using AAV for neuronal tracing

AAV vectors are the first choice of many neuroscientists, with many groups using them to map neuronal networks. [Kohara et al. 2014](#) traced neurons with an AAV9 vector expressing a Cre-dependent fluorescent marker. Injection into a conditional mutant mouse brain that expressed Cre recombinase only in pyramidal cells of the hippocampal



## Using AAV for neuronal tracing (CONT'D)

region CA2 allowed for detailed mapping of the neuronal circuitry involved in learning and memory (Kohara et al., 2014; [plasmids at Addgene](#)).

The [Gradinaru Lab](#) at the California Institute of Technology generated novel AAVs ([Chan et al., 2017](#)) that express one of a few different fluorescent proteins (red, green, or blue). If a researcher injects a cocktail of these different AAV vectors into a brain region, the AAV particles will infect neurons at different ratios, making these neurons fluoresce in different colors. An individual neuron can then be traced throughout the brain based on its unique hue ([plasmids at Addgene](#)).

A large-scale example of neuronal tracing with AAV vectors is the Allen Mouse Brain Connectivity Atlas, a project to map neuronal connections in the mouse brain. For this project, AAV1 was used as an anterograde tracer and injected at several hundred sites across the entire mouse brain. Traced neurons expressed a fluorescent marker in areas close or distal to the injection site across the entire brain, allowing for an unprecedented number of connections, or the “connectome”, to be visualized in a uniform and standardized way ([Oh et al., 2014](#)). Images yielded from this study can be found on the [Allen Mouse Brain Connectivity Atlas website](#).

A similar approach was used by researchers from the Janelia Campus for their MouseLight project but neurons were traced with AAV2. Two-Photon imaging was used to generate a 3D finemap of neurons in the mouse brain. MouseLight images can be found on the [MouseLight website](#).

Together with scientists at the University of California, Berkeley, the Janelia Research Campus also developed a new AAV variant, rAAV2-retro, that migrates retrogradely at higher rates than any other AAV serotype ([Tervo et al., 2016](#); [plasmid at Addgene](#)). Its efficiency is comparable to conventional dye-based tracing while also having the added advantage of lower toxicity, higher specificity and reduced technical variability compared to chemical tracers. rAAV2-retro also has lower affinity for heparin, which possibly explains why it's less likely to be sequestered in the extracellular space. In addition, rAAV2-retro's altered amino acid sequence might facilitate interaction with uptake or intracellular transport machinery. Addgene offers viral preparations of AAV2-retro vectors with a variety of transgene/promoter combinations. Check out our [retrograde AAV section of this eBook](#).

An additional desirable property for network visualization would be the ability to spread trans-synaptically on to subsequent neurons. AAV vectors are replication incompetent and cannot readily be passed on to subsequent cells via replication like RABV or HSV. There are, however, a few reports of trans-synaptic spreading of some AAVs, specifically AAV6 in the retrograde direction and AAV2 in the anterograde direction ([Salegio et al., 2013](#)).

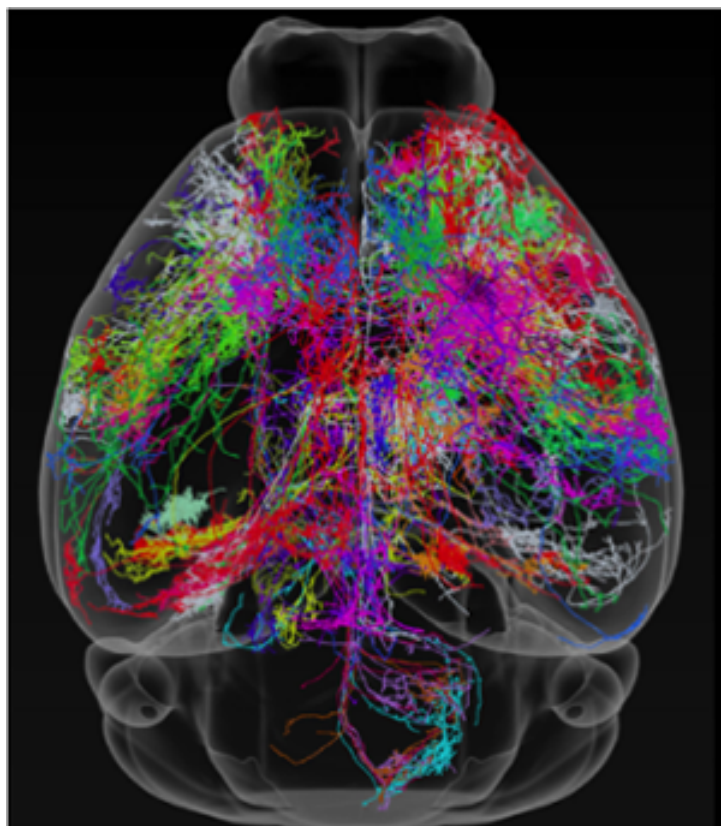


Figure 4: The morphology and position of 75 neurons within the mouse brain. The image was generated via 3D composite reconstruction. Credit: Janelia Research Campus, MouseLight project team [from <https://www.janelia.org/project-team/mouselight/neuronbrowser>].

# Using AAV for neuronal tracing (CONT'D)

[Zingg et al. 2017](#) also provide evidence that AAV1 and AAV9 are capable of synaptic spread. The underlying mechanisms of trans-synaptic trafficking are not fully understood and may be a form of transcytosis where the AAV particle is taken up on one side of the cell and released again at another side of the cell after intracellular transport without actually causing infection or leading to transgene expression within the first cell. While there is always a risk that viral particles will diffuse long distances within the extracellular space along an axon, this does not seem to be the case for either AAV1 or AAV9. It should be noted that AAV9 was shown to cross the blood brain barrier by endothelial transcytosis ([Foust et al., 2009](#); [Merkel et al., 2017](#)), and these two modes of transport may share common mechanisms. Rare observations like these may be a starting point for future developments in the field of AAV vectors.

## Conclusion

An ideal comprehensive toolset for neuronal tracing and network identification requires both antero- and retrograde labelling, synaptic restriction as well as controlled monosynaptic and polysynaptic spreading. AAV vectors cover some of these attributes and their use has allowed for the visualization of single connection networks within the rodent brain. A better understanding of the routes and mechanisms of intracellular transport of AAV could facilitate the development of more powerful AAV serotypes, and perhaps lead to controlled trans-synaptic spread of AAV vectors. Improved neuronal tracing could also be achieved by using optogenetically controlled transgenes, in combination with conditional transgenic mice, to reveal not only the anatomy of neuronal connections, but to visualize neuron connections that are functionally relevant, i.e. activity-dependent neuronal tracing. Apart from mapping neuronal networks, improved AAV vectors could also be powerful tools for the field of gene therapy if they can reach the effected part of the brain in a less invasive manner. For example, in neuromuscular diseases it would be desirable to achieve transgene delivery by targeting peripheral structures (such as injection into muscle) as opposed to direct intracerebral application, which is more invasive and has a much higher risk of complications.

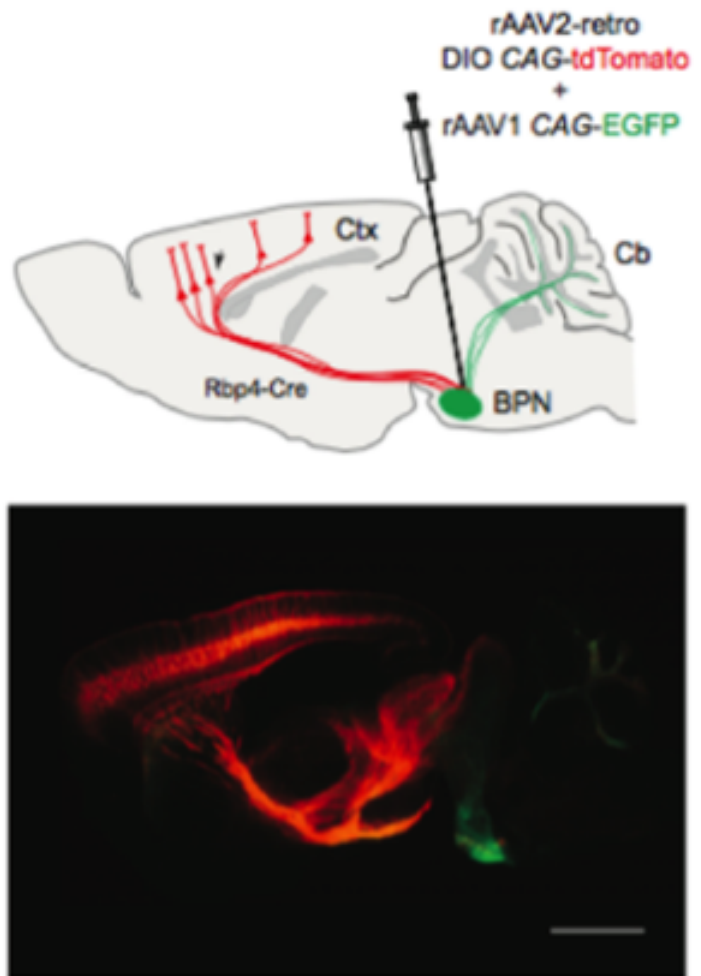


Figure 5: rAAV2-retro-DIO-CAG-tdTomato injection into the BPN (basal pontine nuclei) labels the corticopontine tract throughout the rostro-caudal axis in a Cortical (Ctx) layer V-specific Cre mouse line. Scale bar 1 mm; DIO, double-floxed inverted orientation; image adapted from Tervo et al., 2016 with permission.

# Using AAV for neuronal tracing (CONT'D)

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# Using AAV for neuronal tracing (CONT'D)

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# Retrograde AAV: Making the journey from axon to nucleus

By Leila Haery | May 16, 2017

The concept that the brain has a structure is not obvious. While it's been a long time since Aristotle argued the heart was the thought center of the body, it wasn't until the 1700s that scientists hypothesized and began to gather evidence that the brain has distinct regions with specialized functions. Phineas Gage, a man whose personality changed drastically after an accident where an iron spike was driven through his head, is a famous early example of the link between brain regions and behavior. Also around that time, French scientists Marc Dax and Paul Broca independently discovered the speech production center of the brain when autopsies of speech-impaired patients revealed lesions in a particular brain region, later named the Broca's area. In this section I'll describe a new [virus with retrograde function](#) and how it's enabling scientists to access neurons in a powerful way. Keep reading to find out what retrograde function is and how it gives us better access and ultimately a better understanding of the brain.

## Using brain structure to understand brain function

So far scientists have identified [180 brain regions](#). Both this geography and the way the brain is connected to itself across these regions is important for understanding how the brain functions. The basis for these connections in the brain are (drum roll) neurons! Neurons are really cool because they can transmit information, for example, from one of the 180 regions to another or from the brain to the rest of the body. Therefore, by simply looking at how neurons are physically connected we can begin to understand how the brain works. It's kind of like "the head bone's connected to the neck bone," but with a hundred billion neurons and a hundred trillion connections (synapses), so it's too long to make into a song.

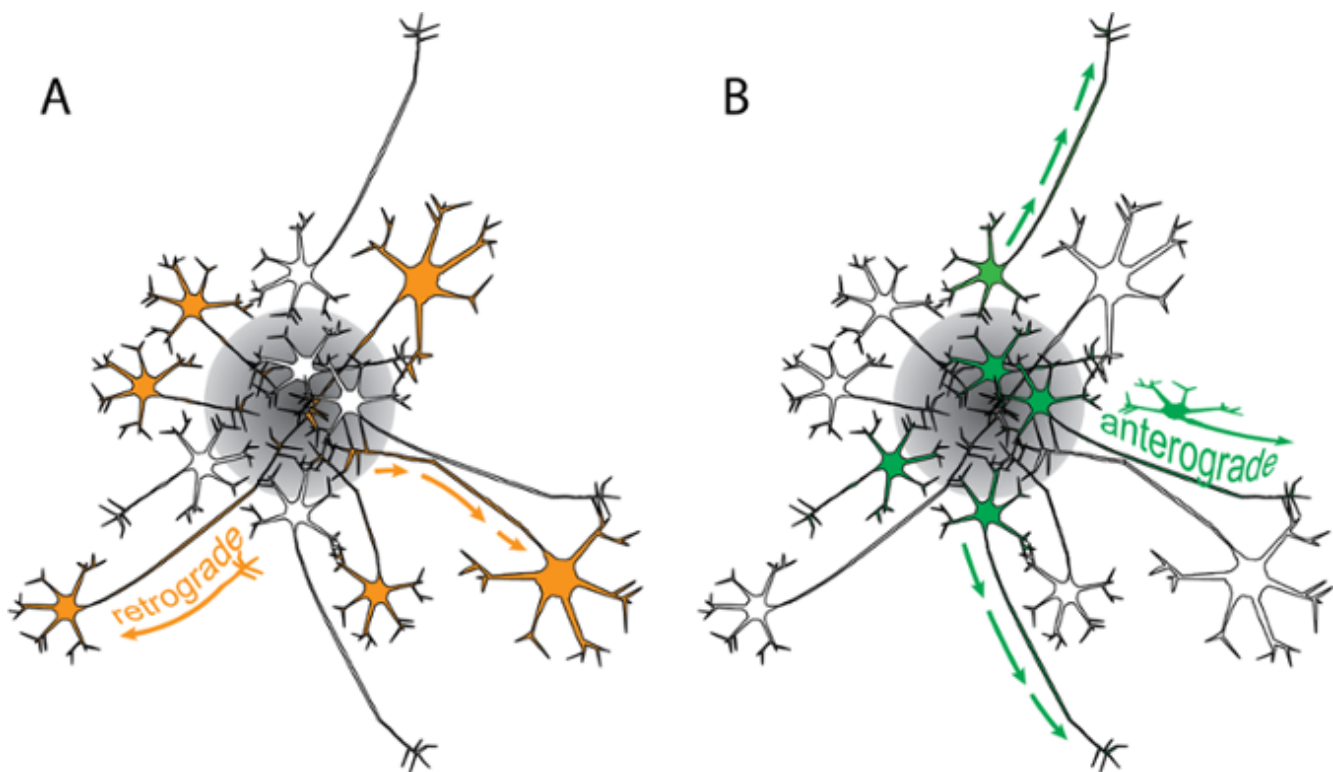


Figure 1: Neuronal tracers are delivered to a particular location (gray). (A) Retrograde tracers mark neurons (orange) that send information to the gray area. (B) Anterograde tracers mark neurons (green) that receive information from the gray area.

## Retrograde AAV: Making the journey from axon to nucleus (CONT'D)

Since neurons are polar, their connections are directional, meaning information typically flows from the cell body (or soma, which contains the nucleus) to the axon terminal. When studying a particular brain region it's important both to know what areas it signals to, and also what areas signal to it. To address this question, there are two methods of neuronal tracing (Fig. 1): one to visualize neurons that send information to a region (retrograde tracer, Fig. 1A), and another to visualize neurons that receive information from a region (anterograde tracer, Fig. 1B). Tracers are typically delivered to a particular location and they either trace downstream from the soma toward the axon (anterograde tracer) or upstream from the axon terminals to the soma (retrograde tracer) (Fig. 2).

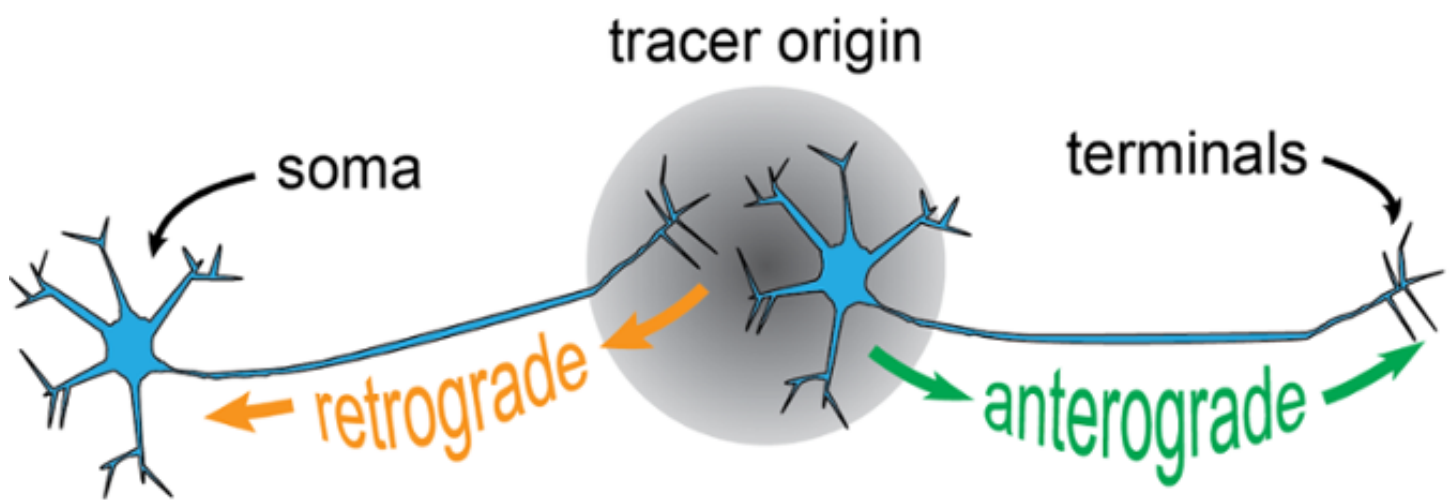


Figure 2: Depiction of neurons and two types of neuronal tracing. Retrograde tracers (left side, orange) travel with a neuron from the neuron terminals to the soma. Anterograde tracers (right side, green) travel within a neuron from the soma to the terminals. The direction of travel is relative to the site of tracer origin/delivery, which is depicted by a gray circle.

### Viruses as neuronal scalpels

Viruses are good tracers because they can be injected into the brain and efficiently deliver genetic information to specific cell types based on [viral tropism](#). Viruses also have a major advantage over traditional tracers because in addition to delivering a visual marker, they can deliver genetic tools that can enable manipulation of these neurons. While some viruses (e.g., rabies) naturally exhibit retrograde transport and thus, can perform retrograde tracing, many of these viruses exhibit technical limitations such as toxicity, low expression, or poor scalability. [Adeno-associated virus \(AAV\)](#) is a preferred virus for *in vivo* use because of its low immunogenicity and high transgene expression, however, most AAV serotypes exhibit relatively low retrograde transport efficiency.

Because efficient retrograde access to neurons is such a powerful technique, scientists at [Janelia Research Campus](#) and the [University of California, Berkeley](#) recently engineered an AAV variant that not only maintains an ability to efficiently infect neurons and direct high-level transgene expression, but which has retrograde functionality that can be used to monitor and map neurons ([Tervo et al., 2016](#)). So, if I think a certain brain region is important for hunger, I can inject it with retrograde AAV to see what neurons and what regions connect to it. Then I can see if stimulating those specific neurons causes an increase in feeding. Manipulating specific neurons has traditionally been challenging because of how physically intermingled neurons are in the brain. With [retrograde AAV](#), scientists can functionally dissect specific neurons within their natural environment, all while reciting their favorite slogan, “look how cool the brain is!”

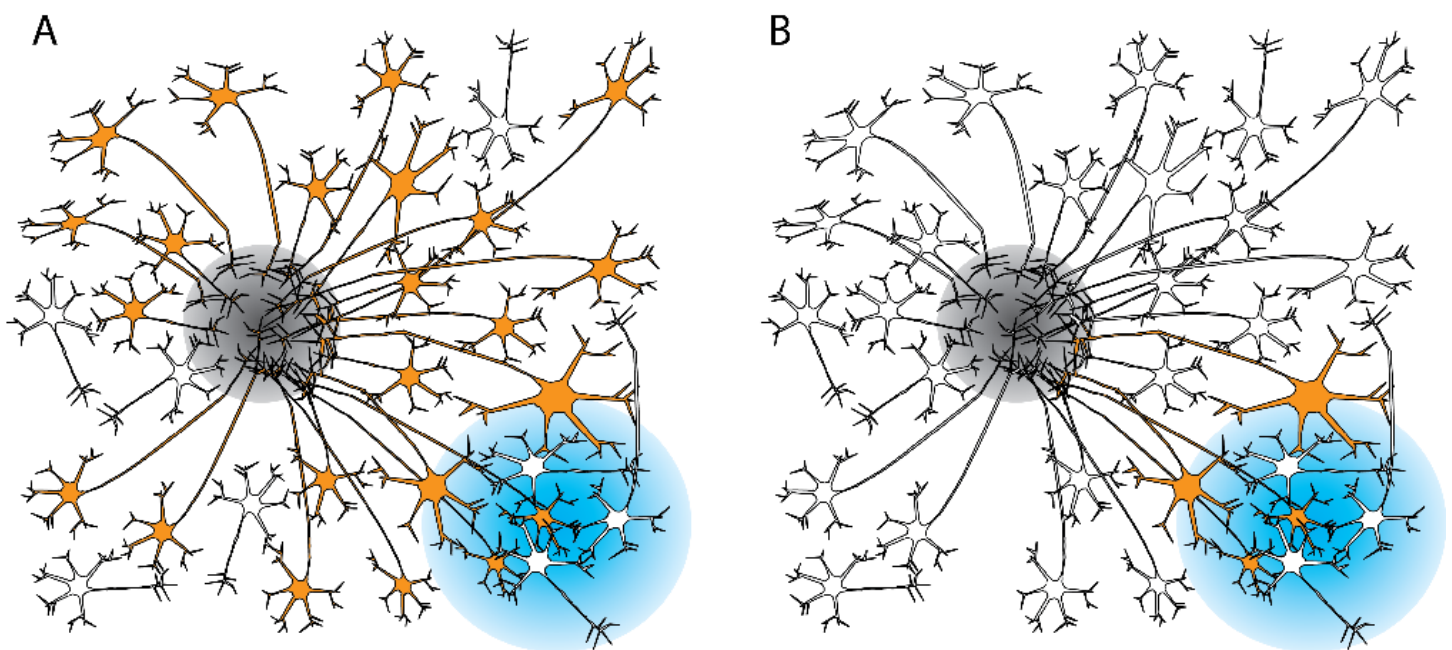


## Retrograde AAV: Making the journey from axon to nucleus (CONT'D)

### Retrograde AAV can be used to interrogate specific neuronal populations

One major advantage of retrograde AAV over traditional tracers is its ability to deliver a wide array of genetic tools for neural interrogation like [optogenetic tools](#), [chemogenetic tools](#), [calcium sensors](#), and many others. Thus, not only can one map what regions are signalling to a particular site, but in the same animal one could then activate, inhibit, detect neuronal signaling, or perform any other genetically controlled function.

By using retrograde AAV in [Cre](#) transgenic mouse lines, one could access and manipulate an even further defined population of neurons. For example, say I wanted to access all the neurons that signalled from the hippocampus to Broca's area. This could be done by injecting a Cre-dependent AAV into Broca's area of a mouse specifically expressing Cre in the hippocampus (Fig. 3). The Cre-dependent AAV would access all the neurons (Fig. 3A) that project to Broca's area (the site of injection) from any other regions of the brain. However, the Cre-dependent AAV cargo would only become activated in the presence of Cre recombinase, which is limited to the hippocampus. Thus, only neurons signaling from the hippocampus to Broca's area would be transduced (Fig 3B). These specific neurons could then be manipulated (e.g., activated or inhibited) by any genetic tool delivered by the AAV.



*Figure 3: Retrograde access to a defined subset of neurons can be achieved by using retrograde AAV to deliver a Cre-dependent construct in a Cre transgenic mouse. (A) Retrograde AAV is delivered to the Broca's area (gray) and transduces neurons in the retrograde direction (visualized in orange) with a Cre-dependent construct. (B) In a hippocampal Cre-transgenic line, this Cre-dependent construct is only activated (visualized in orange) in the Cre-expressing cells of the hippocampus (blue) thus enabling retrograde access to a defined neuronal subset.*

# Retrograde AAV: Making the journey from axon to nucleus (CONT'D)

## Impacts for neuroscience

Retrograde AAV opens a lot of doors in neuroscience by enabling mapping and interrogation of neural networks in a new and specific way. This new serotype also has promising therapeutic applications; it can enhance therapeutic delivery across broader physical areas of the brain and enable access to neurons that are physically blocked, such as those in the spinal cord.

While there are several hypotheses, the mechanism of retrograde transport is still unclear and remains to be discovered. In the meantime, retrograde AAV is likely to have a big impact on neuroscience and enables scientists to interrogate the brain in new and exciting ways.

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# Neuronal labeling with Spaghetti Monster

By Benoit Giquel | August, 2018

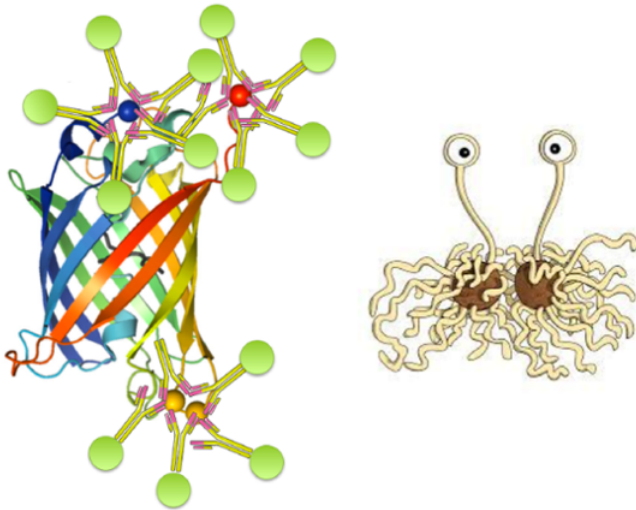


Figure 1: A spaghetti monster FP (smFP) with antibodies bound to it (left). Note resemblance to the flying spaghetti monster diety (right). smFP image adapted from Viswanathan et al., 2015 with permission.

The central nervous system (CNS) orchestrates complex processes enabling organisms to control their movements and behaviour. These functions and others are controlled by collections of neurons that are intricately wired into circuits through synaptic connections ([Shepherd, 2004](#)). Understanding the structure and function of these neural circuits is essential for neuroscience research. The use of genetic tools for visualizing and perturbing circuits together with the development of methodologies to deliver genes to the CNS have recently made it much easier to map these neuronal networks.

For almost three decades, recombinant [Adeno-associated virus \(AAV\)](#) has been widely used to deliver genetic tools to the brain because of its low cytotoxicity, low immunogenicity and the long-term expression of AAV delivered genes. AAV serotypes have been extensively studied in order to define

those that target specific neuronal populations in the brain. The [Allen Institute](#) and the [Janelia Research Campus](#) in the USA have used AAV1 and AAV2 serotypes in order to create an atlas of the mouse brain. This atlas enables researchers to quickly find the best AAV serotypes to target the neurons they're studying. Thanks to this work it is now much easier to express molecules in your favourite neuronal population and track connections to that population.

## Limitations of common genetically encoded neuronal tracers

Neuronal tracers such as epitope tags and [fluorescent proteins \(FP\)](#) have also been important in mapping, monitoring and manipulating neuronal circuits. Epitope tags are short antigenic peptide sequences, attached to a protein of interest (POI), that facilitate immunohistochemistry (IHC) experiments with tag-specific antibodies. The most famous ones are influenza hemagglutinin (HA), myelocytomatosis viral oncogene (myc), simian virus 5-derived epitope (V5), the synthetic peptide FLAG, the synthetic streptavidin-binding strep-tag and, more recently, OLLAS (Escherichia coli OmpF linker and mouse langerin) and Sun Tag ([Viswanathan et al. 2015](#)).

The principal advantage of epitope tags for IHC is the availability of reliable primary antibodies for their detection, particularly when antibodies to the POI are non-specific, cross-react with other antibodies, or are unavailable entirely. The other advantage is that these tags are small (8-12 amino acids) and neither disturb POI folding nor its ability to interact with protein partners within the cell. However, antibodies can have low affinity for these tags and even multimeric tags are frequently insufficient for detection when the POI is weakly expressed. As these epitopes tags have no intrinsic visual signal, they cannot be detected directly in live imaging experiments. It is therefore difficult to use epitope tags to monitor single molecules - something researchers often want to do at neuronal synapses for instance. Also these tags need to be fused to scaffold proteins in order to be expressed stably in neurons.

# Neuronal labeling with Spaghetti Monster (CONT'D)

As compared to epitope tags, FPs are intrinsically fluorescent and can therefore be used in live imaging experiments crucial to studying neuronal circuits and dynamics. FPs are generally bright, stable and well tolerated by cells. They can be used for protein localization, isolation and tracking experiments ([Rizzo et al. 2009](#)). With many different FPs that emit across the visible spectrum, researchers can also use multiple FPs in single experiments and visualize numerous molecules or structures at once ([Shaner et al. 2007](#)). In contrast to epitope tags, FPs are bigger and their fusion to a POI can affect protein folding and protein-protein interactions. Also, overexpression of FPs can trigger protein aggregation which is toxic for the cells and affects the labeling of small structures like neurites. Like epitope tags, low FP expression may be insufficient for monitoring a weakly expressed POI.

## Spaghetti Monster - a new and improved neuronal tracer

With the limitations of epitope tags and fluorescent proteins, it has been crucial to develop new tracers that enable accurate labeling of neurons. As discussed above, weakly expressed proteins in particular are difficult to track with these classic probes. To overcome the limitations of existing FP and epitope tags, researchers in [Loren Looger's lab](#) have developed new molecular tags combining the advantages of both (Viswanathan et al. 2015). For Looger's team, an ideal probe should combine the solubility, cell tolerance and optional fluorescence of FPs with facile antibody recognition of epitope tags. Thus they have created antigenic protein tags called "[spaghetti monster](#)" fluorescent proteins ([smFPs](#)). smFPs are literally composed of FPs fused to multiple epitope tags. Indeed, to create the smFPs Looger's team strategically inserted 10 to 15 copies of single epitope tags into single FP scaffolds with either intact or darkened chromophores. These probes are both intrinsically fluorescent and can be detected with highly epitope-specific, fluorescently-labeled antibodies.

The "spaghetti monster" name is a reference to the deity of the social movement, the church of the Flying Spaghetti Monster; the smFP structure resembles images of the deity (see comparison image above). The church of the Flying Spaghetti Monster is a symbol of opposition to the teaching of intelligent design in public schools and was started in 2005 in Kansas by Bobby Henderson, a physics graduate from Oregon State University.

## Uses of smFPs

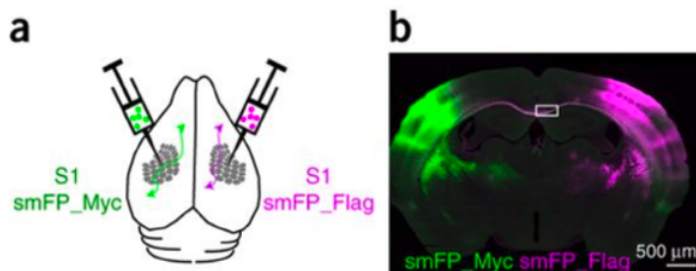


Figure 2: Multicolor labeling of the mouse brain using two different smFP tags. Adapted from Viswanathan et al., 2015 with permission.

In color imaging experiments, the second color is usually provided by red fluorescent proteins such as tdTomato, RFP or blue fluorescent proteins like Cyan. The issue with these FPs is that they are less photostable than

### 1. Multi-channel connectomic tracers

Fluorescent proteins such as GFP have been used for a long time as probes to study connectomics (how neural circuits are organised and how neurons connect to each other). Combined with AAVs, FPs can be easily expressed in the brain and can be detected through their direct fluorescent signals or using anti-FP antibodies.

GFP emits light in only a single channel. In multi-

# Neuronal labeling with Spaghetti Monster (CONT'D)

GFP. In addition, antibody-based amplification of their signals is weak and increases background fluorescence. Furthermore, many of these FPs are cytotoxic, prone to aggregation and cross-react with anti-GFP antibodies, making them difficult to use in a multicolor/multichannel imaging set-up. In contrast, smFP tags can be recognized by distinct, fluorophore-labeled antibodies with very specific reactivities making them more useful for multicolor imaging experiments than standard FPs. The Looger Lab was thus able to easily perform a four-color labeling experiment using smFPs and standard neuronal tracers.

## 2. Visualization of neuronal sub-cellular structures

GFP is limited in its ability to label subcellular structures in the CNS. GFP and other FPs have the tendency to brightly label somata but not neuronal processes. In contrast, smFPs can label fine neuronal structures with high fidelity and at lower concentrations than GFP. smFPs enable better labeling of these fine structures through their multiple, high-affinity binding sites for primary antibodies which can be used to amplify the direct smFP signal within sub-cellular structures. For instance, smFPs have been used to visualize “thorny excrescence” (TE) spines in the Hippocampal CA3 pyramidal neurons which are known to be notoriously difficult to label. It has also been shown that dendrites in the stratum oriens and the stratum lacunosum moleculare were better resolved with smFP\_FLAG than GFP.

## 3. Enhanced ability to distinguish weakly expressed and/or similar proteins

Through their multiple, highly specific antibody binding sites and primary antibody-based detection, smFPs create much brighter signals for weakly expressed proteins than standard tags and can be used to distinguish highly similar proteins. For example, N-cadherin (cadherin-2) is a postsynaptic cell adhesion protein that plays a critical role in neural development. It belongs to a huge family of proteins that are highly similar in terms of sequence and thus very difficult to distinguish using standard antibodies. By fusing N-cadherin to smFPs the Looger team was able to specifically label N-cadherin in neurons and show that smFPs fusions provided better labels than fusions to 3 or more HA tags.

## 4. High-resolutions microscopy

As indicated above, smFPs can be used with conventional microscopes, such as epifluorescence or confocal microscopes, but they can also be used with the most cutting-edge imaging technologies such as array tomography, super-resolution STORM imaging and electron microscopy. Sample preparation for these techniques sometimes makes it more difficult to detect protein tags using antibodies. In contrast, with their many epitopes, smFPs retain their ability to be detected after sample preparation better than standard proteins tags like GFP. Specifically for STORM, the high intensity signal provided by smFPs can result in better resolution images.

## Conclusion

By combining advantages of FPs and epitope tags, smFPs appear to be very effective probes for finely visualising sub-cellular structures or low-abundance proteins. When delivered with AAV, these probes can be used to label neuronal circuits and structures that are usually difficult to see. smFPs are an excellent add-on to the neuroscientist's tool-kit for studying the brain and we looked forward to the expanded use of smFPs in the future.

# Neuronal labeling with Spaghetti Monster (CONT'D)

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# Switch to GECO? An overview of AAV encoded calcium sensors

By Leila Haery | April 26, 2018

## Why monitor calcium?

One approach for monitoring brain activity is to monitor calcium dynamics. In neurons, calcium channels are activated during action potentials, leading to brief (~100 ms) increases in calcium levels (called calcium transients) in the cytoplasm ([Helmchen et al., 1996](#); [Koester and Sakmann, 2000](#); [Hille et al., 2001](#)). The concentration of free calcium in the cytoplasm of eukaryotic cell is about 100 nM and rises to the micromolar range during action potentials ([Wadel et al., 2007](#)). Thus, calcium levels are a proxy for neuronal activation (Broussard et al., 2017).

The main limitations of using calcium as an indicator for neuronal activity are that its quantities are also regulated by other bioprocesses whose rates can be variable depending on cell type. Thus, making inferences about activity from calcium level can become complicated depending on the context ([Badura et al., 2014](#)).

## Single channel calcium indicators

Single-wavelength sensors are based on single [fluorescent proteins](#) with fluorescence intensities that change in response to calcium binding. These sensors are measured at one wavelength, and generally turn on/emit light when calcium is present and binds to the detector, and “off” when calcium is absent and not bound to the sensors (Figure 1). Basically, once introduced into the brain, you can see these sensors light up in neurons that are active. When the neuron becomes inactive, calcium levels return to normal and the sensor turns off. In other words, these sensors are reversible, meaning calcium can dissociate from them, which puts the sensor back to its off state.

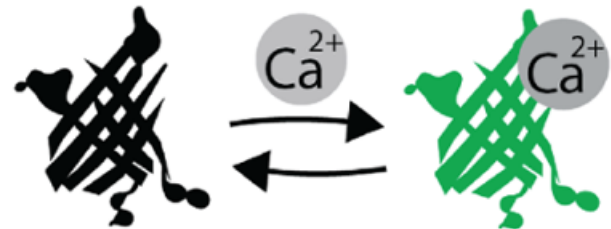


Figure 1: Schematic of single-wavelength calcium sensors. A modified fluorescent protein that fluoresces in response to calcium binding. Once calcium dissociates from the sensor, the fluorescence decreases back to baseline.

How quickly calcium comes off the sensor is particularly important for measuring different types of cell signalling. For example, sensors with higher affinity for calcium take a longer time to turn off after calcium levels return to normal. These sensors aren't good at sensing frequent events, but are sensitive enough to detect the low levels of calcium that are generated from a smaller number of action potentials.

Single-wavelength sensors with differences in overall brightness, on/off kinetics, and calcium affinity have been developed and, based on these properties, are suitable for diverse applications. These variants are described below.

## Switch to GECO? An overview of AAV encoded calcium sensors (CONT'D)

### jGCaMP6:

**Reference:** [Chen et al., 2013](#)

GCaMP indicators are some of the most popular calcium sensors (and have gone through various iterations, up to the current jGCaMP-X). GCaMP indicators are based on a modified GFP, a calcium-binding protein (calmodulin, CaM) and the M13 peptide. Upon binding calcium, CaM undergoes a conformational change that modifies the GFP chromophore, leading to increased GFP brightness. Thus, GCaMP indicators can be used to indicate calcium levels in real time. GCaMP variants have a range of on/off kinetics, and can be used to measure different types of cell activity. Specifically, the fastest variant (GCaMP6f) has a 2.6-fold higher  $\text{Ca}^{2+}$  affinity (lower  $k_d$ ) than the slow variant (GCaMP6s) (Table 1). As a result, GCaMP6f has close to a 4-fold faster rise time and decay time than GCaMP6s. Being more sensitive, GCaMP6s was able to detect single action potentials 99% of the time in mice *in vivo* relative to GCaMP6f, which was able to detect single action potentials 84% of the time.

**Table 1.** Summary of GCaMP6 kinetics for single action potential *in vivo*, in mouse V1 (Adapted from Chen et al., 2013)

| Variant | Change in fluorescence relative to resting | Decay time ( $\tau_{1/2}$ ), ms | Rise time ( $\tau_{\text{peak}}$ ), ms |
|---------|--|---------------------------------|--|
| GCaMP6s | 23 +/- 3.2 %                               | 550 +/- 52                      | 179 +/- 23                             |
| GCaMP6m | 13 +/- 0.9 %                               | 270 +/- 23                      | 80 +/- 7                               |
| GCaMP6f | 19 +/- 2.8 %                               | 142 +/- 11                      | 45 +/- 4                               |

### jGCaMP7:

**Reference:** [Janelia Research Institute](#)

The newest iteration of GCaMP sensors is available from [Douglas Kim](#) and the Janelia Research Institute. These GCaMP7 sensors all respond with greater signal amplitude to a single action potential stimulus than GCaMP6. Advantages of the individual sensors are as follows ([see data here](#)):

- **jGCaMP7s** - is the most sensitive responder to action potentials. For a single action potential, GCaMP7s is approximately twice as bright, relative to baseline fluorescence, as the GCaMP7f variant, and is approximately 5-fold brighter than GCaMP6s.
- **jGCaMP7f** - is the fastest responding variant.
- **jGCaMP7b** - exhibits the brightest resting fluorescence and can be used for imaging small neuronal processes (dendrites and axons).
- **jGCaMP7c** - exhibits high contrast between peak fluorescence and resting fluorescence (achieved



## Switch to GECO? An overview of AAV encoded calcium sensors (CONT'D)

through lowering sensor resting fluorescence, while maintaining high peak fluorescence) and is useful for imaging signaling activity in large populations of densely-labeled neurons because background fluorescence from inactive neurons is reduced. This variant has an approximately 12-fold increase in brightness after ~100 action potentials. This is about twice as bright as GCaMP7f and GCaMP7s.

While useful, GCaMP proteins are limited by their green-ness. The wavelengths of their excitation and emission spectra are scattered by and can damage tissue, limiting imaging depth *in vivo*. To address these limitations, red calcium indicators based on similar architecture were developed (see jRCaMP1a, b and jRGECO1a below).

One advantage of a red indicator is the ability to multiplex in either GFP mouse lines or with mice expressing other GFP-based tools. In this case, the red indicator can be measured independently of other GFP-based tools. Red light imaging in general also allows deeper tissues to be imaged, relative to green light imaging, and can be done with less excitation power ([Rose et al., 2014](#)). This is because light scattering is inversely proportional to wavelength, which applies both to the excitation and the emission lights.

### **jGCaMP1a, b:**

**Reference:** [Dana et al., 2016](#)

These are red calcium indicators based on mRuby. jRCaMP1a and jRCaMP1b are less sensitive than jGCaMP6, but do not show [photoswitching](#) after illumination with blue light (as jRGECO1 does). Since some common optogenetic tools (e.g., Channelrhodopsin, ChR2) are illuminated by blue light, the stability of jRCaMP1a, b to this light makes them suitable for experiments that combine calcium imaging and optogenetics. jRCaMP1a and jRCaMP1b also operate at slightly longer (more red) wavelengths than jRGECO1a, which enables clearer imaging (see note about scattering above).

### **jRGECO1a:**

**Reference:** Dana et al., 2016

This is a red calcium indicator based on mApple, which has comparable sensitivity to jGCaMP6. mApple-based GECIs, such as R-GECO and R-CaMP2, exhibit photoswitching when illuminated with blue light, causing a transient increase of red fluorescence that complicates their use with [optogenetics tools](#) (which are commonly activated by blue light).

## FRET-based calcium indicators

As opposed to single-wavelength sensors, [FRET](#)-based calcium sensors are based on two fluorescent proteins linked by a peptide and a calcium binding domain (e.g., CaM or Troponin). Calcium binding brings the FPs closer together, increasing FRET efficiency between the two proteins. In this way, FRET-based calcium sensors can be quantified by two wavelengths (the donor emission wavelength, and the acceptor emission wavelength).

## Switch to GECO? An overview of AAV encoded calcium sensors (CONT'D)

### **Twitch-2B:**

**Reference:** [Thestrup et al., 2014](#)

Twitch indicators comprise two fluorescent proteins (FPs) connected by linkers and the Troponin calcium-binding domain (Figure 2). In the absence of calcium, Twitch forms an elongated structure in which the two FPs are farther apart (~5.2 nm). Upon binding of calcium, the FPs move closer together (~15 Å), increasing FRET efficiency between the donor and acceptor FPs. The donor and acceptor FPs in Twitch2B (mCerulean3 and cpVenusCD, respectively) have the advantage of particularly bright donor emission spectra.

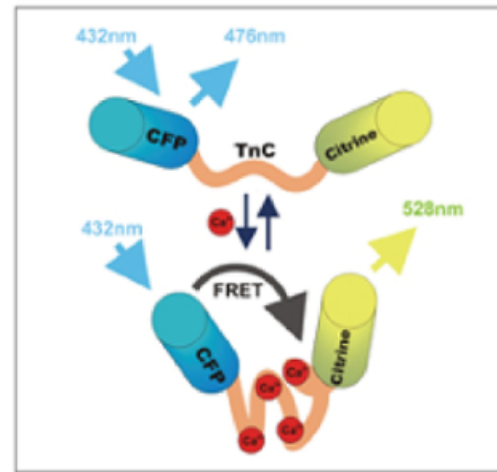


Image credit: MPI of Neurobiology / Griesbeck

Figure 2: Schematic of FRET-based calcium sensors. Two fluorescent proteins bound by a linker sequence move closer together, increasing FRET between the two proteins upon binding calcium. Once calcium dissociates from the sensor, the proteins move apart and FRET decreases.

FRET-based calcium indicators have the advantage of being brighter than single-wavelength sensors at rest, allowing better visualization in cells (Thestrup et al., 2014), and have been shown to detect calcium within cellular compartments ([Russell, 2011](#)). Relative to FRET-based indicators, however, the GCaMP sensors have the advantage of greater fluorescence changes in response to  $\text{Ca}^{2+}$  binding (Russell, 2011). For example, upon calcium binding, Twitch-2B has about a 2-fold increase in acceptor FP fluorescence (Thestrup et al., 2014), while jGCaMP6 sensors showed a dynamic range of about 10-fold (Chen et al., 2013).

## Ooops, I blinked - Benefits of irreversible calcium sensors

The ability to track brain activity in real time is great when you're watching the brain in real time. But these real-time signals turn off and can only be detected if your microscope is focused on them at the exact time that they're activated (which is basically limited by the field of your microscope). Essentially, real time sensors have the advantage of enabling long-term detection of brain activity, but only in small areas. On the other hand, using irreversible calcium sensors, it's possible to detect activity in the entire brain without directly monitoring it, but only for a few seconds.

### **CaMPARI:**

**Reference:** [Fosque et al., 2015](#)

Calcium-modulated photoactivatable ratiometric integrator (CaMPARI) differs from the reversible calcium sensors because it gives a permanent record of calcium activity during a user-defined time period. This integrator works by converting from green to red only in the simultaneous presence of high calcium levels and user-supplied violet light (Figure 3). This permanent conversion provides the ability to record calcium levels over broad areas of the brain over a user-specified time period, and the red fluorescence intensity inversely correlates with calcium concentration. CaMPARI complements existing calcium indicators by allowing total calcium activity

## Switch to GECO? An overview of AAV encoded calcium sensors (CONT'D)

measurement over large areas of cells and tissues.

### Bottom line

Overall, there is no shortage of available calcium indicators, though each type of indicator has its own advantages and limits. The best indicator will vary based the type of biological activity being measured.

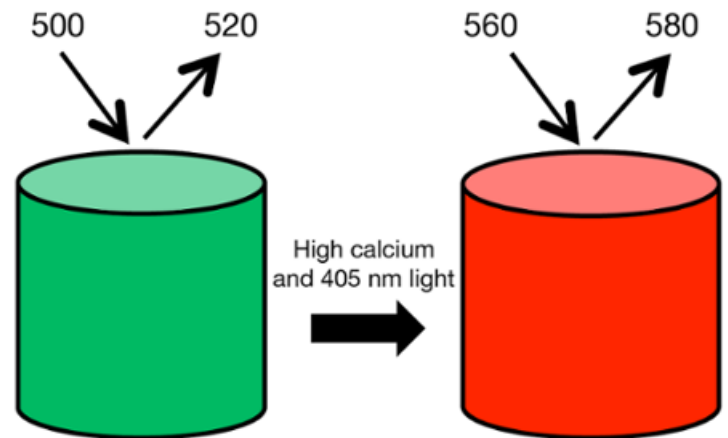


Figure 3: Schematic of CaMPARI calcium integrator. A green fluorescent protein converts permanently to red in the simultaneous presence of calcium and the photoconversion (PC) light at 405 nm. The calcium integrator remains red even after calcium levels decrease. Adapted from Fosque et al., 2015.

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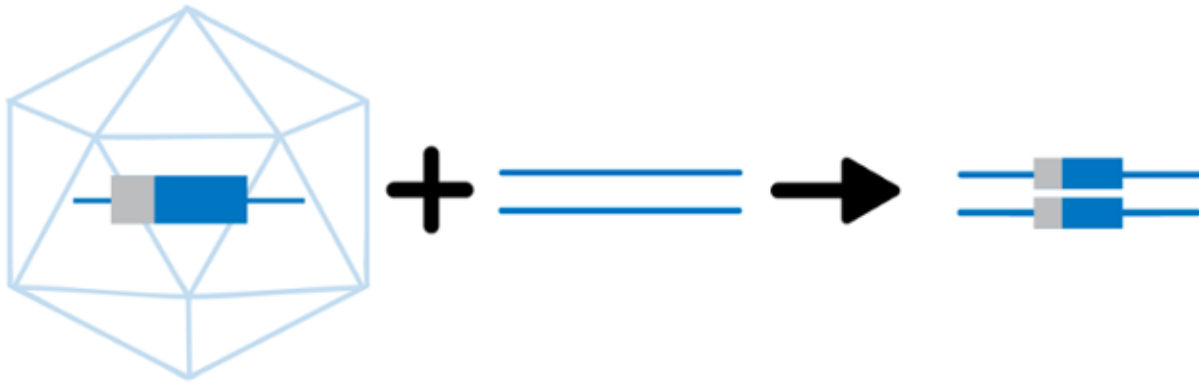
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## Switch to GECO? An overview of AAV encoded calcium sensors (CONT'D)

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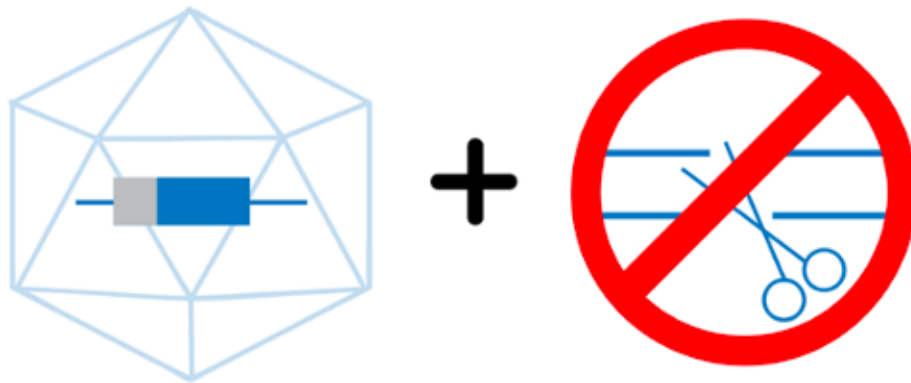
# AAVs for genome editing

By Tyler Ford and Todd Waldman | Mar 27, 2018



[Adeno-associated viruses \(AAVs\)](#) make fantastic gene delivery vehicles for episomal gene expression and are particularly useful for gene delivery to the nervous system. For many years they have also been used to enhance the efficiency of genome editing. In this post we'll walk through a variety of ways you can use AAVs to improve your genome editing experiments (with and without targeted nucleases).

## AAV genome editing without a nuclease



Although the mechanism of incorporation is not well understood, researchers have been using AAV vectors for gene editing in mammalian cells since 1998. In that year, [Russell and Hirata](#) from the University of Washington reported a surprising finding - when delivered via AAV infection, gene targeting vectors with relatively short (1 kb) homology arms underwent homologous recombination at a frequency three orders of magnitude higher than identical targeting vectors delivered through transfection of naked DNA. Other investigators who had been working to develop high efficiency gene targeting approaches improved on this finding with the addition of selectable markers that are only expressed when the targeting vector is inserted downstream of a functional promoter (gene traps). The resultant gene targeting system could be used to routinely deliver sequences of interest to cells with efficiencies of 1-40% after G418 selection ([Kohli et al. 2004](#), [Kim et al. 2008](#)). While the biology behind this AAV effect remains unclear, it is likely not a coincidence that the AAV genome is composed of single stranded DNA, which is particularly well suited for strand invasion, a key step in the homologous recombination pathway.

The Waldman lab at the Georgetown University School of Medicine has used this gene editing system frequently

# AAVs for genome editing (CONT'D)

over the past fifteen years. Some examples from their lab include using AAV to introduce epitope tags into the endogenous alleles of the p53 and PTEN tumor suppressor genes in human cells (Kim et al 2008). They have also used AAV gene editing to introduce naturally occurring cancer-causing mutations into the endogenous allele of the STAG2 tumor suppressor in human cells ([Kim et al, 2016](#)).

The Waldman lab has designed their AAV tagging vectors ([pAAV-SEPT-Acceptor](#) and [pAAV-TK-Acceptor](#)) so that they can be easily adapted to edit your gene of choice. These vectors are very similar, both with polylinkers for the addition of gene-specific 1 kb homology arms flanking a FLOxed neoR gene. pAAV-SEPT-Acceptor, the preferred vector, contains a promoterless splice acceptor-IRES-neoR gene, which enhances the efficiency of homologous integration via gene trap enrichment, whereas pAAV-TK-Acceptor has a conventional heterologous promoter-driven TK-neoR gene.

To modify these tagging vectors for your specific gene, the lab recommends designing homology arms with the modification of interest, ordering them from a gene synthesis company such as IDT or Genscript, and cloning them sequentially into the chosen acceptor vector. The vectors are then packaged into AAV virions via transient transfection of 293T cells with helpers, and used to infect recipient cells. After G418 selection, individual gene edited clones are identified by PCR. Then, if necessary for subsequent experiments, the floxed neoR gene is removed via infection with adeno-cre.

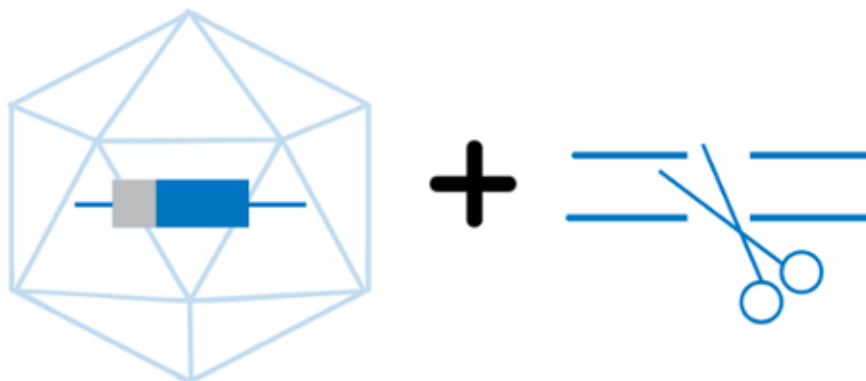
## AAV-based gene editing compared to CRISPR editing

[CRISPR](#), of course, has started a revolution in gene editing. However, while CRISPR is remarkably effective for introducing non-sequence specific frameshift mutations through aberrant double strand break repair, it has proven more challenging to develop high efficiency CRISPR-based approaches for the generation of knockins via homologous recombination with a homologous repair template. It is this application - the creation of sequence-specific knockins - for which the AAV approach is most well suited, since it routinely generates knockin efficiencies of 1-40% with G418-selected clones.

However, there are important disadvantages to the AAV-based approach that must be considered when deciding whether to use AAV or CRISPR for a given experimental approach. First, unlike CRISPR, the AAV-based approach is only able to target a single allele of a single gene at once. Therefore, applications that require simultaneous modification of all alleles of a gene, or which require modifying multiple genes simultaneously in the same cell, are more appropriate for a CRISPR-based approach. Second, the AAV preps generally used for gene editing are low titre, and are therefore able to infect only a small subset of the cells used in a given experiment. For this reason, the AAV-based approach requires G418 selection of infected clones, making it unsuitable for most *in vivo* applications. Third, even in the most optimized tissue-culture based system, the efficiency of gene editing is occasionally less than 1%, and virtually never exceeds 40%. While this is comparable or better than the efficiency of most current CRISPR-based approaches for the generation of knockins, it is not always ideal. Because of these limitations, the AAV approach is best suited for tissue culture based approaches that require modification of only a single allele of a gene, such as the introduction of heterozygous endogenous epitope tags, or the introduction of dominant mutations of a gene into the endogenous allele in cultured cells.

# AAVs for genome editing (CONT'D)

## AAV genome editing combined with targeted nucleases



In an effort to combine the respective advantages of the AAV and CRISPR-based approaches, several groups have recently worked to enhance the efficiency of AAV-based gene editing via the introduction of a double strand break by using a targeted nuclease. [Asuri et al](#), for example, used zinc fingers to increase the efficiency of AAV mediated genome editing in human ESCs and iPSCs from ~0.2% to ~1.5%. In more recent work, researchers have combined AAVs with CRISPR systems. [Ohmori et al 2017](#) use a dual vector system composed of SaCas9 and a gRNA in one AAV and a repair template in a second AAV to deliver their repair template to mouse zygotes and thereby correct a mutation that causes Hemophilia B.

Unfortunately, work combining CRISPR with AAVs for improved genome editing is hampered by the fact that even small Cas9 homologs like SaCas9 and their requisite gRNAs take up essentially all of an AAV's ~4.5 kB packaging capacity. Luckily, researchers have devised methods to deliver CRISPR components as ribonucleoproteins (RNPs) that can be supplied separately from the viral vector. This delivery method frees up space in the AAV allowing a researcher to fill it with the repair template.

[Bok and Porteus 2017](#) recently showed that by delivering CRISPR components as RNPs and delivering two halves of a repair template spread across two AAVs, it is possible sequentially repair a target site with the contents of the two AAVs. Using this technique, Bok and Porteus deliver a repair template that would take up ~6.5 kB if delivered in a single AAV. They report roughly 40% repair efficiency in the K562 leukemia cell line and ~8 and 9% efficiencies in T-cells and hematopoietic stem cells respectively. Future researchers could apply this technique to efficiently deliver large repair templates for applications that require large gene fusions. The current state-of-the-art in combining the AAV and CRISPR-based approaches is described in several recent reviews ([Howes and Schofield, 2015](#); [Moser and Hirsch, 2016](#); [Epstein and Schaffer, 2017](#))

Hopefully you've been able to check out some of the many applications of AAV in our [viral vector blog posts](#) and have also been able to browse some of the ready to use AAV available through our [viral service](#). In addition to being the vector of choice for gene delivery to a variety of cell types, now you know that AAVs can provide a powerful boost to genome editing experiments. We're excited to see how you continue to use and develop AAVs for your own research and encourage to [deposit](#) any new AAV tools you create.



# AAVs for genome editing (CONT'D)

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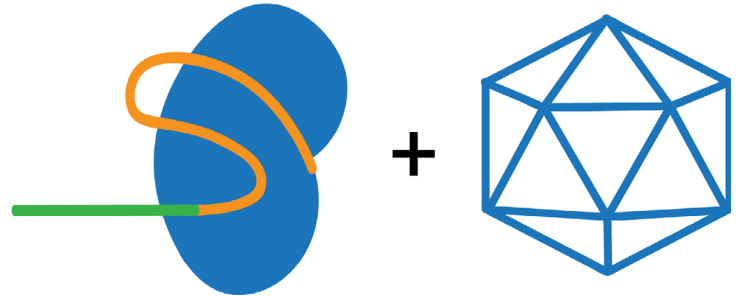
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# A match made in heaven: CRISPR and AAV

By Mary Gearing | July 14, 2015

[CRISPR genome editing](#) has quickly become the most popular system for *in vitro* and germline genome editing, but *in vivo* gene editing approaches have been limited by problems with Cas9 delivery. Adeno-associated viral vectors (AAV) are commonly used for *in vivo* gene delivery due to their low immunogenicity and range of serotypes allowing preferential infection of certain tissues. However, packaging *Streptococcus pyogenes* (SpCas9) and a chimeric sgRNA together (~4.2 kb) into an AAV vector is challenging due to the low packaging capacity of AAV (~4.5 kb.) While this approach has been proven feasible, it leaves little room for additional regulatory elements. [Feng Zhang's](#) group previously packaged Cas9 and multiple gRNAs into separate AAV vectors, increasing overall packaging capacity but necessitating purification and co-infection of two AAVs.



## Cas9 orthologs: shorter, but just as potent and specific?

The previous two AAV strategies described above showed successful target modification, indicating that AAV is a good delivery vehicle for Cas9. To maximize the genetic capacity of AAV, [Gang Bao's](#) group has developed a split-intein Cas9 that can be separated into two AAV cassettes, providing even more room for regulatory sequences and additional gRNAs in each cassette. However, to fit Cas9 and gRNAs into one AAV construct, the construct must be made even smaller. Previous attempts to “shrink” Cas9 include the use of St1Cas9 (~3.3 kb) from *Streptococcus thermophilus* and a rationally-designed truncated Cas9. Unfortunately, certain drawbacks limit the utility of these systems: St1Cas9 requires a very specific PAM sequence that limits the number of targetable loci, and truncated Cas9 has much lower efficiency than its wild-type counterpart.

[Ran et al.](#) recently developed a new strategy to overcome these drawbacks. To discover a shorter, but equally potent Cas9 enzyme, they analyzed over 600 Cas9 orthologs and found that they could be divided into two groups: one with orthologs of ~1350 amino acids, which includes SpCas9, and one with orthologs of ~1000 amino acids. From the pool of shorter orthologs, only *Staphylococcus aureus* Cas9 (SaCas9, 1053 aa) displayed cleavage activity in mammalian cells. SaCas9 produced indels at a similar efficiency to SpCas9, leading the group to focus their efforts on SaCas9 characterization for *in vivo* studies.

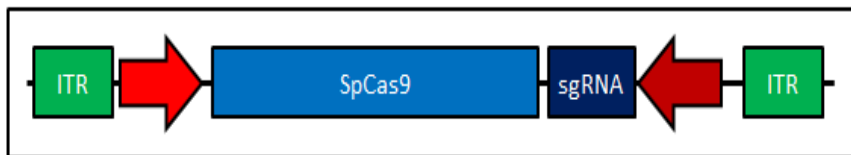
One of the pitfalls of CRISPR/Cas9 genome editing is the potential for [off-target effects](#). To compare the off-target effects of SpCas9 and SaCas9, Zhang's group used an approach called BLESS (direct *in situ* breaks labeling, enrichment on streptavidin and next-generation sequencing). Using this sensitive method, [Ran et al.](#) found that SaCas9 did not display higher levels of off-target activity than SpCas9, confirming its suitability for *in vivo* studies.

## Testing AAV-SaCas9 *in vivo*

To test the efficiency of AAV-SaCas9 *in vivo*, Ran et al. created an all-in-one SaCas9 and sgRNA construct using the liver-specific serotype AAV8. Since the efficiency of CRISPR/Cas9 genome editing varies across targets, they tested two genes in mice. For both genes, they observed indel formation and phenotypic changes as early as 1 week post-injection. Livers from these mice were histologically normal and liver injury markers were

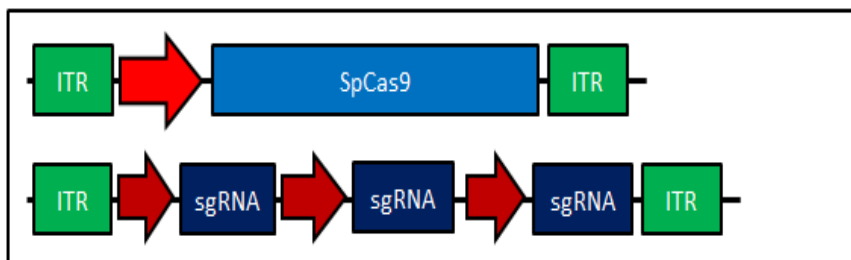
# A match made in heaven: CRISPR and AAV (CONT'D)

## Comparing CRISPR-AAV strategies



### SpCas9/sgRNA vector

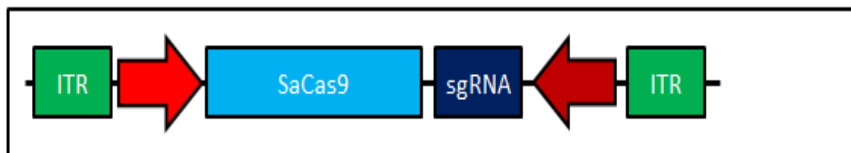
No room for additional regulatory elements



### SpCas9 vector + sgRNA vector

Multiple sgRNAs can be used

Must purify two viruses for coinfection



### SaCas9/sgRNA vector

Use of SaCas9 adds >1 kb of space for other elements

not increased compared to a control AAV-GFP. Not only did AAV-SaCas9-sgRNA constructs mediate genome modification, but they did so without a substantial immune response or toxicity.

The work of Zhang's group illustrates the potential of combining an advantageous vector delivery system (AAV) with a potent genome modification technique (CRISPR). In this "best of both worlds" scenario, *in vivo* genome editing without substantial toxicity or off-target effects will likely become much easier than we could have imagined.

If you're interested in using SaCas9 in your research, the [AAV targeting constructs are available from Addgene](#).

## Other AAV-based CRISPR systems

SaCas9 isn't the only CRISPR enzyme to be successfully packaged into AAV. At 984 amino acids in length, Cas9 from *Campylobacter jejuni* ([CjCas9](#)) is the smallest Cas9 ortholog characterized to date. [Kim et al.](#) successfully used CjCas9 with AAV to target genes in mouse muscle and eye tissue.

In 2016, [Chew et al.](#) developed a [split spCas9-AAV toolbox](#) that retains the gene-targeting capabilities of full-length SpCas9. This set of plasmids includes [AAV-Cas9C-VPR](#) for targeted gene activation.

REPAIR (RNA Editing for Programmable A to I Replacement), the new [CRISPR-based RNA editing system](#), is also compatible with AAV delivery. This system fuses catalytically dead dCas13b to the catalytic domain of RNA deaminase ADAR2. Constructs containing the ADAR2 truncation ADAR2DD(delta984-1090) are approximately 4.1 kb in length, allowing them to be packaged in AAV.

# A match made in heaven: CRISPR and AAV (CONT'D)

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# A match made in heaven: CRISPR and AAV (CONT'D)

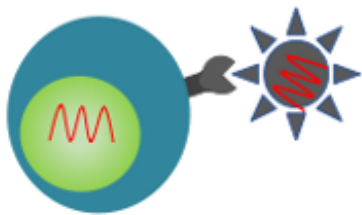
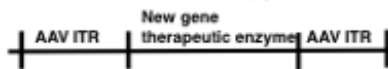
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# AAVs for cell and gene therapy

By Harshana S De Silva Feelixge. | Nov 7, 2017

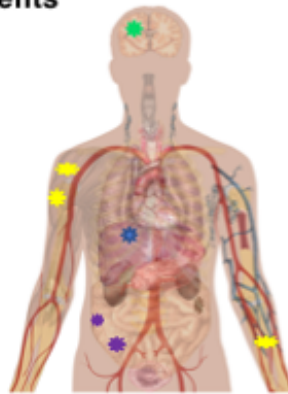
## Adeno Associated Virus (AAV): a promising vector for gene therapy

### AAV Gene Therapy



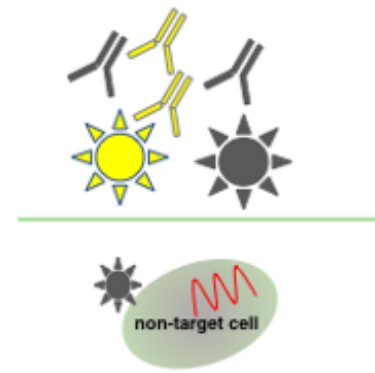
- Delivery of non-mutated genes
- Genome editing for curative therapies

### Benefits



- Infect wide range of cell types and organs
- Cell type specific infection
- Non pathogenic
- Multitude of capsid Variants

### Challenges in the Clinic



- Neutralization by anti-AAV antibodies
- Transduction of non-target cells

Gene therapy technologies hold great promise for improving or potentially curing human diseases that were previously thought to be incurable. Rapid advances in next generation sequencing technologies have allowed scientists to quickly identify underlying genetic causes of some human conditions, opening up new avenues for therapeutics that treat disease at the molecular level. For instance, if a disease is caused by a mutation in a single gene, it can potentially be treated by correcting the mutation or replacing the gene. A notable example is the treatment of Severe Combined Immune Deficiency disease (SCID-XI), also known as bubble boy syndrome. This disease is caused by mutations in the common cytokine receptor gamma chain (c) and is characterized by a lack of immune cell development and function. To date, gene therapy has been used to treat 10 infants with this disease. To do so, their T-cells were grown *in vitro*, their mutations corrected, and the T-cells were transferred back into the infants. Almost all patients have achieved persistent immunological reconstitution with a normally functioning T cell repertoire (1).

In this example, it was possible to transduce the T-cells outside the body and thereby correct the mutations using retroviral vectors. However, many diseases require techniques that can deliver gene therapies directly to the cells that need them. [Adeno-associated virus \(AAV\)](#) – based delivery techniques may hold the key.

In the simplest form, translating gene therapies from conceptual design to clinical trials involve identifying a therapeutic gene, finding a means to deliver it and identifying a suitable route of administration. However, until recently, safe delivery of nucleic acid cargo (DNA or RNA) to target cells has been challenging. A variety of vector delivery technologies have been investigated including [lentiviruses](#), [adenovirus](#), and inorganic delivery tools such as nanoparticles. These technologies can have serious adverse effects, and their immunogenic or

# AAVs for cell and gene therapy (CONT'D)

carcinogenic profiles have limited their usability in clinical settings. On the other hand, vectors derived from AAV have recently gained popularity as they are uniquely suited gene delivery vehicles with great safety profiles as well as other benefits.

## Properties that make AAV vectors great gene delivery tools

There are a variety of key properties that distinguish AAVs as gene delivery tools:

1. Unlike most naturally occurring viruses used in clinical trials, wild-type AAV is not associated with any pathogenicity and has a low immunogenic profile. AAV was first discovered as a contaminant of adenovirus preparations. It is an icosahedral, non-enveloped virus that carries a small, approximately 4.7 kb single stranded genome. AAV belongs to the family Parvoviridae and requires co-infection of helper viruses like adenovirus for productive infection. AAV vectors are engineered to provide additional safety benefits: they lack all viral genes, including those that are responsible for integration into host chromosomes, further minimizing potential activation of innate immunity.
2. AAV vectors are also capable of infecting a wide range of cell types including but not limited to muscle, liver, and brain cells. There are a multitude of known AAV serotypes that have slight variations in their viral capsids. Novel forms of AAV capsids are discovered frequently and recent advances in AAV technology have enabled us to generate new and refined forms of capsids with improved specificity.
3. AAV vectors are able to facilitate and enhance DNA repair via [homology directed repair \(HDR\)](#). This characteristic makes AAV vectors especially amenable to correcting disease-associated mutations.
4. AAV vectors maintain persistent transgene expression over many years in postmitotic, long-lived cell types.

## AAVs in the Clinic

Thus far, AAV vectors AAV1, AAV-2, AAV1-AAV2 hybrids, AAV-6, AAV-7, AAV-8, AAV-9 and AAVrh10 have been used in more than [183 gene therapy trials](#). These clinical trials have been directed at diseases including but not limited to hemophilia B, LPL deficiency, Cystic fibrosis, Muscular Dystrophy, Parkinson's disease, and HIV. A notable landmark study showed significant clinical efficacy against a form of blindness known as Leber's congenital amurosis (LCA). This disease is caused by mutations in the gene RPE65. It is characterized by childhood onset of blindness and was thought to be untreatable until 2008. Following a single dose of subretinal administration of rAAV2 carrying the human gene RPE65, patients with clinical diagnosis of LCA and mutations in RPE65 (n=3), showed significant improvements in vision over a period of 1 to 3 years – the treatment appears to be both safe and effective.

## Challenges for AAV gene therapy

Although AAV-based gene therapies are promising, scientists are working to overcome key challenges to their efficacy:

1. Pre-existing immunity: Many individuals already have wild-type AAV in their bodies and may therefore already have ways to prevent AAV infection. For example, prevalence of neutralizing antibodies against some AAV



# AAVs for cell and gene therapy (CONT'D)

types, AAV1, 2, 3 and 5 are thought to be as high as 70% among the human population. These pre-existing antibodies can interfere with virus-cell interactions and seriously hinder gene therapy outcomes.

2. Anti-AAV antibodies developed by the immune system: patients' own immune systems can develop antibodies that neutralize the AAV vectors used to treat them. Scientists are working to overcome this issue by developing new capsids through direct evolution, capsid shuffling, and peptide displays. These capsids will be selected both for their ability to evade the immune system and for enhanced abilities to infect target tissues. Improved specificity through control of gene expression and the selection of specific [promoters](#) may also help AAV vectors evade host immunity.

While basic research and other ongoing clinical trials support the utility of AAV as a broadly applicable gene delivery tool, aspects of AAV biology should be taken into account when developing AAV-based therapies. Furthermore, deeper understanding of disease pathogenesis will enable robust therapeutic outcomes. In contrast to other gene delivery tools that are under consideration, recent advancements in various AAV technologies have vastly improved AAVs' potential to become ideal vectors for gene delivery.

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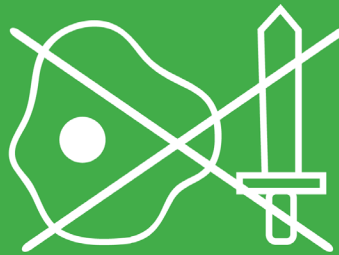
# AAVs in retinal gene therapy

By Karen Guerin | July 17, 2018

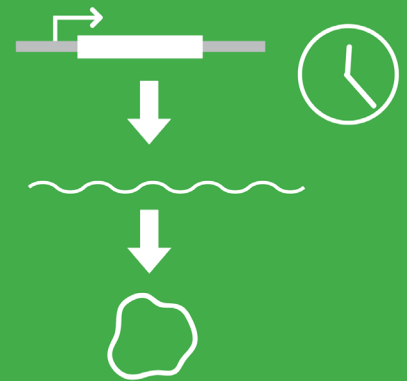
## AAVs in retinal gene therapy



The eye is easily accessible and observation is non-invasive



The eye is immune privileged - destructive inflammatory responses are not triggered



Gene expression from AAVs is long-lived

It was by serendipity that I got into the field of gene therapy, more specifically AAV-based retinal gene therapy. The year was 2001 and I started a job as a technician in a lab using [adeno-associated viral vectors \(AAVs\)](#) to treat an inherited retinal degenerative disease called Retinitis Pigmentosa. I quickly became fascinated by this emerging technology and its potential for the treatment of some genetic diseases.

I didn't know it at the time, but gene therapy had fallen - plummeted really - from grace 2 years prior with the death of [Jesse Gelsinger](#) in an adenovirus-based gene therapy clinical trial gone horribly wrong. It brought the whole field to a grinding stop and only now, 2 decades later, is it finally back in the limelight. On December 19, 2017 the FDA approved [Luxturna™](#) the first gene therapy for the treatment of a rare genetic eye disease, Leber's congenital amaurosis. This is the first of what I, scientists, doctors and patients hope are many more clinical successes for gene (and cell) therapies.

One may wonder why the first AAV-based gene therapy treatment for a genetic disease approved by the FDA is for a rare eye disease and not another genetic disease more prevalent or life-threatening (cystic fibrosis, hemophilia, muscular dystrophy)?

## AAV gene therapy started in the mid-90's with a lot of work and a little bit of luck

A pioneer in the field, Dr. Jean Bennett started working on ways to deliver genes to correct genetic conditions over 25 years ago when completing her PhD and medical training in Ophthalmology. Gene therapy was in its infancy, but the eye was identified early on as an ideal target. It is an easily accessible organ that can be monitored non-invasively over time. Importantly, it is an immune privileged compartment, meaning that it can



# AAVs in retinal gene therapy (CONT'D)

tolerate the presence of antigens (such as viral vectors) without triggering a destructive inflammatory immune response. At the time, adenoviral vectors were the most commonly used viral vectors for gene delivery, and they had been shown to transduce the retina but only transiently. However new viral vectors capable of providing long-term gene expression in retinal cells -AAV- were becoming available.

At the same time advances in human genetics led to the identification of several disease-causing genes, including the [RPE65 gene](#) responsible for an autosomal recessive, severe, childhood-onset form of blindness name Leber's Congenital Amaurosis (LCA). Shortly after the cloning of the RPE65 gene, a naturally occurring animal model, the RPE65<sup>-/-</sup> dog, was characterized and found to suffer from early and severe visual impairment that faithfully recapitulated the human condition.

Luck brought all these key elements together at the right time and place. But it is the hard work and persistence of a dedicated team of scientists led by Dr. Jean Bennett (UPenn) that resulted in the groundbreaking 2001 study showing that AAV-mediated delivery of the RPE65 gene restores vision in the dog model of LCA (Lancelot, one of the dogs who received the AAV-RPE65 treatment, famously visited Congress to help increase awareness about the potential of gene therapy!). It would take another 6 years before the 1st clinical trial started, and 10 more years to obtain FDA approval and make history in the form of Luxturna<sup>™</sup>.

## A better vector for ocular gene therapy: Adeno-Associated Virus (AAV)

While other viral vectors such as [adenovirus \(AdV\)](#) and [lentivirus \(LV\)](#) are known to transduce retinal cells, AAV quickly became the vector of choice for ocular gene therapy.

AdV and LV have been used successfully in preclinical and clinical settings for ocular conditions. However, AdV was abandoned early on due to its considerable immunogenicity and its transient expression. LV results in long term expression in dividing and non-dividing cells due to its (random) integration into the genome, and its large DNA packaging capacity is well suited for larger transgenes. Unfortunately it has limited tropism in the retina, primarily infecting cells of the retinal pigmented epithelium (RPE).

AAVs, on the other hand, have broad tropism in the eye. AAV-2, the first identified serotype and the one used in Luxturna<sup>™</sup>, can efficiently transduce various types of retinal neurons. Nowadays a toolbox of naturally occurring and engineered AAV serotypes that further expands the range of AAV properties is available. While only natural serotypes are currently used in clinical trials, a variety of engineered ones have shown great promise in preclinical studies and could reach clinical trials soon.

The AAV safety profile is excellent and further strengthens its position as the vector of choice for *in vivo* gene therapy. It is not known to cause any disease in humans, and its low immunogenicity is critical when systemic delivery of high doses of vectors are needed. Additionally, AAVs do not integrate in the host genome but still lead to long-term (>10 years) transgene expression in non-dividing cells because they remain in episomal form in the cell's nucleus.

But since nothing's ever perfect, neither is AAV. Its main limitation is the small size of its DNA cargo - 4.8 kb at most - which means that a gene larger than 3 kb cannot be delivered using this vector. For example, although

# AAVs in retinal gene therapy (CONT'D)

researchers know that Usher Syndrome (a disease that results in combined deafness and blindness) is caused by defects in the gene MYO7a, at ~7 kb this gene is too large to deliver via AAV. Dual vectors strategies are being optimized to overcome this limitation, with limited success so far. Luckily for patients, this gene can be targeted to RPE cells using a lentiviral vector and a clinical trial is ongoing.

## Moving forward with ocular gene therapy

The pipeline of AAV-based ocular gene therapies is impressive: clinical trials for choroideremia, X-linked retinitis pigmentosa, achromatopsia, and age-related macular degeneration are ongoing and some will hopefully follow in the footsteps of Luxturna™. However, despite promising results in preclinical studies some potential candidates for rare to ultra-rare ocular diseases are being benched due to the high cost of clinical development. As a result, Luk Vandenberghe and colleagues at MEEI co-founded a [non-profit](#) to help push forward candidate therapies for these diseases.

Riding on the early successes of retinal gene therapy, an ever-growing number of clinical trials for devastating genetic diseases such as hemophilia, muscular dystrophy, spinal muscular atrophy, neurodegenerative diseases and metabolic diseases are also underway and reported results are encouraging.

Importantly, a significant amount of basic research is being done to further improve the technology: engineering of new capsid variants, designing expression cassettes to regulate and optimize transgene levels, and large scale manufacturing of viral vectors to support trials for prevalent diseases - to name a few.

[CRISPR](#)-based tools are also particularly well suited to be combined with AAV for *in vivo* gene editing, and reports suggest that the first clinical study combining these tools may start as early as next year.

Twenty years after falling from grace, gene therapy is back, better and safer. It is now one of the most active and exciting areas of science. This is hopefully only the beginning of a gene therapy revolution and the development of personalized medicine.

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# AAVs in retinal gene therapy (CONT'D)

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# AAV Titers: Where do they come from and what do they mean?

By Leila Haery | Nov 15, 2017

Remember the game show “The \$25,000 Pyramid” where one player tries to get the other to guess a category by listing off things that fall into that category? Okay, let’s play! I’ll list the examples and you try to guess the category:

ELISA...  
qPCR...  
Digital droplet PCR...  
DNA dot blot...  
Transduction assay...  
SDS-PAGE...  
Electron microscopy...

Any guesses?

That’s right... ways to titer [AAV](#)!



The point is there are many ways to quantify the composition of a [viral vector](#) solution, and generally these methods measure different characteristics of the solution. These characteristics give us information on three important factors:

**1. Physical titer:** the concentration of viral particles containing viral genomes. Physical titers are measured by quantifying the concentration of viral genomes (by [qPCR](#) or other DNA quantification methods - see below), since each viral particle typically contains one viral genome. Other than a theoretical maximum value, physical titers don’t necessarily give any indication of the infectious titer. The infectious titer of a viral vector stock can vary based on transduction target and can also be altered by freeze-thaw of the viral solution ([Lock et al., 2010](#)).

**2. Infectious titer:** the concentration of viral particles that can transduce cells. Infectious titers are typically quantified by cell transduction assays. Wild-type AAV2 has been reported to have a near-perfect physical-to-infectious particle ratio of 1:1 ([Zeltner et al., 2010](#)). However, for recombinant AAV2, the same study reported a physical-to-infectious particle ratio of 50:1 (Zeltner et al., 2010). The specific infectivity of viral preparations is defined by the ratio of physical viral particles to infectious viral particles.

**3. Ratio of full to empty viral capsids:** the ratio of genome-containing viral particles relative to the total number of viral capsids (which can include empty capsids that are devoid of a viral genome). Empty viral capsids can be formed during viral production depending on the experimental conditions used to generate AAV. One study showed that approximately 50% of viral capsids were genome-containing (“full”) (Zeltner et al., 2010), while another reported that only 20% of recombinant AAV2 particles were full, compared to 50% of wild-type AAV2 stocks ([Grimm et al., 1999](#)). The percentage of genome-containing viral capsids is typically quantified by electron microscopy of a viral vector solution. Since this technique is intensive and requires an electron microscope, it is not routinely performed on all new viral vector preparations. Addgene has used this method to generally

## AAV titers: Where do they come from and what do they mean? (CONT'D)

validate our viral vector preparation protocol, and we are pleased to report that over 95% of viral capsids in representative viral vector preparations are genome-containing (Figure 1).

### Which titer do people usually report?

Addgene and other viral vector manufacturing facilities report the physical titer of a viral solution (Figure 2). Because physical titers are used for dosing purposes in preclinical studies, it is important to understand what these values mean and how they can be compared.

Physical titer is typically calculated by two popular PCR-based methods:

- Quantitative PCR (qPCR)
- Digital droplet PCR (ddPCR)

Historically, a quantitative DNA hybridization method (DNA dot blotting) had been used to titrate AAV, but this method is not widely used today ([Fagone et al., 2012](#)).

PCR-based methods are robust, easy, fast, and convenient. However, these methods are not necessarily precise or accurate for quantifying AAV because PCR can be affected by many experimental factors.

To list some complicating factors for quantifying AAV:

- qPCR efficiency can be affected by second-order structure of the AAV genome, caused by repeats and self-complementarity of the AAV genome and the ITRs.
- qPCR efficiency can be affected by the temperature parameters. For example, we found that changing the annealing temperature of the PCR from 60° C to 61° C improved assay reliability.
- Different primers can have different annealing efficiencies ([Wang et al., 2013](#)). Although you can optimize your primers for each individual sample, this will reduce convenience and comparability because each sample will be quantified with a different primer pair.

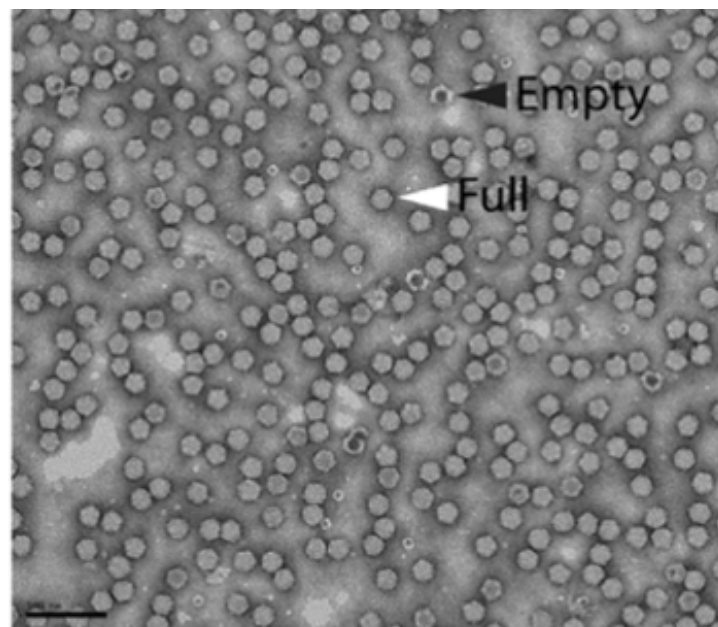


Figure 1: Electron micrograph of an Addgene AAV viral vector preparation after negative staining. Empty vector particles can be identified after negative staining and appear darker than full vector particles. This image shows that the vast majority of the vectors consist of full particles (white arrowhead) relative to empty particles (black arrowhead). Scale bar = 100 nm. \*Thank you David Bell and Svetla Stoilova-McPhie, Center for Nanoscale Systems, Harvard University.

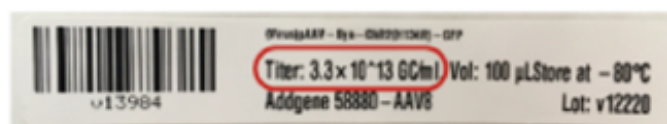


Figure 2: Label that comes on Addgene's AAV aliquots. Our titer values (circled in red) report physical titer, which is measured by qPCR.

## AAV titers: Where do they come from and what do they mean? (CONT'D)

- Ct values vary based on the amount of starting material in the PCR because the sample must fall within the linear region of the standard curve. In addition, high and low-concentration samples can also behave differently in PCR, even if both samples fall within the linear region of the assay. This may be due to competition for binding sites between the primers and the AAV genome repeats.
- Primer annealing can be affected by the presence of protein contaminants, which is a factor in AAV titration since the starting template is the purified viral vector solution (which contains viral capsid proteins) as opposed to purified DNA. This may be mitigated by using purified viral DNA as the template instead of the intact AAV particle.
- Finally, the precision of the assay can vary up to two fold for the final titer value, which is simply due to the noise of the assay. In other words, repeating the exact same PCR twice can give titer measurements that vary up to two-fold.

### How can we get more reliable AAV titering?

For our [viral service](#), we currently titrate AAV vector preparations by qPCR. To ensure accurate and reliable results, we have optimized our assay in a few ways:

- Absolute quantification by qPCR requires generating a standard curve of known concentration. We regularly make and validate fresh plasmid standards.
- We validate our absolute quantification by using the universal AAV Reference Standard Material (AAV SFM), which is an AAV sample that has been quantified by 16 labs around the world and can be used by researchers to validate their qPCR assay (Lock et al., 2010). In addition to the AAV RSM, we include a second AAV reference sample of known titer, which is at a higher titer than the AAV RSM. By using these samples in all our qPCR assays, we ensure that the standard curve generated is reliable, and can thus be used to accurately infer titer values of new AAV samples.
- We also typically quantify our AAV sample by two qPCR assays and compare the values to determine accuracy and reliability. When doing this, we make a judgement call on whether the two titer values are within the error of the assay and thus are deemed reliable, or if the sample needs to be quantified again.

Some of the issues with titrating by qPCR are addressed with [ddPCR technology](#) (Hindson et al., 2011, Hindson et al., 2013), which has been shown to be more precise and more repeatable than qPCR (Taylor et al., 2017). However, because purified AAV preps contain similar and low levels of background protein and chemical components, ddPCR may not be an improvement over qPCR (Taylor et al., 2017).

### What do Addgene AAV titers mean for your experiments?

Because (physical) AAV titers are approximations and they depend highly on the experimental conditions used, titer values of samples from different sources should not be compared. If using AAV from different sources in the same experiment, consider [retitering both AAV](#) in your lab. The absolute titer values may not be as important as the relative titer values. Finally, because physical titer does not give an indication of infectious titer, consider



## AAV titers: Where do they come from and what do they mean? (CONT'D)

titrating each lot of AAV *in vivo* to determine the optimal dosage. At Addgene, we don't reserve lots, but if you're reordering the same virus feel free to email us and we can do our best to send you a particular lot (if we still have it available).

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# Important considerations when using AAVs

By Katrina Armstrong | June 13, 2017

1. Location, Location, Location!
2. Failure to Plan (for Storage) Is Planning to Fail
3. Patience Is Bitter but Its Fruit Is Sweet
4. The Future?

I knew little about [adeno-associated Viral Vectors \(AAVs\)](#) before starting my graduate program at the University of Manitoba. Our lab has been utilizing chemogenetics (Designer Receptors Exclusively Activated By Designer Drugs, [DREADDs](#)) and [optogenetics](#) as tools to investigate the roles of certain cell types in locomotion. We have relied heavily upon AAV vectors to deliver chemogenetic/optogenetic constructs into our cells of interest. Although they have a small packaging capacity, AAV vectors were suitable for our needs for the following reasons:

- **Safety:** Since AAVs are nonpathogenic, we are able to use AAV vectors without specific facilities (Biosafety Level-1). Other possible tools, lentiviruses for example, are not approved for use *in vivo* at the University of Manitoba due to their Biosafety Level-2 or 2+.

**\*Please Note\*** *Despite the fact that AAVs themselves are not pathogenic, precautions should still be taken when using them. There are suspected cases of insertional mutagenesis in humans (1) and AAVs are non-enveloped viruses, so they are resistant to alcohol-based disinfectants. A 10% bleach solution should be used to clean AAVs from workspaces.*

- **Speed:** AAV transduction experiments are relatively quick. We have found that expression occurs quickly and results in long and stable expression (up to 9 months in mice [2]).
- **Low Immune Response:** Compared to other viral vectors (e.g. [Lentivirus](#), [Adenovirus](#)) AAVs cause the least amount of immune response. Any investigator who is using an *in vivo* animal model should consider the possible elicited immune response from viral infection. Animal safety is a top priority in our lab so we opted for AAV.

While these benefits make AAVs useful for a wide variety of experiments, care still needs to be taken when selecting which particular AAVs to use for your research. Below I'll provide some tips that will help you think about how to get started with AAVs. Since my research is rooted in the central nervous system (CNS), my focus will be mostly on the brain, but many of these tips apply to all tissues.

## 1. Location, Location, Location!

What do buying real estate and using AAVs have in common? The three most important items when looking for a house and choosing an AAV are... location, location, location! Just like this common real estate mantra, the choice of serotype (which determines which tissues and cells an AAV can infect) can influence an AAV's value greatly. Applying this mantra to AAV selection, there are three important factors related to location.

Location (1): What tissue?

Location (2): Where in the tissue/which cell type?

Location (3): Traveling from the original location - e.g. Axonal transport in the CNS

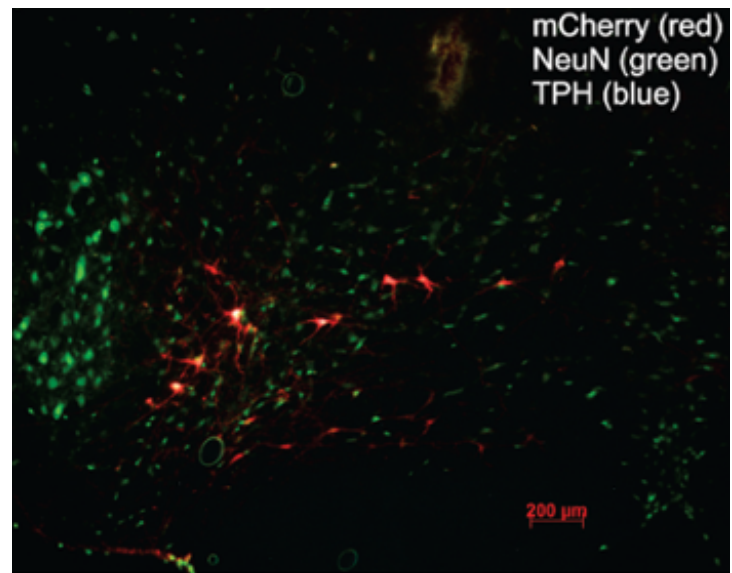
# Important considerations when using AAVs (CONT'D)

## Location (1): What tissue?

What is commonly known is that different serotypes have different tropisms but, as you can see in table 1, AAVs of each serotype can transduce multiple tissues. So how do you choose the right serotype? Keep asking questions!

## Location (2): Where in the tissue/what cell type?

In the CNS, for instance, there are marked differences among serotypes in their efficiency at transducing different brain regions. Although AAV2 can transduce many tissue types, it has low expression in several brain regions where other serotypes may perform better (3).



Portion of the rat brain triple labeled with AAV vectors. mCherry labels the hM3Dq DREADD, NeuN (green) is a neuronal marker, and TPH (blue) is tryptophan hydroxylase, an enzyme involved in serotonin production.

**Table 1: AAV Serotypes and tissue tropism**

| Tissue type                      | AAV for targeting                  |
|----------------------------------|------------------------------------|
| CNS                              | AAV1, AAV2, AAV4, AAV5, AAV8, AAV9 |
| Heart                            | AAV1, AAV8, AAV9                   |
| Kidney                           | AAV2                               |
| Liver                            | AAV7, AAV8, AAV9                   |
| Lung                             | AAV4, AAV5, AAV6, AAV9             |
| Pancreas                         | AAV8                               |
| Photoreceptor Cells              | AAV2, AAV5, AAV8                   |
| RPE (Retinal Pigment Epithelium) | AAV1, AAV2, AAV4, AAV5, AAV8       |
| Skeletal Muscle                  | AAV1, AAV6, AAV7, AAV8, AAV9       |

Modified from <https://www.addgene.org/viral-vectors/aav/aav-guide>

This preferential tropism for different brain regions is largely due the ability of different serotypes to infect different cell types. In the CNS, it has been shown that there is variable transduction of oligodendrocytes, microglia, astrocytes, neurons, and inhibitory neurons. Your choice of AAV will depend upon which mouse brain section you're looking at (striatum, hippocampus and cortex) for example (Table 2, [ref 4](#)). An example from the cortex is outlined in the table below.

# Important considerations when using AAVs (CONT'D)

**Table 2: AAV serotypes and brain region tropism**

| Brain region    | Optimal serotype              |
|-----------------|-------------------------------|
| Striatum        | AAV5>AAV9>AAV8>AAV1>AAV6>AAV2 |
| Hippocampus     | AAV9>AAV5>AAV8>AAV1>AAV6>AAV2 |
| Auditory cortex | AAV9>AAV8>AAV6>AAV5>AAV1>AAV2 |

**Table 3: AAV Serotypes and Cell Type Tropism in the Cortex**

| Cell type in the cortex | Optimal serotype   |
|-------------------------|--------------------|
| Oligodendrocytes        | AAV8 > AAV2 & AAV6 |
| Microglia               | AAV9 > AAV2 & AAV6 |
| Astrocytes              | AAV8 > AAV2 & AAV6 |

*Modified from Aschauer et al. (2013)*

What does this mean to us? Well, an AAV user must determine which serotype is most efficient at transducing the cell type under study. Many AAVs will show significant expression in a variety of cell types, but you should try to find the best AAV for your particular experiment!

## Location (3): Traveling from the Original Location - Axonal Transport

It has been demonstrated that certain serotypes can undergo axoplasmic transport. These AAVs may move toward the cell bodies of neurons (retrograde transport) or towards the synapse (anterograde transport), which is a possible mechanism for the AAV to infect other cell types not within the vicinity of the injection.

Examining serotypes 1, 8 and 9, not only do they undergo retrograde and anterograde transport, but they also have similar transport characteristics (velocities of movement, distance traveled, etc.) (4). This has also been identified with AAV2 (5), AAV6 (6) and AAV5 at excitatory connections (3).

These facts beg the question: **is axonal transport desirable or not desirable?**

If diffuse gene expression is tolerated, then axonal transport is fine and a researcher should aim to inject a larger amount of vector using a serotype with strong CNS tropism (4).

If axonal transport is not desirable, transport can be reduced by injecting a smaller dose of AAV and using a serotype with weak CNS penetrance (such as AAV2) (4). Otherwise, using a specific system for controlling gene expression such as [Cre-lox](#), may be sufficient to overcome the transport issues.

# Important considerations when using AAVs (CONT'D)

## 2. Failure to plan (for storage) is planning to fail

### Freeze/Thaw Cycles

Of course, one cannot truly predict the perfect titer, but one of the main recommendations when using AAVs is to avoid freezing and thawing. Although more stable than other viruses, multiple freeze thaw cycles can still reduce AAV titer. For long term storage, make > 25 µl aliquots in 0.5 mL tubes, snap freeze in liquid nitrogen or a dry ice/ethanol bath, and store at -80° C. Once thawed, the AAV aliquot should be used quickly.

## 3. Patience is bitter, but its fruit is sweet

### Time

If you require a quick onset of expression, AAV may not be the best tool to use. However, we are starting to see that gene expression really does not take much time at all. Much of the previous literature using AAVs for DREADD delivery indicates ~7-21 days are required for the onset of gene expression. However, it has yet to be determined what gene expression looks like at 3 days vs 7 days vs 14 days etc. To decrease the time until expression, it is possible to use AAV constructs with self-complementing structure (scAAV) [7]. As single stranded DNA viruses, AAVs normally require second strand synthesis before gene expression begins. scAAV avoid this rate limiting step and can immediately undergo replication and transcription. However, with scAAV the effective genome size usable is shortened (from ~4.8 kB [8] to ~2.3 kB [9]).

## 3. The future

### What new types of research may be enabled by the use of AAVs?

#### 1) Combined functional and anatomical mapping of circuits:

In our lab, we use [AAV-DREADD](#) complexes to study neural function. Often the reporter proteins associated with these complexes are so robustly expressed that anatomical assessments can be easily performed too. While I implied that axoplasmic transport of AAVs is a potential problem in many experiments, a group of researchers has identified this feature of AAVs as a new tool for neuroanatomical tracing [11]. With the ability to produce neuron specific transfection using Cre-lox, this new method could allow for labeling of neurons showing a very specific phenotype. Previous neural tracing methods used viruses that were potentially toxic and would result in cell death. With several new constructs becoming available, AAVs could be much better tools for neural tracing in very specific cell populations.

#### 2) Comparison of genetically engineered receptors (i.e. chemogenetics vs. optogenetics):

I'm particularly interested in using chemogenetic and optogenetic AAV-based tools to study neural control. Surprisingly, little research has compared and contrasted optogenetic and chemogenetic receptors delivered by AAV within the same cell types. Not only can we compare these methods in specific applications, we may also be able to use these comparisons to enhance our understanding of receptor physiology and the effects of metabotropic and ionotropic influences on overall cell function. AAVs are the most appropriate vehicle for this type of research, as they are safe to use *in vivo*.

# Important considerations when using AAVs (CONT'D)

## 3) The use of AAVs in humans:

AAVs are already being used to treat disease in humans and additional improvements are being made to make them even more useful for therapeutic purposes. The [University of Pennsylvania](#) and the biotechnology firm [Biogen](#) have recently collaborated to develop “[AAV 3.0](#)”, vectors that will have substantially improved performance profiles and should be suitable for [genome editing](#). These vectors can be used to improve targeted therapies for the eye, skeletal muscles, and the central nervous system.

Currently, AAVs are being used to deliver [CRISPR/Cas9](#) but have the potential to deliver other therapeutic molecules such as microRNAs (miRNAs), short hairpin RNAs ([shRNAs](#)), antisense oligonucleotides (ASOs), zinc finger nucleases, and many others ([12](#)).

The most attractive potential of AAVs is their ability to be used with new exciting technologies (such as CRISPR/Cas9) as they allow for gene delivery in an efficient and non-pathogenic fashion. Work improving AAV tissue tropism, transduction efficiency, and safety profiles should strengthen its appeal for the scientific and medical communities.

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# Important considerations when using AAVs (CONT'D)

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# The importance of high titer for AAV transductions

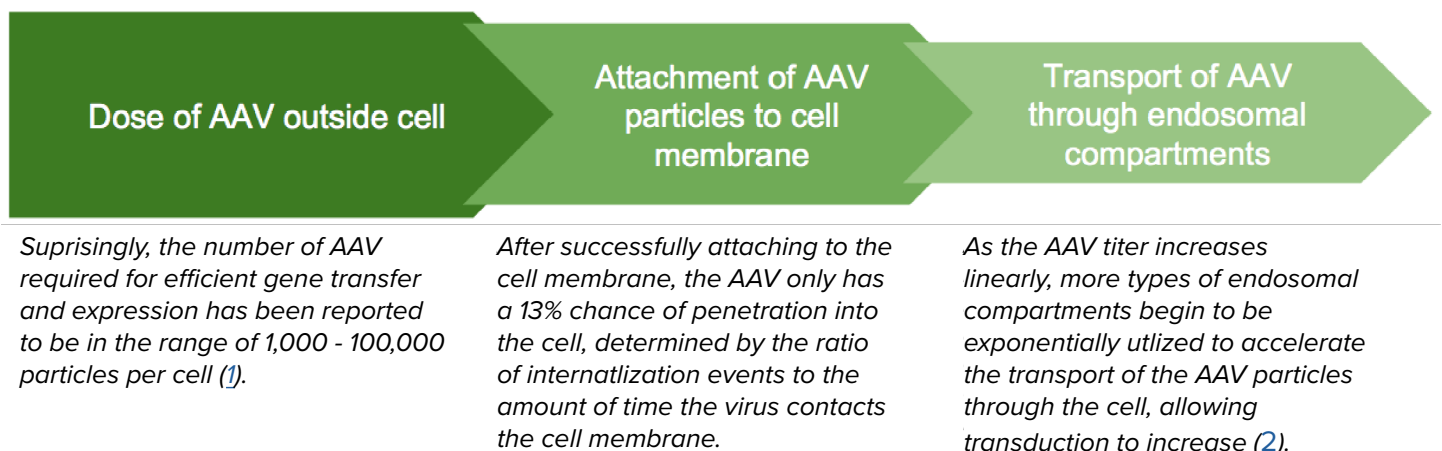
By Luke Hanley | March 1, 2018

Many of us take comfort in the fact that it's often not quantity, but quality that really matters. Well, it turns out this isn't the case for using AAV. When it comes to infecting cells, titer, the amount of virus used, really does matter. (\*psst\*, quality definitely also matters).

## Making contact with a cell

Although an individual AAV particle's ability to infect a cell is dependent on its capsid construction and the cell it's infecting, getting these particles to infect cells is also very titer-dependent. In order for an AAV particle to infect a cell, it has to come into physical contact with that cell. Since there is a limited amount of surface area on each cell, the way to get more AAV in contact with the cell is to increase the AAV concentration.

In this flowchart, you can see the dwindling proportion of AAV particles that make it through each part of the cell on their way to the nucleus where their gene cargoes can be expressed.



The bottom line is that a lot of AAV particles are needed to get a few good ones into a cell to produce expression. The higher the titer, the better.

## AAV transport and transgene expression

It's clear that we need high titers, as contact between the cell and any individual AAV particle is rare. But simply getting into the cell may not always be enough and therefore higher titer will be required. For example, retrograde AAV gets transported up the cell's axon. While the mechanism for this function is not yet fully understood, we can imagine that this transport is probably not 100% efficient. So the few AAV particles that have entered the cell now have to travel up the axon, through the cell, and potentially spread out over an even larger area, thus necessitating even higher retrograde AAV titers for successful transduction.

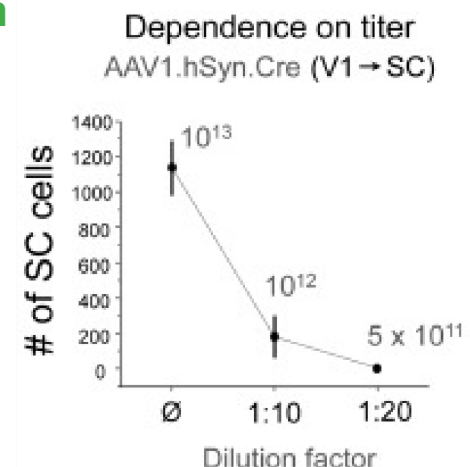


Figure 2: Dependence of transneuronal labeling on AAV1 titer (3).

# The importance of high titer for AAV transductions (CONT'D)

In another example, neuronal gene expression by an anterograde AAV was shown to drop off drastically with lower titers. As you can see in figure 2, the expression from the  $5 \times 10^{11}$  titer was essentially nonexistent, as there wasn't enough virus to produce any observable expression. Doubling this titer to  $10^{12}$  resulted in expression in roughly 200 cells. Increasing the titer to  $10^{13}$  resulted in a roughly 6-fold increase in expression (3, Figure 2). Note how expression increases exponentially with an increase in titer. That means that once you drop below a certain (very high) threshold of viral particles per cell, you may not be able to detect AAV-mediated gene expression in a target cell.

## Practical Considerations

If you have high quality AAV, you can address the issue of low titer by concentrating your virus. See our [Concentrating AAV Protocol](#) (Step 10) for instructions on how to do this. Concentrating your virus will allow you to add many more viral particles to your solution while still using a volume that's compatible with your experimental setup.

Some experiments require higher gene expression and therefore higher titer than others. High titers are often necessary to visualize expression of [fluorescent proteins](#), for example, as many viral particles must infect a cell to achieve visible expression of the protein. However, expression of receptors may not need to be as high to achieve an observable signal in your experiment, therefore titer may not need to be as high when delivering receptor constructs with AAV. In the case of channelrhodopsins, fewer channel proteins are required for the transduced cell to become light responsive than fluorescent proteins required to get a good fluorescent signal. It may not be necessary to concentrate this type of vector to such a high titer when looking for effective gene expression.

## How do you achieve high titers in the first place?

Some AAVs are known to be difficult to produce in high titer. These include AAV2, which is known to adhere to the cell interior, causing less particles to be released into suspension and lowering the number of viral particles that can be harvested from the surrounding media.

Nonetheless, there are a variety of ways you can optimize AAV production. We've listed some simple steps you can take below but you should read our [AAV Production Protocol](#) for additional best practices.

- Keep your cells happy - Make sure your cells are as healthy as can be to prepare them for the arduous process of generating viral particles.
- Limit tube transfer - the fewer opportunities your viral particles have to stick to your tubes, the higher titer you will have. When possible, use low-bind or siliconized tubes to store samples as it is more difficult for viral particles to adhere to these tubes.
- Rinse off the filter - When using a concentration filter column, make sure to rinse off the surface of the filter membrane with the surrounding solution to ensure excess viral particles do not remain adhered to the membrane.
- Be careful when diluting - if you end up concentrating your AAV above your desired titer and want to dilute it, err on the side of caution when adding buffer back to the solution. Add a bit less buffer than you would to achieve the exact desired concentration to avoid over-diluting, and make sure to remeasure your titer after diluting to confirm.

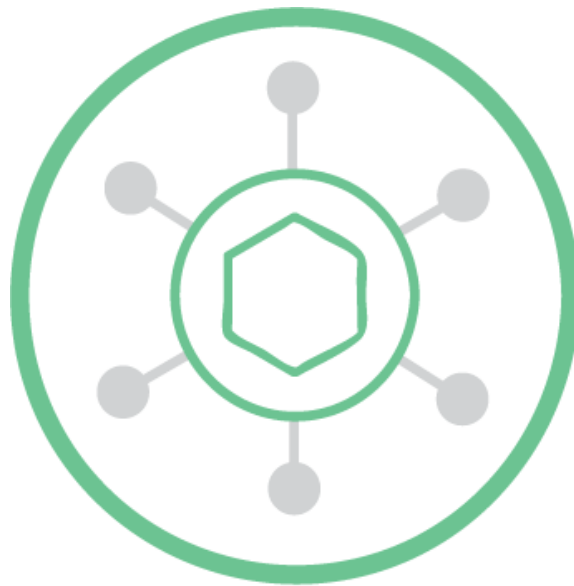
# The importance of high titer for AAV transductions (CONT'D)

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# CHAPTER 3

## Retroviral and lentiviral vectors



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# γ-Retrovirus

By Various Addgenies

## γ-Retroviral packaging systems

The widespread use of γ-retroviruses (gamma-retroviruses) in [cancer](#) and [stem cell research](#) has prompted the development of multiple virus packaging methods. Across these methods, the following components are needed:

- **γ-Retroviral transfer plasmid encoding a transgene, sgRNA, or shRNA of interest:**

The transgene sequence is flanked by long terminal repeat (LTR) sequences, which facilitate integration of the transfer plasmid sequences into the host genome. Typically it is the sequences between and including the LTRs that are integrated into the host genome upon viral transduction. Most transfer plasmids available from Addgene were derived from MoMLV (Moloney Murine Leukemia Virus) or MSCV (Murine Stem Cell Virus) sequences.

- **Packaging genes (viral Gag-Pol):** Gag is a structural precursor protein, and Pol is a polymerase.
- **Envelope gene (may be pseudotyped to alter infectivity):** The use of the VSV-G envelope provides the widest tropism or range of cells a virus can infect.

**\*Note\*** While both [lentiviruses](#) and γ-retroviruses use the same gene products for packaging (i.e., Gag, Pol, and Env), the isoforms of these proteins, as well as the viral LTRs, differ. As a result, lentiviral and γ-retroviral packaging plasmids are not interchangeable. General envelope plasmids, such as VSV-G, however, may be used across both systems.

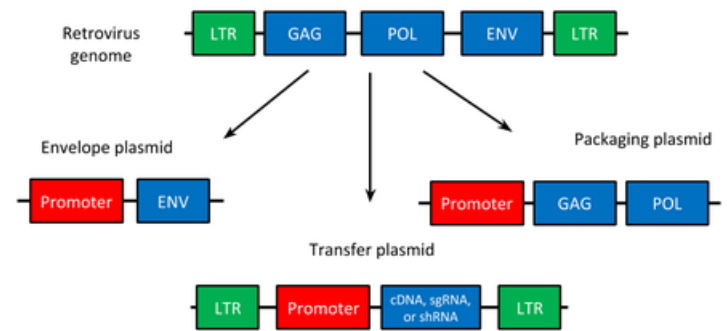
Please see our [Biosafety page](#) for information on working with retrovirus.

## Packaging using 293T cells

This method is very similar to lentiviral packaging methods. The three components described above (envelope, packaging, and transfer) are supplied by three types of plasmids, which are cotransfected into the 293T packaging cell line. This system provides the greatest flexibility to pseudotype γ-retrovirus using different envelopes to modify tropism. Briefly, different envelope plasmids can direct the production of virus with various tropisms.

## Packaging using helper-free packaging cell lines

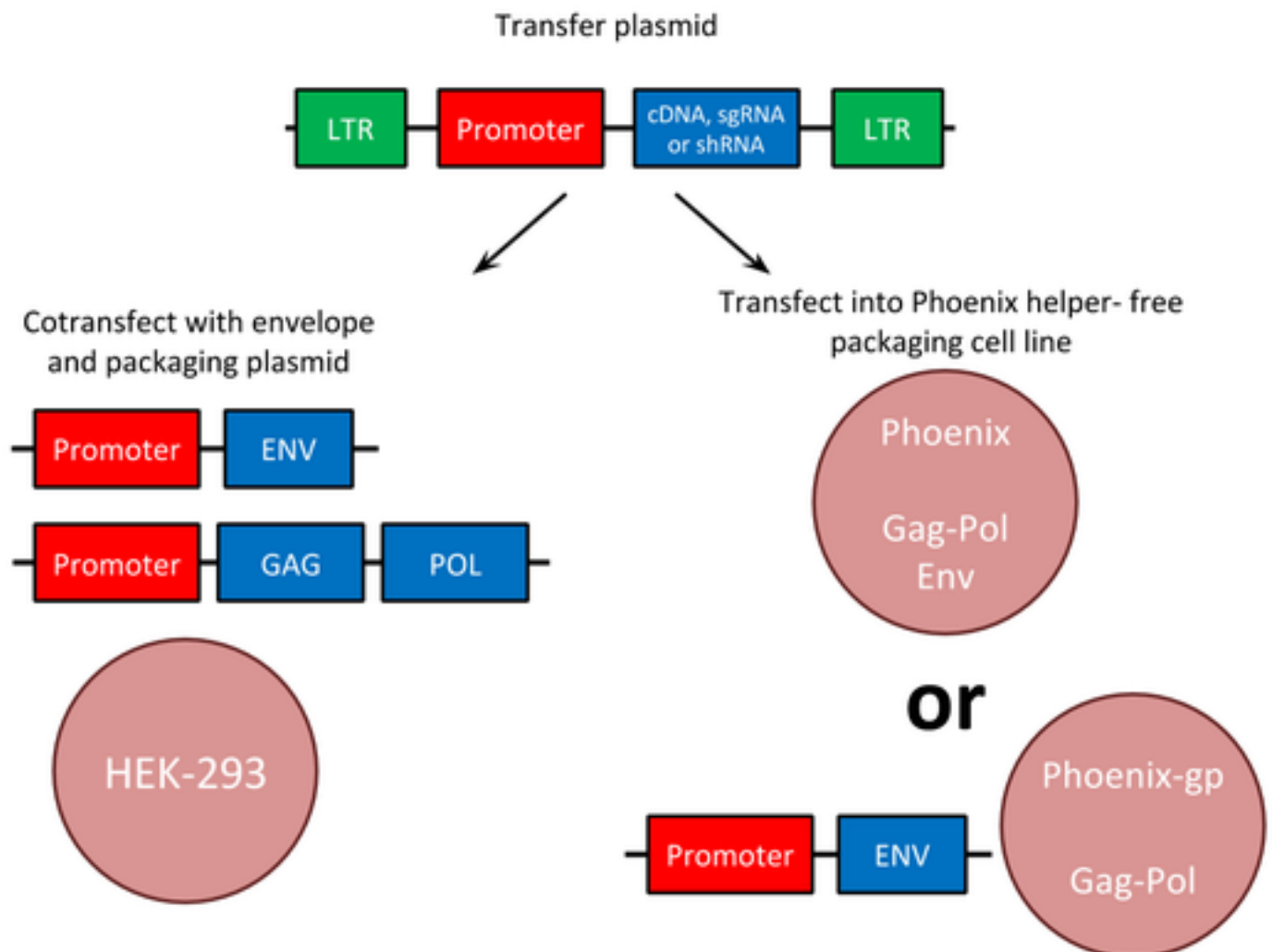
This method utilizes a packaging cell line that stably expresses Gag-Pol and/or Env, eliminating the need to deliver these genes *in trans* by specific plasmids. This method, therefore, reduces the number of plasmids that are required in the transfection step of the viral packaging cell line. For example, Phoenix, a second generation γ-retrovirus packaging cell line developed by [Garry Nolan](#) at Stanford contains Gag-Pol and either an ecotropic envelope, Phoenix-ECO, (for infection of mouse and rat cells), or an amphotropic envelope, Phoenix-AMPHO



Overview of retroviral plasmid system

## γ-Retrovirus (CONT'D)

(for the infection of mammalian cells.) Using this system, virus is produced in just a few days. Another variant, Phoenix-gp, contains only Gag-Pol and enables additional flexibility in pseudotyping.



## Frequently asked questions about γ-retroviral plasmids

### How do engineered γ-retroviruses differ from lentiviruses?

There are a few important differences between engineered γ-retroviruses and lentiviruses. First, these viruses were derived from different genomes (MoMLV and MSCV for γ-retrovirus; HIV for lentivirus). Additionally, γ-retroviruses can only transduce dividing cells because they are only able to enter the nucleus during mitotic breakdown of the nuclear envelope. Lentiviruses can transduce both dividing and non-dividing cells because once in the host cell cytoplasm, the viral components are small enough to enter the nucleus through the nuclear pore complex. This feature may be useful for the transduction of terminally differentiated (non-dividing) cells.



# γ-Retrovirus (CONT'D)

Both lentiviruses and standard γ-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, lentiviruses may not be efficiently packaged by γ-retroviral packaging systems, and vice versa. Envelope plasmids may be used interchangeably.

For more information about lentivirus, please read the subsequent sections on lentivirus.

## **Are γ-retroviruses replication incompetent?**

Yes, as with lentiviruses, the genes required for γ-retroviral packaging and transduction are not encoded by the transfer plasmid, but instead are provided in trans by other plasmids or the packaging cell line. γ-Retroviruses can transduce target cells, but the transduced target cell does not produce additional virus.

## **Are there various generations of γ-retrovirus technologies?**

No, the different “generations” associated with lentiviral transfer plasmids are described in the [next section](#).

## **Are γ-retroviruses self-inactivating (SIN)?**

They can be. Plasmids that encode SIN γ-retrovirus lack most of the U3 region of the 3' LTR and are primarily used in gene therapy research to reduce promoter interference and/or drive high expression of a transgene from an internal promoter. An internal promoter must be included as the wildtype LTR promoter is no longer present in these constructs.

## **Why do many γ-retroviral plasmids have similar names?**

The nomenclature of the plasmid backbone is meant to be descriptive of the components found within the backbone. For example: pLXSN has the wild type LTR, an MCS for cloning X gene, an SV40 promoter, and Neomycin selection.

**See the glossary on the next page for definitions of important γ-retroviruses terms**

# γ-Retrovirus (CONT'D)

## γ-Retrovirus glossary

| Plasmid Type | Element   | On Same Plasmid as Transgene? | Purpose   |
|--------------|---|-------------------------------|---|
| Envelope     | VSVG  | <i>in trans</i>               | Vesicular stomatitis virus G glycoprotein; broad tropism envelope protein; pseudotyped to alter infectivity.  |
| Packaging    | Gag   | <i>in trans</i>               | Precursor structural protein of the retroviral particle containing Matrix, Capsid, and Nucleocapsid components.   |
|              | Pol   | <i>in trans</i>               | Precursor protein containing Reverse Transcriptase and Integrase components.  |
|              | cPPT  | <i>in cis</i>                 | Central polypurine tract; recognition site for proviral DNA synthesis. Increases transduction efficiency and transgene expression.  |
|              | Psi (Ψ)   | <i>in cis</i>                 | RNA target site for packaging by Nucleocapsid.  |
|              | WPRE  | <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>Subcomponents:<br><br>-U3<br>-R<br>-U5 | <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>Subcomponents:<br/>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 plasmids.</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.   |
|              | 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.   |

# Lentiviral vector uses and overview

By Mary Gearing | May 19, 2016

Lentiviral vectors are one of the most popular and useful viral vectors in the lab. Advantages of lentivirus include a large genetic capacity and the ability to transduce both dividing and non-dividing cells. Lentiviral vectors are the vector of choice for many CRISPR applications, and they've also had success in clinical gene therapy applications. Read on to learn more about the current (and future) applications of lentiviral vectors!

## Lentiviral vector history and components

In the early 1990s, researchers developed viral vector systems based on [retroviruses](#) like Moloney murine leukemia virus (MMLV). The vectors could integrate into the genome, permitting long-term transgene expression, but they could only infect actively dividing cells. Another type of vector, based on [adenovirus](#), could infect non-dividing cells, but without sustained transgene expression. To design a viral vector system that could do both, Addgene depositor [Didier Trono](#) and collaborators turned to the lentivirus HIV-1, well-known to infect non-dividing cells.

The first lentiviral vector system consisted of three plasmids: the packaging, envelope, and transfer plasmids. The packaging plasmid carried a mutated HIV-1 provirus that couldn't package itself due to a few missing proteins. The envelope plasmid contained a viral envelope to dictate the tropism, or types of cells the vector could infect. Finally, the transfer plasmid encoded the desired transgene flanked by HIV-1 long terminal repeats (LTRs) that facilitate viral packaging and host genome integration. After co-transfection of these plasmids, 293T cells released transgene-containing lentiviral particles into the media, which could be collected for experimental use. In 1996, [Naldini et al.](#) transduced rat neurons *in vivo* with beta-gal and other reporters, and they showed that expression persisted for at least thirty days. Although adeno-associated viral vectors can also target non-dividing cells, lentiviral vectors can carry much more genetic cargo (8 kb vs <4.8 kb), and they remain popular for targeting and tracing cells in the brain (Figure 1).

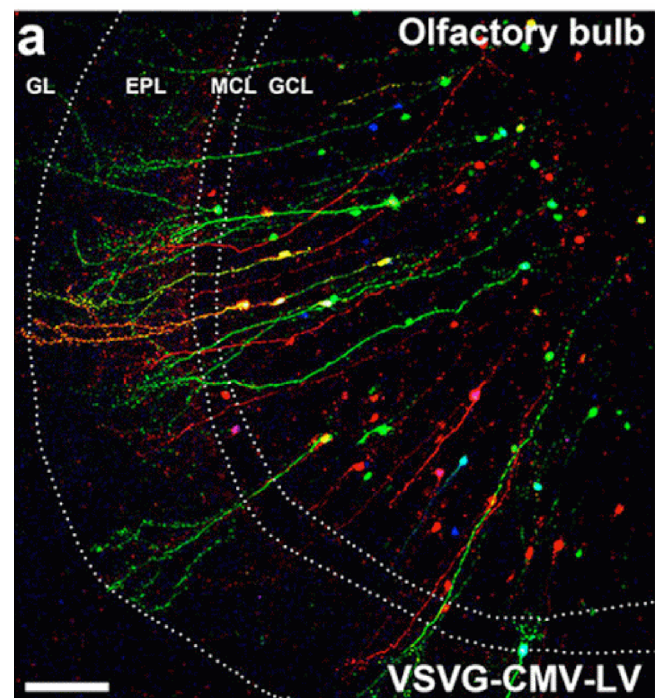


Figure 1: Lentiviral RGB vectors allow researchers to mark neurons and trace their migration. Lentiviral CMV-RGB (red, green, blue) mixed vectors were injected intraventricularly to label cells in the subventricular zone. The olfactory bulb was imaged six weeks post-injection, showing the cell's migration to and differentiation in this area of the brain. GL, glomerular layer; EPL, external plexiform layer; MCL, molecular cell layer; GCL, granular cell layer. Scale bar: 100  $\mu$ m. Image from Gomez-Nicola et al., 2014, licensed under a Creative Commons Attribution 4.0 International License.

## 2nd generation lentiviral packaging system

The graphic below shows how the lentiviral genome was condensed to create the 2nd-generation lentiviral system (Figure 2). The HIV genes that do remain are very important for viral production: Gag (structural precursor protein), Pol (polymerase), Tat (viral transactivator for transcriptional activation from the 5' LTR) and Rev (facilitates nuclear export of transcripts). A different viral envelope protein (Env), usually from VSV-G due to wide infectivity, is often used as a substitute for HIV-1 Env, which can only infect CD4<sup>+</sup> cells.

# Lentiviral vector uses and overview (CONT'D)

## 3rd generation lentiviral vector packaging systems

Although the 2nd generation system is safer than the original system, the possibility of creating a replication-competent virus via recombination between the transfer and packaging plasmids does exist. To further reduce this possibility and enhance biosafety, [Dull et al.](#) created the 3rd generation system (Figure 3), which differs from the 2nd generation in two key ways. First, the packaging system is split into two plasmids: one encoding Rev and one encoding Gag and Pol, increasing the number of recombination events necessary to create a replication-competent virus. Second, Tat is eliminated through the addition of a chimeric 5' LTR containing a Tat-independent promoter. Although safer, this system may be more cumbersome to use and lead to lower viral titers due to higher plasmid number. For more information on the key differences between the 2nd and 3rd generation packaging systems, see the [Lentiviral Plasmid FAQ](#) section of this eBook.

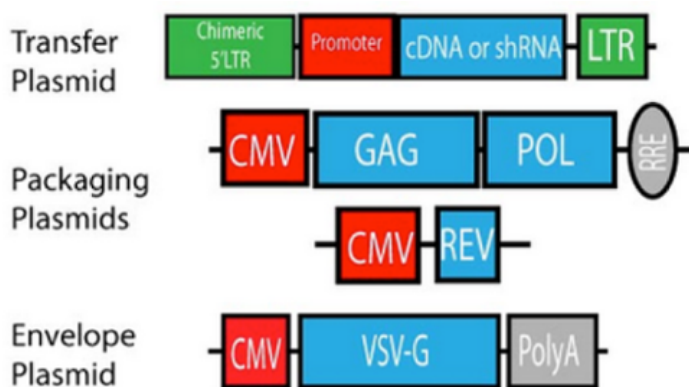


Figure 3: 3rd generation lentiviral packaging system. In this system, the chimeric 5' LTR on the transfer plasmid eliminated the need for Tat transactivation. This transfer plasmid can be packaged by either a 2nd generation or a 3rd generation packaging system.

[lentiCRISPR v2](#), used both for targeted modification and genome-wide screening.

## Lentiviral vectors in the clinic

Since lentiviral vectors can deliver a large amount of DNA (~8 kb) with a relatively low immune response, it should come as no surprise that researchers are interested in developing these vectors for gene therapy. In 2003, VIRxSYS began the first clinical trial with a lentiviral vector. CD4<sup>+</sup> T-cells from patients with HIV-1 were [transduced with a lentiviral vector](#) containing an antisense sequence against the HIV-1 envelope. As of 2013,

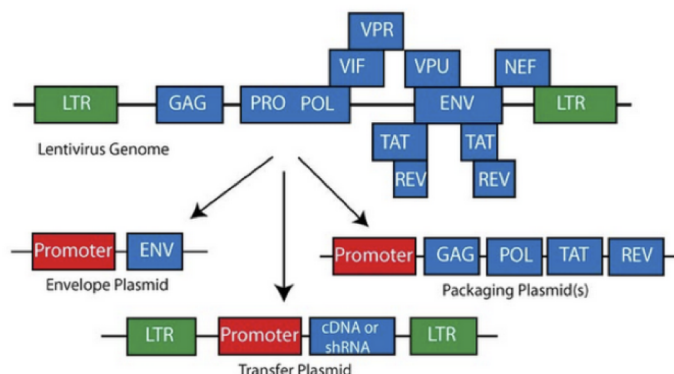


Figure 2: 2nd generation lentiviral packaging system. In this system, lentiviral genes are split across three plasmids. 2nd generation transfer plasmids require Tat transactivation so they must be used with 2nd generation packaging plasmids.

## Lentiviral vectors in the lab

Lentiviral vectors are incredibly popular in the lab - the Trono lab's lentiviral [packaging and envelope](#) plasmids are two of Addgene's most requested plasmids! Lentiviral particles with the VSV-G envelope have high infectivity in a wide range of cell types, so they're ideal for work with primary cells or other difficult-to-transfect cells. Lentiviral barcoding libraries have been used for complex applications like lineage tracing in heterogeneous tumors.

Lentiviral vectors are also commonly used to make stable transgene-expressing or knockout cell lines. Many CRISPR plasmids are designed for lentiviral use, including Addgene's top requested plasmid in 2015:

# Lentiviral vector uses and overview (CONT'D)

65 patients had been treated with this therapy, which lowered viral load and did not cause severe adverse reactions. Clinical benefits of lentiviral vectors have been observed in small trials for other diseases, including the common hematopoietic disorders [sickle cell anemia](#) and [beta-thalassemia](#). Lentiviral vectors are promising agents for [cancer immunotherapy](#), but this research is still in the pre-clinical/early clinical stages.

Researchers are also working to make lentiviral vectors safer. Since lentiviral vectors integrate into the genome, they could promote oncogenesis by altering local gene expression. [Self-inactivating \(SIN\)](#) lentiviral vectors contain a deletion in the 3' LTR that prevents aberrant activation of nearby genes. These safer vectors have become standard in gene therapy. Researchers have also developed [integrase-deficient lentiviral vectors](#) for applications that require the large genetic capacity of a lentiviral vector but for which transient transgene expression is sufficient.

Lentiviral vector technology has come a long way since the 1990s, both in terms of research impact and safety. Many of Addgene's most requested plasmids are used with lentiviral expression systems.

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# Quick guide to all things lentivirus

By Benoit Giquel | Mar 21, 2017

If you are interested in using lentiviral vectors to introduce your favourite gene into your favourite cell line or into primary cells, this section will give you some tips to plan your experiment using the lentiviral vector system.

Viral vectors have been increasingly popular in fundamental and applied research since their first use in the early 90's to genetically modify primary cells. Amongst the different vectors used, lentiviral vector constructs have proven very useful due to their ability to infect both dividing and non-dividing cells, including [stem cells](#). These properties make lentiviral vectors fantastic options for delivering [shRNA](#), [CRISPR/Cas9](#) components, and [fluorescent sensors](#).

## Retroviral vector background

Until the development of viral vectors, gene transfer into mammalian cells often relied on chemical (CaCl<sub>2</sub>, cationic liposome) or electrical (electric current forming membrane pores) [transfection](#). These techniques are still used and efficiently transfer plasmid DNA into dividing immortalized cells lines but reach their limits when it comes to transferring DNA into primary cells or non-dividing cells. To target these un-transfectable cells, labs use viral vectors and particularly retroviral vectors because they target a broader range of cells and their integration into the genome is more efficient.

The first retroviral vectors used were derived from the Moloney Murine leukemia virus (MoMLV) and consisted of two plasmids: the [packaging plasmid](#) containing all the structural genes necessary for the production of a viral particle (Gag, Pol, Tat, Rev and Env) and the transfer plasmid encoding the insert of interest as well as sequences necessary to encapsulate that insert into a viral particle (the packaging signal Psi). Because the packaging plasmids lacked the psi signal, the genes encoding the structural proteins could not be integrated into the new viral particles and the virus particles produced could not replicate. However, Mother Nature found a way to re-create a functional virus. Indeed, by testing several vectors and packaging cell line combinations, [Miller et al.](#) showed that infectious wild-type viruses were produced in high-titer retroviral stocks generated by the transfer of a retrovirus into the packaging cell line PA12 (containing the psi deletion). A double crossover event between the vector plasmid and the packaging plasmid caused the Psi sequence to be placed on the packaging plasmid forming a vector that could create replication competent virus. Even though this system was efficient at transferring the gene of interest to the appropriate cells, it was obvious that its safety was a disadvantage particularly for clinical development. In addition, MoMLV is inefficient at infecting non-dividing cells and so researchers set out to create a better, safer system.

Unlike the MoMLV, lentiviruses, a separate genus of the Retroviridae family including human immunodeficiency virus type-1 (HIV-1), can infect both dividing and non-dividing cells. The ability to infect non-dividing cells is not restricted to *in vitro* cell culture as lentivirus-derived vectors are capable of transducing certain quiescent or terminally differentiated cells such as macrophages and microglia. This property makes the lentivirus an attractive choice for a gene transfer vector.

## Lentiviral vectors

Although retroviruses are still in use, considerable efforts have been devoted to develop efficient and safe HIV-1-derived lentiviral vectors due to their ability to infect non-dividing cells. Thus three different generations of lentiviral vectors have been established so far, with safety increasing in each generation.



# Quick guide to all things lentivirus (CONT'D)

- The 1st generation of lentiviral vectors consisted of three plasmids encoding 1) the lentiviral vector genome which was composed of the wild-type 5' and 3' LTRs, the  $\psi$  sequence, a part of the env gene containing the rev response element (RRE), an internal promoter, and the desired gene (transfer vector plasmid), 2) the HIV-1 genome containing all viral genes with the exception of the env gene (packaging plasmid), and 3) the vesicular stomatitis virus G protein (VSV-G) that improves the stability and broadens the cellular tropism of the viral particles produced. However, in this vector production system, there is still a potential risk for the generation of replication competent lentiviruses (RCL) especially if you are working on HIV positive human cells from the clinic.
- The 2nd generation is similar to the first one with the exception that the HIV accessory proteins that are not essential for the production of the lentiviral particle have been removed. This generation is safer than the first one and can be used routinely in a research laboratory. The risk of generating RCL is low but extra care has to be taken especially if you are working with proto-oncogenes or with human samples that have not been tested for HIV. If not using proper technique, there's always a small chance that a researcher could infect him or herself with the oncogenic virus, or that the accessory factors in HIV positive cells could make the virus replication competent.

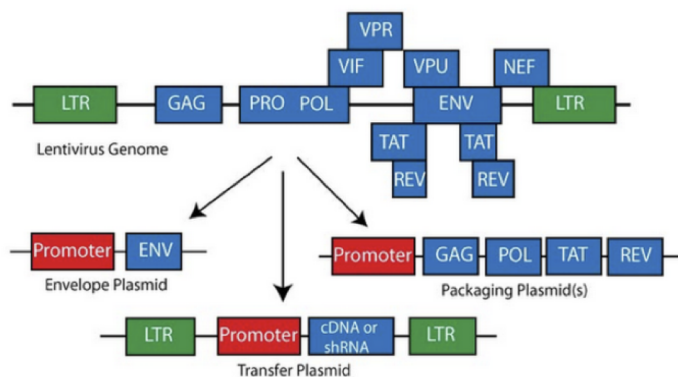


Figure 1: 2nd generation lentiviral packaging system. In this system, lentiviral genes are split across three plasmids. 2nd generation transfer plasmids require Tat transactivation so they must be used with 2nd generation packaging plasmids.

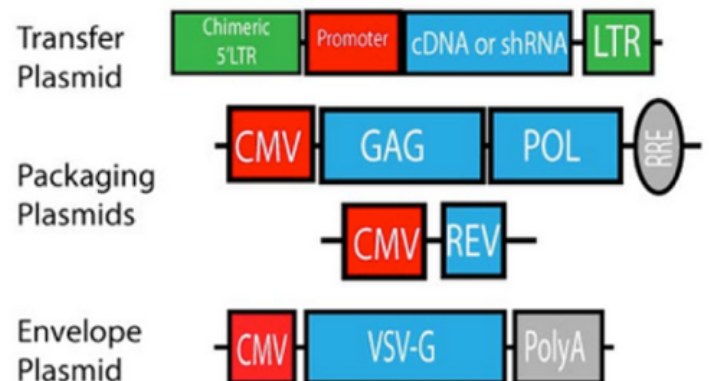


Figure 2: 3rd generation lentiviral packaging system. In this system, the chimeric 5' LTR on the transfer plasmid eliminated the need for Tat transactivation. This transfer plasmid can be packaged by either a 2nd generation or a 3rd generation packaging system.

- The 3rd generation of lentiviral vectors was developed for safe use in a clinical setting. In this generation, the HIV Tat gene (previously used to drive expression from the LTRs) has been removed, Rev (which facilitates nuclear export) is expressed from a separate plasmid, and the promoter of the 5'LTR has been deleted to reduce its activity. A CMV or an EF1 $\alpha$  promoter has been inserted in this LTR so you don't need Tat to transcribe the viral genome in producing cells. Currently, the third-generation lentiviral vector system offers the best safety profile in terms of RCL generation because this vector requires only three HIV-1 genes (gag, pol, and rev) for production. Third generation lentiviral vectors can be used for research and for clinical purposes, however improving the safety of these vectors is still an active area of research due to the possibility that mutation or recombination with human retroviruses could lead to RCL

Regarding transduction efficacy, no differences between the 2nd and the 3rd generation of vectors have been shown. However, with the 3rd generation you get lower viral titer than with the 2nd generation. Knowing this you can scale up your production set-up and purify and concentrate your lentiviral particles to get them to the optimum concentration to infect your cells of interest.

# Quick guide to all things lentivirus (CONT'D)

| Advantages   | Disadvantages  |
|--|--|
| Can carry large transgenes (>8 kb)   | Potential for generation of replication-competent lentivirus (RCL)   |
| Efficient gene transfer  | Potential for insertional mutagenesis: Replication-incompetent lentiviruses with human tropism can still infect the researcher and insert into her/his genome - this is a biosafety risk |
| Infect dividing and non-dividing cells                                     |  |
| No immunogenic proteins generated  |  |
| Stable integration into host genome and stable expression of the transgene |  |

Table 1: Advantages and disadvantages of lentiviral vectors.

## Transfer plasmids

The [transfer plasmid](#) is the vector you clone your gene of interest into and is an important feature for your experiment; it must be chosen carefully. Often researchers want a simple set-up where the gene of interest is expressed together with a marker ([fluorescent protein](#) or [selection marker](#)) allowing for selection of the transduced cells. In this case your gene and the marker can be separated by an internal ribosome entry site (IRES) and expressed under the same promoter. Alternatively, your gene and the marker can be placed under the control of two different promoters. Both the single and dual promoter transfer vectors can be used to express your gene of interest either constitutively or inducibly. If your gene of interest is toxic or needs to be expressed at a certain point in the cell cycle, a transfer vector with an [inducible promoter](#) (see below) should be used.

## Inducible lentiviral transfer vectors

The most widely used inducible lentiviral vector system is the tetracycline (Tet)-regulated system. You can choose either the Tet-off system or the Tet-on system. In the Tet-off system a Tet-response element (TRE) is placed upstream of the promoter in your transfer vector. In the absence of tetracycline or a derivative such as Doxycycline (Dox), the Tet-controlled transactivator (tTA, also expressed by your transfer vector) binds to the TRE and activates the expression of your gene. The addition of Dox in the culture will then repress your gene of interest. This system is efficient but you have to be aware that there is often background activity and it requires continuous administration of Dox to repress transgene expression. In contrast, in the Tet-on system, tTA has been modified (and renamed rtTA) to bind TRE only in presence of Dox. In this case, your gene is repressed in steady state and is expressed when Dox is added to the culture. This system is now used as it shows rapid gene expression kinetics as compared to the Tet-off system. One drawback of the Tet system is that it often requires the delivery of two distinct expression vectors to target cells - one containing the gene of interest ([pTet-IRES-EGFP](#), [pPRIME-Tet-GFP-FF3](#)) and one with either tTA or rtTA ([FUW-M2rtTA](#)). In this kind of approach, a population of cells might express only the tTA or your transgene resulting in low inducibility and potentially making it difficult to interpret your results. This can be a bottleneck for your experiment. Single vector systems containing both your transgene and tTA/rtTA ([pINDUCER20](#), [pINDUCER21](#)) have been developed, but these vectors are quite large and you might get lower viral titer when using them.

# Quick guide to all things lentivirus (CONT'D)

Regarding biosafety, it is also important to be aware that lentiviral particles are a powerful way to transduce cells - even your own! You should therefore be cautious about inserting potentially harmful genes into a transfer vector. Lentiviral particles expressing proto-oncogenes or oncogenes for instance should be manipulated with care. See the NIH's biosafety guidelines for more information and always be sure to consult your institution's Biosafety Committee before beginning any work.

## Lentiviral production and transduction

Once you have cloned your gene of interest into a lentiviral transfer vector, the next step is to produce the viral particles themselves. For this you will first need to transfect producing cells, usually 293T cells, with your transfer plasmid and your packaging plasmids. For this, you can use the transfection reagent you are most comfortable with. If you don't have any experience in transfection, [CaCl<sub>2</sub> transfection](#) is a simple and cheap technique that should work well.

Quickly after transfection (4-8 h), cells start releasing viral particles into the culture supernatant and you can generally harvest the supernatant containing viruses 24 h after transfection. This supernatant can be stored at 4 °C or can be directly added to your target cells. However in that case you don't know how many viral particles you put in your culture, as viral production can change from one batch to another. For experimental consistency it's a good idea to purify and titer your virus. Virus purification can be a tricky process. You have to centrifuge the culture supernatant in order to precipitate the viral particles. Some protocols suggest adding sucrose to the supernatant to create a gradient of density that favours virus precipitation. My tip for this process is to test several protocols that have been described ([protocol 1](#), [protocol 2](#), [protocol 3](#)) to find the one that will give you the best production for your system.

Once purified, you must titrate your viral particles to determine their concentration. Three methods are broadly used for virus titration: the p24 ELISA dosing method detects the expression of viral capsid antigen p24, the FACS dosing method correlates viral titre to the number of cells transduced by a GFP encoded virus, and the reverse transcriptase activity assay uses qPCR to quantify viral RNA. All of these methods are described in the "[Tips for titering your lentiviral preps](#)" section of this eBook.

Once you know your viral titre, you can incubate your cells with virus at different multiplicity of infection (MOI=ratio of virus to cells). Again, my tip here is to test several protocols as the set-up depends on the type of cell you would like to infect. Most of the protocols you will find will recommend using a polycation, like polybrene ([protocol](#)) or DEAE dextran ([protocol](#)), which help the viral particle bind to your target cells. If you are transducing non-adherent cells you might need to centrifuge your cells with the virus in order to aid viral binding. This method, called spinoculation, has been found very effective for [T cell transduction](#) for instance.

I hope you will find this information useful for your experiments. Don't hesitate to check our [lentiviral vector guide](#) to get more information and to get access to additional protocols and plasmids.

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# Quick guide to all things lentivirus (CONT'D)

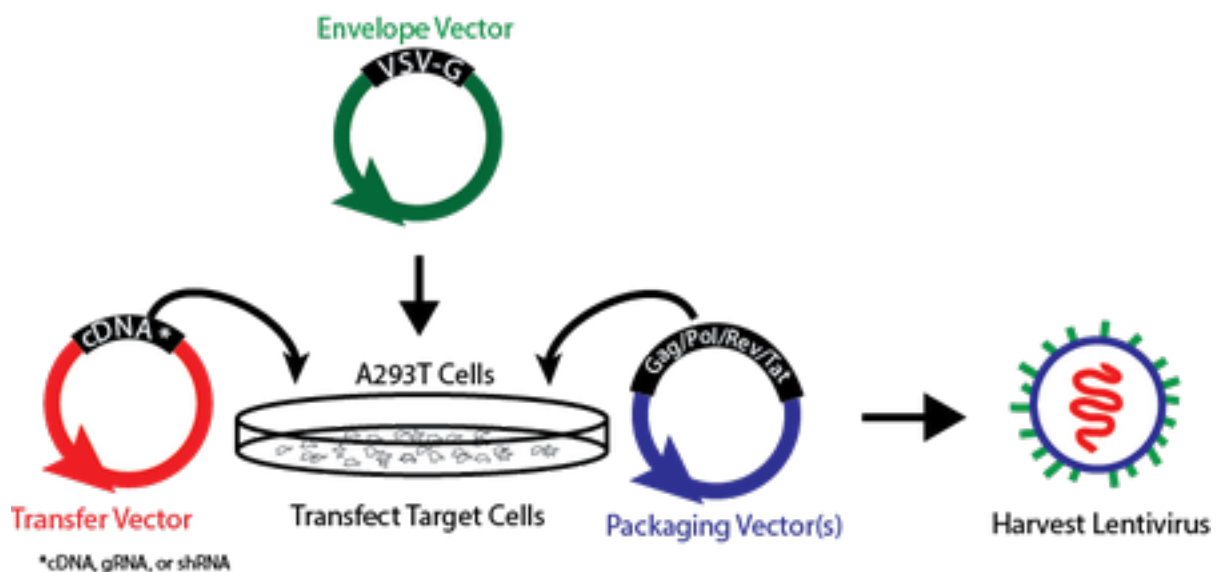
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# Your lentiviral plasmid FAQs answered

By Kendall Morgan | April 23, 2014

Lentiviruses are useful and efficient tools to introduce your gene of interest into cells. Unlike gamma-retroviruses that can only infect dividing cells, lentiviruses can infect dividing and non-dividing cells.

Addgene has an [extensive collection of lentiviral plasmids](#) created for a variety of applications including cDNA expression, shRNA-mediated knockdown, Tet and Cre-regulated expression, [CRISPR genome editing](#), and more. Not surprisingly, we receive many questions from scientists all over the world looking for some additional information or clarification on these vectors. Read on to find the answers to our most frequently asked lentiviral questions.



[This useful resource](#) from Addgene Depositor (and lentiviral expert) Didier Trono's lab webpage explains how lentiviral particles are produced by co-transfecting three essential ingredients: the lentiviral packaging vector, a transfer vector including the gene of interest, and the plasmid coding for an envelope. Three generations of lentiviral packaging systems have been developed over the years based on HIV-1; however, the 1st-generation vectors are never used as they pose too much of a biosafety risk to scientists. Second-generation vectors are likely sufficient for most experiments; however, the 3rd-generation packaging systems offer the maximal biosafety available for the technology. The minor limitation in using the 3rd-generation system is that it involves transfecting four different plasmids into the producer cells (two packaging plasmids, an envelope plasmid, and the lentiviral transfer vector), rather than the three used for 2nd-generation systems.

## Frequently asked questions

Now let's get into some of the nitty gritty and answer the questions those of us at Addgene get asked most often:

**Q1:** Are retroviruses and lentiviruses the same thing? Can I package lentivirus with retroviral packaging plasmids (and vice versa)?

**A1:** Lentiviruses are part of the retroviral family. What scientists often refer to as "retroviruses" are technically

# Your lentiviral plasmid FAQs answered (CONT'D)

[γ-retroviruses](#) - another, separate member of the retroviral family. While both lentiviruses and gamma-retroviruses use the same genes for packaging (that's gag, pol, and env), the isoforms of these proteins, as well as the viral long terminal repeats (LTRs) are different. As a result, lentiviral and retroviral packaging vectors are not interchangeable. While these differences may seem subtle they result in a key physiological difference between lenti- and retroviruses. Lentiviruses are able to enter an intact nuclear membrane allowing them to infect both dividing and non-dividing cells while retroviruses can only infect dividing cells. When choosing the appropriate gene delivery system, the specific target cell or tissue needs to be considered.

Q2: Can I use lentiviral transfer vectors for transient transfections?

A2: Technically, yes. Will it actually work? That depends. The majority of transfer plasmids use the weak viral LTR promoter to drive expression of the gene of interest. Consequently, the levels of protein expression tends to be much lower than those seen when using a plasmid backbone that was designed specifically for transient expression (find some useful backbones [here](#)) or from viral transduction. While not ideal, transient expression of viral constructs is a useful tool as it provides a means to quickly check that a construct is functional before investing time and resources into making a stable cell line. When expressing a viral construct transiently care needs to be taken to ensure that the proper cell line is used; several common laboratory cell lines including 293 were immortalized with adenoviral protein E1A, which has been shown to repress expression of HIV-1 LTRs (more information is available [here](#)).

## 2nd vs 3rd generation lentiviral plasmids

Q3: How can you tell 2nd- and 3rd-generation transfer vectors apart?

A3: Addgene defines 2nd- or 3rd-generation transfer vectors based on whether or not they have a chimeric 5'LTR. Second-generation transfer plasmids have a wildtype 5'LTR, which requires the presence of the Tat protein to work, while 3rd-generation transfer plasmids have a chimeric 5'LTR that includes a CMV or RSV promoter as well as a portion of the 5'LTR. Including a chimeric 5'LTR removes the requirement for the HIV Tat protein, thus decreasing the probability of creating replication-competent lentivirus (RCL) in your target cells.

Q4: How do the packaging systems differ between 2nd- and 3rd-generation vectors? What does this mean for transfer vectors?

A4: The [lentiviral packaging systems](#) differ in two ways. Firstly, 2nd generation systems consist of an envelope plasmid and a packaging plasmid encoding HIV proteins gag, pol, rev, and tat. 3rd generation systems require an envelope plasmid, and two packaging plasmids, one encoding gag and pol and a second encoding rev. Secondly, 3rd generation packaging systems have modified the 5' LTR of the transfer plasmid to eliminate the need for the HIV Tat protein and consequently are considered safer.

Second-generation transfer plasmids MUST be packaged with a 2nd-generation system because the wildtype 5'LTR promoter requires Tat to function. Third-generation plasmids can be packaged with either system. Please note that the generation used to package the virus does not change the generation of the transfer vector.

Q5: What envelope glycoprotein will be expressed on virus particles?

# Your lentiviral plasmid FAQs answered (CONT'D)

A5: This depends on the envelope plasmid you use as the choice of envelope dictates the tropism of the virus. VSV-G is very common due to its broad host range, and increased particle stability; however, the protein is cytotoxic precluding the long-term expression of lentiviral vectors in producer cell lines. Identifying less toxic envelope proteins or envelope proteins that target specific tissues are areas of active study (for a review click [here](#)). Alternative envelopes are powerful tools as they allow researchers to target transduction to a particular cell type or tissues; this is useful in gene therapy applications where a distinct subset of the cellular population needs to be targeted.

Q6: Are transfer plasmids replication competent? What is SIN?

A6: Most (if not all) Addgene transfer plasmids are replication-deficient meaning they can be used to create virus that is capable of infecting target cells, but cannot produce any new viral particles after the initial infection.

SIN is shorthand for self-inactivating, which is achieved by deleting a large portion of the 3'LTR in the transfer plasmid. The deletion prevents full-length viral RNA from being produced in target cells and minimizes the risk of generating RCL. In addition the deletion reduces the risk of insertional mutagenesis by limiting the interaction between viral LTR enhancers and the transgene; such interactions can alter expression of not only the transgene but also that of adjacent cellular genes.

Q7: Which genes are deleted or modified to result in replication deficiency? What percent of the vector components are HIV-based?

A7: No single plasmid contains all the components necessary to produce viral particles. The components are divided as follows:

- Transfer vectors contain minimal cis-acting HIV components: LTRs, PPT, RRE, and  $\Psi$  packaging signal. Viral components typically total <1.5kb, which is rarely more than 30% of the transfer plasmid. Many transfer vectors are SIN.
- Packaging plasmid(s) contain the minimal number of HIV genes required for virus production (3 or 4). Third-generation vectors contain gag, pol, and rev. Second-generation contains those three genes plus tat.
- The envelope plasmid provides a heterologous envelope for pseudotyping and is not HIV-derived.

## Considering safety of lentiviral transduction

Q8: You mentioned biosafety. What are the major risks when it comes to lentiviruses in terms of biosafety?

A8: According to the American Biological Safety Association, “the two major risks of lentiviral vectors are: 1) the potential generation of replication competent virus [usually HIV-1]; and 2) the potential for oncogenesis through insertional mutagenesis. These risks are largely based upon the vector system used and the transgene insert encoded by the vector.”



## Your lentiviral plasmid FAQs answered (CONT'D)

The table below breaks down the biosafety concerns associated with various aspects of lentiviral transduction along with suggestions on how to lower the risk.

| Biosafety Concern | High Risk   | Lower Risk   |
|-------------------|---|--|
| Vectors           | Two plasmids (1st gen) used to produce virus; expression of viral genes | Three (2nd gen) or four (3rd gen) plasmids used to produce virus; removal of viral genes |
| Insert/transgene  | Oncogenic, apoptotic, or toxic  | Non-oncogenic, non-apoptotic, non-toxic  |
| Envelope/host     | Permissive/ amphotrophic  | Ecotropic/ non-permissive  |
| Propagation       | Large scale   | Lab scale (less than 100 mL)   |

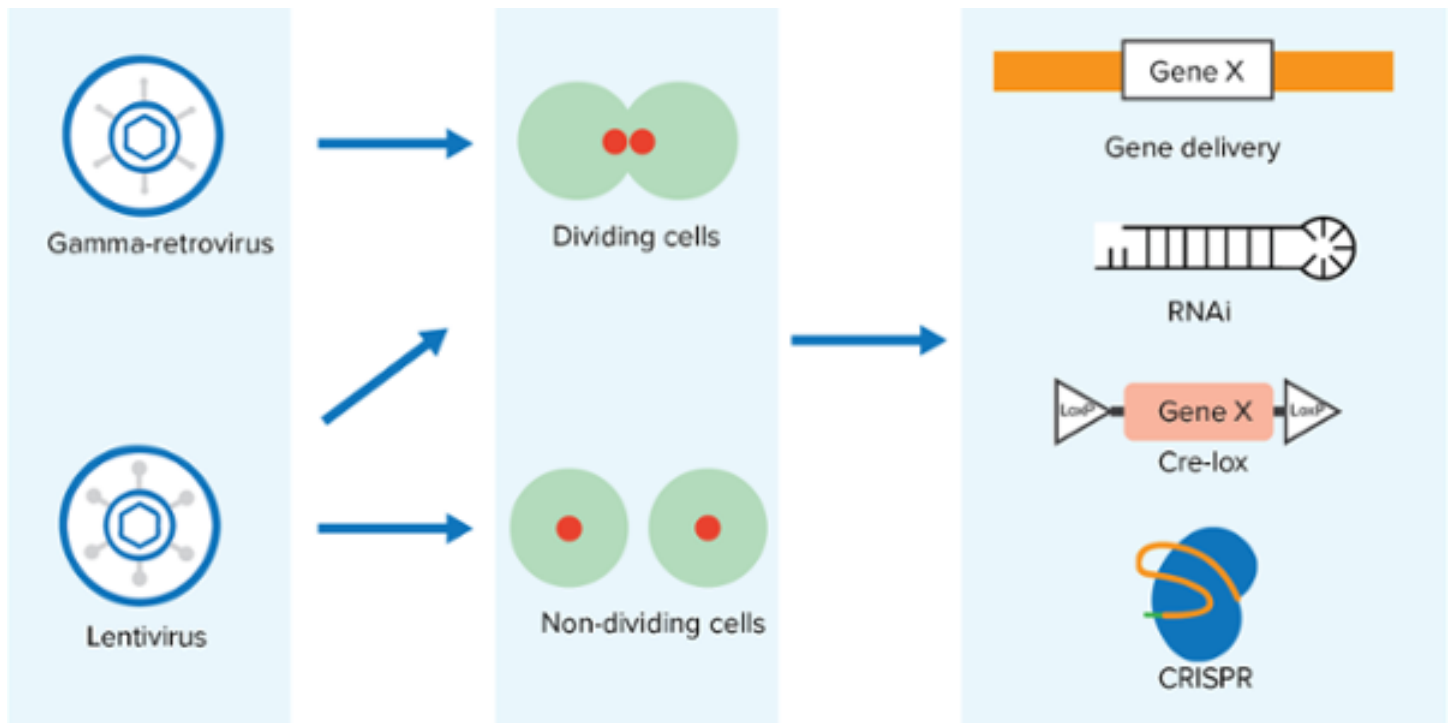
Biosafety should always be considered with respect to the specific experiments being performed and you should understand the guidelines for using such reagents as outlined by your institution or country.

Have any other questions? Feel free to email [help@addgene.org](mailto:help@addgene.org) and we'll be happy to answer them as best we can!

*Note: A. Max Juchheim, Marcy Patrick, and Meghan Rego contributed to the writing of this article.*

# Popular retroviral vectors and their uses

By Susanna Bachle | June 29, 2018



Viruses are intracellular parasites and natural vehicles for genetic information. Therefore they make excellent tools for genetic engineering. There are several different viral vectors to choose from for example gamma-retrovirus, lentivirus, Adenovirus and Adeno-associated virus (AAV). If you are wondering which virus fits your experiments best, have a look at this [viral vector overview](#).

Viruses of the Retroviridae or Retrovirus family, which includes the gamma-retrovirus and lentivirus genera, have the unique ability to integrate permanently into the host genome and thereby enable long-term stable gene expression. Gamma-retroviral vectors are derived from the Moloney Murine Leukemia Virus (MoMLV, MMLV, MuLV, or MLV) or Murine Stem Cell Virus (MSCV) genomes whereas lentiviral vectors are derived from the human immunodeficiency virus (HIV) genome.

For research purposes, retroviral vectors have been developed into 'split-vector systems' by separating viral genes and transgene expression across several plasmids. The most commonly used viral vector systems are made up of separate envelope and packaging plasmids as well as transfer plasmids. This concept increases safety when these vectors are used in experiments.

Retroviral vectors make up a large toolbox used by researchers for (among other things) gene delivery, generating pluripotent stem cells, and developing gene therapies for monogenic diseases. Additionally, retroviral vectors can regulate gene expression by delivering shRNA or miRNA. The ever expanding CRISPR field makes wide use of retroviral delivery systems, especially lentiviral vector-based [CRISPR pooled libraries](#) that allow functional screening of thousands of genes in genome-wide experiments.

# Popular retroviral vectors and their uses (CONT'D)

## **γ-Retroviral vs lentiviral vector systems**

Gamma-retroviral vectors have a relatively high transduction efficiency, but in contrast to lentiviral vectors, their infectivity is limited to dividing cells. Find tips on using gamma-retroviral vectors in the previous section on [γ-retrovirus](#) and find popular packaging and gamma-retroviral envelope plasmids [here](#).

Lentiviral vectors are very versatile tools as they are able to transduce dividing and non-dividing cells. More detailed descriptions of lentivirus elements and the 2nd and 3rd generation lentiviral systems can be found in [previous section of this eBook](#). Popular packaging and lentiviral envelope plasmids are listed [here](#). Please note that both lentiviruses and gamma-retroviruses use the same gene products for packaging, however, the isoforms of these proteins, as well as the viral LTRs differ. Therefore, lentiviral and gamma-retroviral packaging plasmids are not interchangeable. General envelope plasmids, such as VSV-G, may be used across both systems.

Below I provide a brief overview of a few popular gamma-retroviral and lentiviral vectors that are used for gene regulation, gene of interest expression, knockout and screening. There are many more useful viral vectors available at Addgene: Follow our [viral vector blog posts](#) for updates on the most recent deposits, protocols, and tips for using retroviral vectors in your experiments.



### **γ-Retroviral Vectors**

Popular γ-retroviral vectors can be found [here](#).

#### **Expressing your gene of interest**

[pBABE-puro \(Plasmid #1764\)](#)

Retroviral vector for cloning and expressing your gene of interest with puromycin selection. One of the scientists who created pBabe writes how its name came about [here](#).

#### **Cloning and expressing RNAi**

[pMKO.1 puro \(Plasmid #8452\)](#)

RNA interference (RNAi) is an RNA-mediated gene silencing mechanism. Scientists can express siRNAs to direct the cellular RNAi machinery to silence target genes. This empty MuLV vector from the [Weinberg lab](#) enables researchers to express siRNAs of their choice in mammalian cells. Learn more about [Mammalian shRNA Tools](#) for RNAi!

#### **Cre-Lox technology**

[CAG-GFP-IRES-CRE \(Plasmid #48201\)](#)

# Popular retroviral vectors and their uses (CONT'D)

This retroviral vector from the [Gage lab](#) encodes GFP and Cre-recombinase. The [Cre-lox system](#) is a technology that can be used to induce site-specific recombination events and is widely used in the field of mouse transgenics. Experimental uses include: Cre-dependent gene expression, gene knockout, and selection marker removal.

## CRISPR technology

[pdCas9-humanized \(Plasmid #44246\)](#)

Expression of a catalytically inactive (dead), human codon-optimized Cas9 under the control of Murine Stem Cell retrovirus promoter for mammalian gene knockdown. Vector provided by the [Qi lab](#). Learn more about [CRISPR plasmids for transcriptional inhibition](#).

## Retroviral Plasmid Kit

[Retroviral Barcoding Library \(Kit # 1000000097\)](#)

The [Winslow lab](#) barcoding library consists of 96 retroviral plasmids (based on the Murine Stem Cell Virus, MSCV) each with a unique 6 nucleotide barcode. This collection can be used for *in vivo* phenotypic screening of small-molecule libraries and can be combined with high-throughput sequencing to perform multiplexed analysis of cells pre-treated with a compound of interest.



## Lentiviral Vectors

[Popular lentiviral vectors can be found here.](#)

## Expressing your gene of interest

[pLenti-puro \(Plasmid #39481\)](#)

You can clone your gene of interest into this tetracycline inducible, 3rd generation lentiviral vector backbone from the [Shih lab](#). You can find more lentiviral expression vectors (empty backbones) [here](#).

## Cloning and expressing new shRNA sequences

[pLKO \(Plasmid #10878\)](#)

This plasmid has been used by [The RNAi Consortium](#), also known as the [Genetic Perturbation Platform](#), to produce their shRNA library to support functional investigations of the mammalian genome that can reveal how genetic alterations lead to changes in phenotype. The plasmid comes with a 1.9 kb stuffer sequence that can be removed and replaced with an shRNA sequence of your choice. More information and a detailed protocol on how to use the pLKO.1 vector can be found [here](#).

# Popular retroviral vectors and their uses (CONT'D)

## Cre-Lox technology

### [Puro.Cre empty vector \(Plasmid #17408\)](#)

This is a lentiviral vector deposited by [Dr. Tyler Jacks](#), it carries CRE recombinase and a puromycin resistance cassette.

No time to produce virus? Get the ready-to-use [lentiviral prep](#) from the Addgene viral service.

## CRISPR technology

### [lentiCRISPR v2 \(Plasmid #52961\)](#)

This is a 3rd generation lentiviral backbone from the [Zhang lab](#) expressing *S. pyogenes* Cas9 and a gRNA cassette. It can be used to produce a ~10-fold higher titer of lentiviral particles than the original [lentiCRISPR v1](#).

Special note from the Zhang lab: We are constantly improving our CRISPR reagents. Please check [www.genome-engineering.org](#) for the most up-to-date information.

Check our [CRISPR guide](#) and [blog](#) to learn more about CRISPR and the most recent developments in the genome editing field.

## Lentiviral Plasmid Kit

### [Multiple Lentiviral Expression System Kit \(Kit #1000000060\)](#)

The MuLE (Multiple Lentiviral Expression) system facilitates the simultaneous introduction of multiple genetic alterations into mammalian cells. The MuLE system kit from the [Frew lab](#) consists of 91 building block Entry vectors and 5 lentiviral Destination vectors. These vectors can induce combinatorial constitutive or inducible gene overexpression, knockdown (shRNA, miR-30-shRNA), mutation/editing (CRISPR-Cas9) or deletion (Cre), together with expression of fluorescent or enzymatic reporters for cellular assays and animal imaging studies. The ability to simultaneously alter multiple genes via infection with a single ecotropic or amphotropic MuLE lentivirus provides significant genetic power and allows high throughput genetic modulation studies in mammalian cells and tissues.

This modular and flexible system can be used to construct complex lentiviruses for modification of mammalian cells with a single viral infection.

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# Popular retroviral vectors and their uses (CONT'D)

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# Genome-wide screening using CRISPR

By Joel McDade | August 18, 2015

What genes are important in your phenotype of interest? Many scientists study diseases for which the underlying genetic cause is not entirely known. Identifying which genes are important for a phenotype can lead to a wealth of additional experiments investigating the role of individual genes or entire pathways in a particular disease process. While CRISPR is certainly not the first means to carry out so-called “forward genetic screening experiments”, it is certainly the most robust. In this blog post, we will discuss how [CRISPR libraries](#) are being used to perform genome-wide screens and highlight some of the reagents that have been made publicly available through Addgene.

## What makes CRISPR so special?

A major advantage of CRISPR/Cas9 over previous genome editing techniques is its simplicity and versatility. CRISPR/Cas9 consists of two components: the non-specific endonuclease (Cas9) and a single stranded guide RNA (gRNA). The ~20 nucleotide targeting sequence within the gRNA is defined by the user, and it can be easily modified to target Cas9 to virtually any genomic locus, provided the target is unique compared to the rest of the genome and located immediately 5' to a protospacer adjacent motif (PAM) sequence. Co-delivery of wild-type Cas9 and a gRNA generates a double-strand break in the target DNA, which, when repaired through error-prone [non-homologous end joining \(NHEJ\)](#), usually results in a loss-of-function mutation within the target gene. CRISPR can also be used to [activate](#) or [repress](#) target genes without permanently modifying the genome. If you'd like to brush up on the various CRISPR technologies, check out Addgene's [CRISPR Guide](#).

## What CRISPR-based reagents are available for genome-wide screens?

The goal of a genome-wide screening experiment is to generate and screen a population of mutant cells to identify genes involved in a particular phenotype. CRISPR can be readily scaled up for genome-wide screening due to the broad range of potential target sequences and ease of generating gRNA-containing plasmids. CRISPR genome wide-screening experiments commonly use lentivirus to deliver a pooled population of gRNAs to Cas9 expressing cells. Pooled lentiviral CRISPR libraries (referred to simply as “CRISPR libraries”) consist of a heterogeneous population of gRNA-containing lentiviral transfer vectors, each targeting a specific gene within the genome (see Fig. 2). Individual gRNAs are designed *in silico* using publicly available gRNA design software and synthesized. Pooled gRNAs are then cloned into a lentiviral transfer vector to create the CRISPR library.



Figure 1: Like this jar of jellybeans, a pooled CRISPR library is a complex mixture. Pooled libraries are composed of many different gRNA-containing plasmids, each with a different genomic target. The goal of a CRISPR screen is to use Cas9 and a pool of gRNAs to identify genes that are essential for a given phenotype. Image Credit: <https://www.flickr.com/photos/72005145@N00/5600978712/>.

CRISPR libraries have been created to knock out, activate or repress target genes by combining a gRNA library with the aforementioned derivatives of Cas9. Several CRISPR libraries are publicly available through Addgene, with more being added all the time. Keep in mind that libraries are only as good as the experiments you use them for! A well-developed biological question and experimental system are absolutely necessary to ensure that



# Genome-wide screening using CRISPR (CONT'D)

you select the correct CRISPR library.

## Choosing the library that is right for you

There are several factors to consider when selecting a CRISPR library for your experiments.

- 1) What species are your cells derived from? Currently, Addgene carries CRISPR libraries that target mouse, human, fly, and *T. gondii* genes.
- 2) What genetic modification are you trying to make? Addgene carries CRISPR libraries for gene knockout, activation, repression, and barcoding.
- 3) Are you trying to target every gene in the genome, or a specific class of genes? Addgene currently carries several genome-wide CRISPR libraries and a selection of sub-libraries targeting specific classes of human genes.

## What are the steps involved in a CRISPR screen?

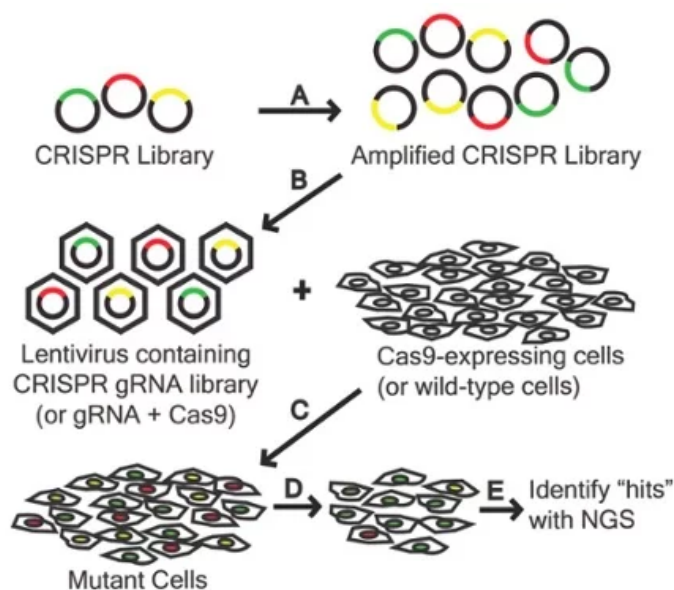


Figure 2: The CRISPR library must be amplified (A) to be used to generate lentivirus (B). Cas9-expressing cells or wild-type cells are treated with lentivirus containing the gRNA library or gRNA library plus Cas9 to generate mutant cells, respectively (C). Mutant cells are screened (D) and “hits” are identified using next-generation sequencing (E).

a particular phenotype. For example, mutant cells can be used in drug screens to identify genes that confer drug-resistance. Here, mutant cells are treated with a drug of interest and gRNA distribution is analyzed in the drug-resistant population compared to a non-treated control group. In this scenario, gRNAs that are “enriched” correspond to genes that confer drug resistance when mutated. Findings from this type of experiment can shed light on the mechanism by which cells gain resistance to drugs and can identify future therapeutic targets for diseases causing uncontrolled cell growth, such as cancer.

Performing a forward genetic screen using CRISPR libraries is a multi-step process (see Fig. 2). In most cases, CRISPR libraries are provided at a concentration that is too low for experimental use. Thus, the first step is to amplify your library to a concentration that is sufficient to generate lentivirus. Be sure to use next-generation sequencing to check the quality of your amplification. If you’ve obtained a [ready-to-use lentiviral preparation](#) from Addgene, you can skip the steps above!

Cells are then transduced with lentivirus containing the CRISPR library to generate a heterogeneous population of mutant cells, with each cell or set of cells containing a mutation in a different gene. Libraries may be available in a 1-plasmid system, in which Cas9 is included on the gRNA-containing plasmid, or a 2-plasmid system in which Cas9 must be delivered separately.

Mutant cells are enriched using either drug selection or fluorescence-based cell sorting and screened for

# Genome-wide screening using CRISPR (CONT'D)

## Considerations and tips for successful screens

**Next-generation sequencing** - CRISPR libraries contain thousands of gRNA plasmids, discerned only by a unique barcode on each plasmid. As such, sequencing CRISPR libraries after amplification and after a screen requires the use of [next-generation sequencing](#).

**Representation** - Most libraries contain 3-6 gRNAs per target gene, and maintaining the distribution of each gRNA within the population is key. Loss of representation due to enrichment or depletion of specific gRNAs can lead to skewed results.

**Selecting a cell type** - Theoretically, any cell type can be used in a CRISPR screen. However, maintaining sufficient representation within your mutant population requires a massive amount of cells as starting material. Therefore, cell types that are of low abundance are not particularly well-suited for genome-wide screening.

**Avoid false positives and false negatives** – As with any experiment, the use of appropriate controls, multiple replicates and several cell types can strengthen your results. Enrichment or depletion of multiple gRNAs targeting the same gene can be strong evidence that a particular gene is actually important for a given phenotype. Each hit from the screen should be independently validated to ensure that the desired modification produces the phenotype you screened for in the first place.

With the proper experimental design and validation practices, CRISPR libraries can help you learn a lot about your phenotype of interest. To learn a bit about how CRISPR/Cas9 can be used in other types of experiments, check out our [CRISPR Plasmids and Resources Page](#).

# New tool for lineage tracing: the ClonTracer library

By Tyler Ford | September 22, 2015

*This article is based on an interview with Novartis researcher, Carrie Bhang.*

The [ClonTracer Library](#), deposited by Carrie Bhang, a research investigator in the In Vivo Pharmacology group at [Novartis](#) Oncology, is an exciting new tool that allows researchers to individually label millions of mammalian cells through [lentiviral infection](#) and to monitor their abundance and clonal dynamics over time using next generation sequencing (NGS). The library was developed when Carrie was a post-doc in [Frank Stegmeier's lab](#) in Novartis Oncology.

Bhang did her PhD work at [Johns Hopkins](#) and came to Novartis for her post-doc in January 2012. She worked for three years as a postdoc in the Target ID and Validation Group and says that she decided to do a [post-doc at Novartis](#) because she wanted first-hand experience in industry research. When she came to Novartis, she was happily surprised that “Novartis was actually really research oriented [and] somewhat similar to an academic research environment” with a strong focus on publishing, but still made it so she could “get exposure to the drug discovery process.”



*Some of the many flasks Bhang and coworkers used when testing the ClonTracer system*

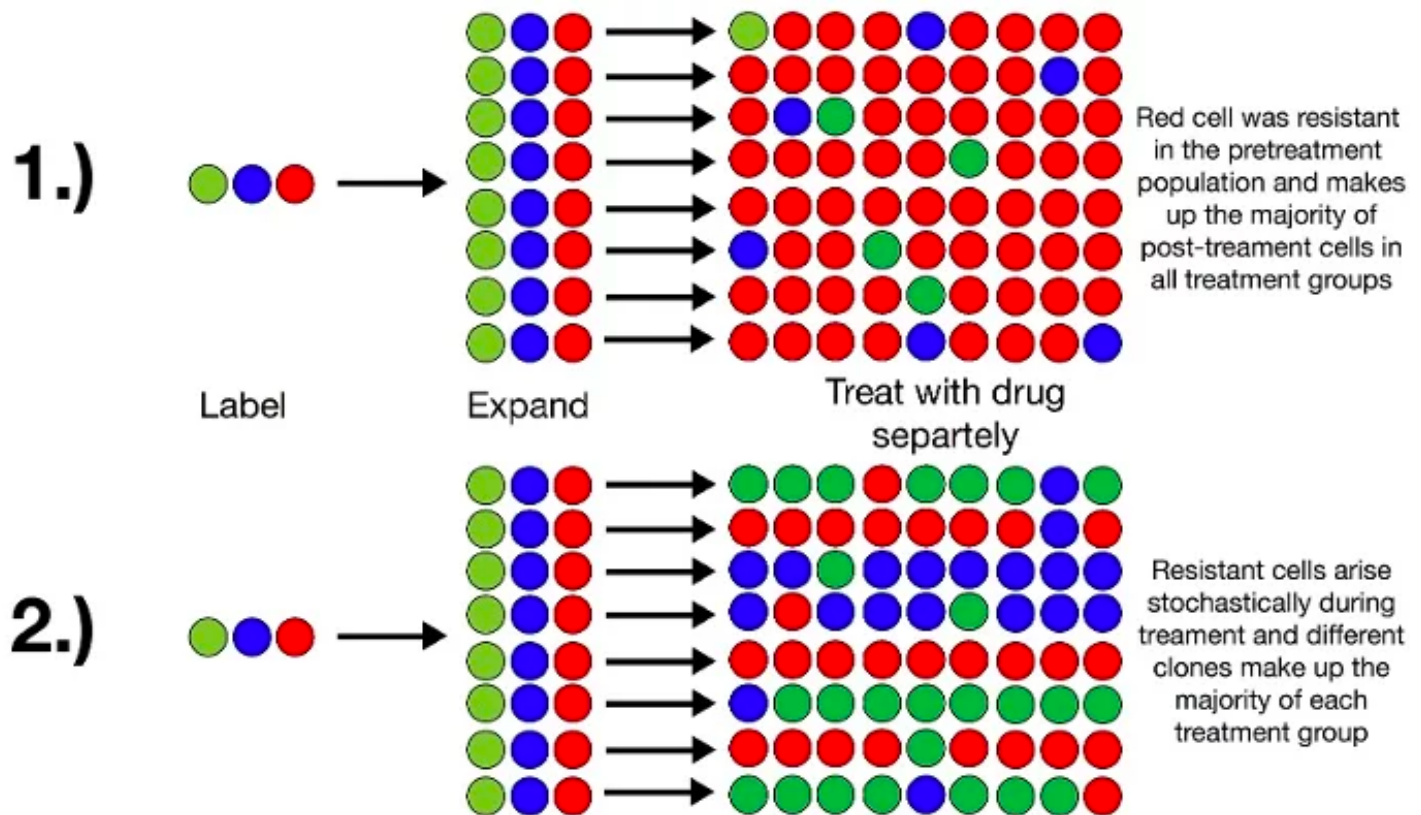
The ClonTracer library originally came about as Bhang and co-workers were trying to develop a way to quantify the take rate of mouse xenografts after cell implantation. Their goal was to figure out how to optimize protocols for *in vivo* screening with lentiviral [pooled shRNA libraries](#). Later, Bhang says, “When we developed this tool, there were so many interesting questions that we could apply [it to].”

## Using ClonTracer to study drug resistance

Although Bhang and co-workers started using the tool to look at a variety of questions surrounding cancer development, metastasis, and drug resistance, Bhang says “the [drug] resistance story panned out quickly,” and was summarized in a recent [Nature Medicine paper](#). In the publication, Bhang and coworkers used the ClonTracer library to determine whether or not cancer cell populations resistant to drug treatments arise during treatment or are preexisting in the parent population prior to the start of treatment. They hypothesized that, if they labeled the parent cell population using the library, propagated it, separated it into roughly identical replicate groups, and treated these groups with the same drug regimen, they would find one of two outcomes (see schematic depiction below):

1. Resistant cells among the separate replicates would contain the same labels - i.e. they would always come from the same parent cells that represent the preexisting resistant subpopulation
2. Resistant cells among the separate replicates would have different labels - i.e. they would stochastically arise from different cells throughout the treatment regimen and were not preexisting.

# New tool for lineage tracing: the ClonTracer library (CONT'D)



This was previously debated in the [cancer field](#) and was difficult to determine using NGS because the error rate of current NGS approaches allows for a detection limit of roughly 0.1%. Thus, rare, pre-existing clones that are present at less than 0.1% of the total population are easily missed.

The ClonTracer library allows a researcher to label individual members of a starting population of cells with specific, easily-read sequences that can be used to measure their clonal abundance before and after a treatment. The library consists of a pool of lentiviral vectors with roughly 73 million semi-random, 30 bp DNA barcodes that are integrated into target cells' genomes upon infection. When using the ClonTracer system, lentiviruses containing these barcodes infect the target cell population at low [multiplicity of infection \(MOI\)](#) such that each individual cell receives only one barcode. The abundance of each barcoded clone can then be monitored over time by sequencing the barcodes in the population (all barcodes can be amplified using the same sets of forward and reverse primers). The abundance of a particular barcode sequence then corresponds to the abundance of a specific clone; lineage tracing is performed via barcode sequencing.

Using the ClonTracer library, Bhang et al. found that antibiotic resistant cancer cells exist prior to drug treatment. They infected a non-small cell lung cancer cell line containing an activating mutation in epidermal growth factor receptor (EGFR) with the ClonTracer library and monitored population dynamics after treatment with EGFR inhibitor, erlotinib. Looking at barcode abundances among multiple replicate populations, they found that similar barcodes consistently made up the majority of the resistant populations. In fact, 88% of the barcodes within the resistant populations were shared by at least one other replicate; prior to treatment, there are small (previously

# New tool for lineage tracing: the ClonTracer library (CONT'D)

undetectable) populations of cancer cells that are resistant to drug treatment and that these are selected for during the treatment process. The authors went on to show that combination treatment with multiple drugs reveals even smaller preexisting populations with resistance to both drugs.

## The future of ClonTracer

Since starting her new role as an investigator in the In Vivo Pharmacology group at Novartis Oncology, Bhang has been working on using the ClonTracer library to monitor clonal dynamics following drug treatment in cell line and primary tumor xenograft models. She hopes that the library can be used to identify appropriate, non-overlapping targets for drug development - such that initial resistant populations can be eradicated up-front quickly. This approach is in contrast to the current treatment paradigm where resistant populations are treated as they arise. In their *Nature Medicine* paper, Bhang and coworkers also looked at cancer cell resistance to ABL1 inhibitors in chronic myeloid leukemia. Bhang says the work highlighted the importance of developing more potent ABL1 allosteric inhibitors at Novartis.

The ClonTracer library has been distributed all over the world and was recently used in a study focusing on tumor cell resistance to EGFR inhibitor ([Hata et al., 2016](#)). Bhang deposited the library with Addgene because she wanted to make it as easy as possible for researchers to use this tool in their own work. She says, “I spend a lot of time packing, shipping, and sharing protocols one by one,” and she is excited to deposit the library with Addgene because it is a “really great way of broadly sharing these kinds of reagents.”

Here at Addgene, we're excited to see the creative new ways you put the ClonTracer library to use!

*Many thanks to Carrie and Novartis for taking the time to talk with us and for providing this powerful resource to the Addgene community!*

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# Tips for titering your lentiviral preps

By Meghan Rego | March 15, 2016

The day has arrived; you've [painstakingly cared for your packaging cell line, prepped your DNA, transfected and harvested your lentivirus](#). Now it's time to move ahead with your infection and make your stable cell line. While we've all experienced the pressure to move a project forward, transductions should not be rushed into. Before you start any transduction, you should always titer your virus - that is, determine the amount of virus you actually have in your prep. Taking time to properly titer your virus will not only ensure that your infection is designed in the best possible way, but it may also save you time in the long run. Read on for an overview of the titering options as well as the benefits and drawbacks of different methods (for comprehensive protocols for all of the methods discussed here refer to [Kutner et. al.](#)).

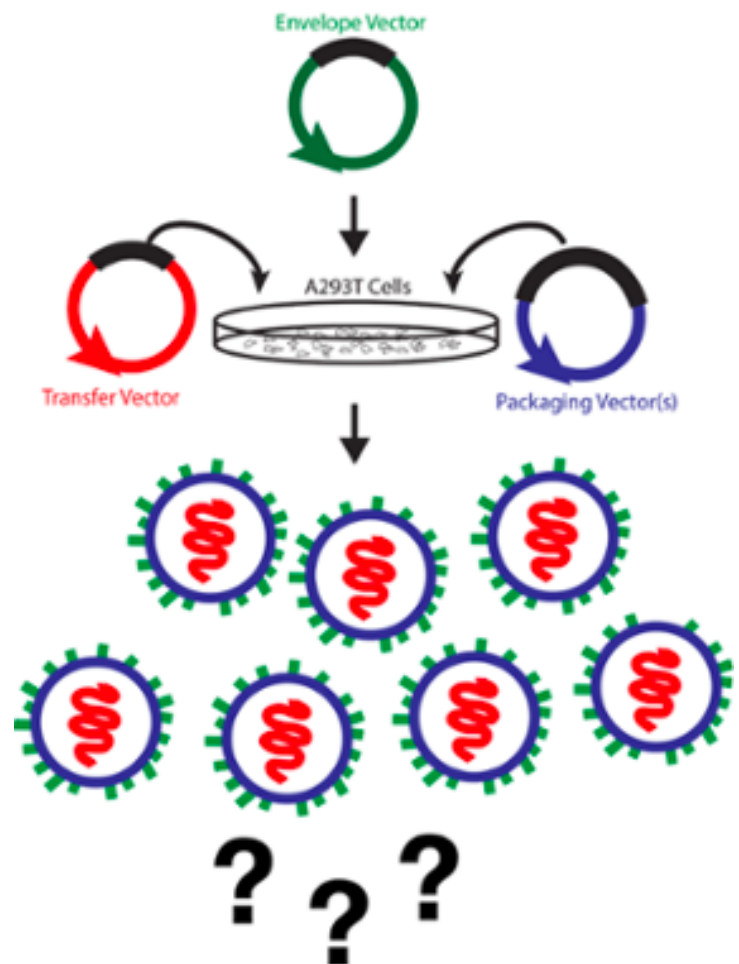
## Physical versus functional titer

Viral titers usually come in two flavors, physical or functional. Physical titers measure the amount of viral particles in a sample and are usually based on the presence of a viral protein, such as p24, or viral nucleic acid. Functional titers, or infectious titers, measure how many of the viral particles produced can actually infect cells. These assays typically involve infecting a target cell line with your virus and assaying for expression of a gene carried on the transfer plasmid or quantifying the number of viral copies that have integrated into the target cell's genome.

## Physical titer

The two most common assays for physical titer are direct p24 measurements via ELISA and qPCR for viral RNA. Measuring p24 levels in a viral prep is straightforward and rapid as there are several commercially available [p24 ELISA](#) kits that can quantify p24 levels in a matter of hours. A major drawback of this method, however, is that a p24 ELISA measures all p24 in the sample regardless of whether or not it is incorporated into a viral particle. Consequently, titers based off of p24 quantification tend to be overestimates since they can include free p24 and defective viral particles.

Direct measurement of lentiviral RNA is one alternative to direct p24 measurement. Like a p24 ELISA, this method is relatively quick, providing results in less than a day, but tends to be less expensive than an ELISA kit. In this approach, viral RNA is first converted to cDNA and then quantified using qPCR primers targeting specific [viral components](#) such as LTRs, gag, WPRE, antibiotic resistance-genes, or the transgene itself. Many researchers prefer to design primers that target the common features of viral vector backbones; once validated these universal primers can be used



# Tips for titering your lentiviral preps (CONT'D)

to titer any lentiviral prep that shares that specific feature. Primers targeting the transgene are also beneficial as they ensure that the correct transfer plasmid was used in the transfection; if preparing several different lentiviruses in parallel, this approach should be considered.

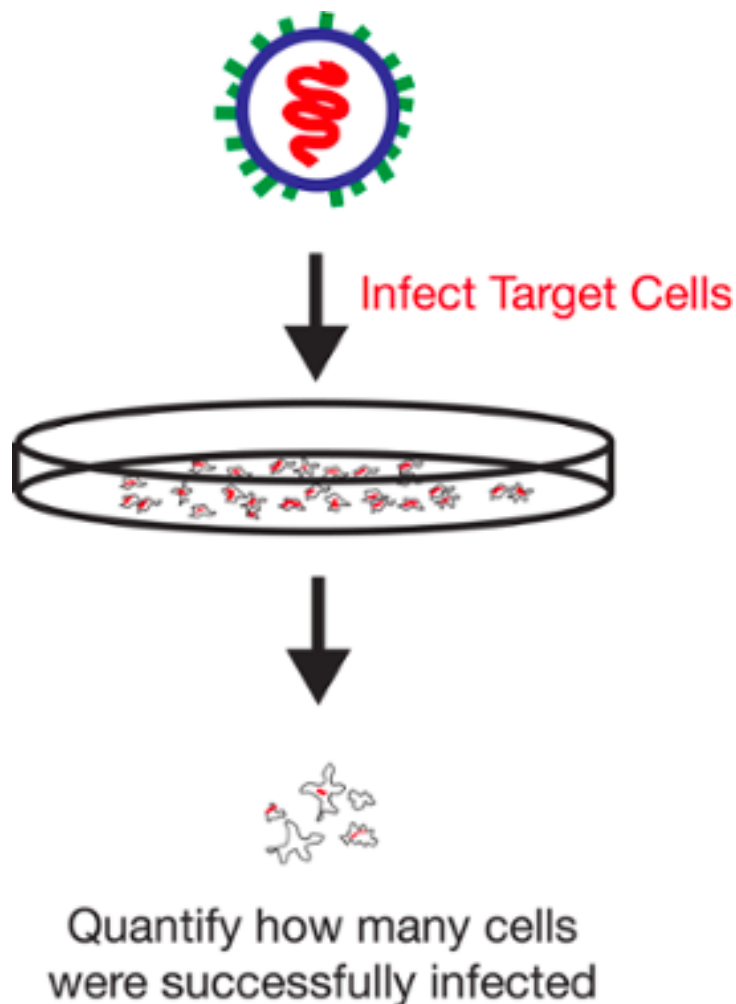
Similar to the p24 assay, titering via measurement of viral RNA can overestimate the amount of infectious virus in a prep as the measurement will include defective particles. Studies have found that values can be [10-1000 fold higher](#) than for functional assays depending on the vector backbone. When using qPCR based methods for viral titration consider using a reverse transcriptase mutant in a separate control reaction. This will allow you to quantify the amount of PCR product that comes from amplification of the original plasmid, which can inflate results. A [packing vector deficient in reverse transcriptase](#) will produce low levels of virus and can be used to track carryover.

## Functional titer

Functional titer is considered a more accurate method to quantify virus as it only measures infectious viral particles. One of the simplest methods of measuring functional titer uses FACS to count the number of target cells that are positive for vector encoded transgene expression following transduction with serial dilutions of a viral prep. This method is particularly useful when vectors carrying fluorescent markers are used as it eliminates the need for antibody staining.

Another popular approach utilizes the [antibiotic resistance genes](#) carried on transfer plasmids. Target cells are transduced with serial dilutions of a viral prep, treated with the antibiotic, and colony-forming units quantified. While these methods tend to be more accurate than measuring physical titer, there is a risk that they may underestimate viral titer as these assays cannot distinguish between a cell that has had one viral particle integrated into its genome and a cell that has had multiple integration events. In addition, promoter choice can also impact transgene expression.

The most accurate method of lentiviral titration uses qPCR to measure the number of proviral copies that have integrated into a target cell's genome. This approach can discriminate multiple integration events, reducing the possibility of underestimating the viral titer. The main drawback of this approach is that it can





# Tips for titering your lentiviral preps (CONT'D)

be quite labor intensive. Target cells are transduced with serial dilutions of a viral prep, genomic DNA isolated, and primers targeting viral components or the transgene itself are used to quantify the number of integration events. Another drawback to this method can be the absence of suitable controls. The best control for this type of assay is a clonal cell line known to contain 1 integrated copy of the qPCR target gene. Developing the proper control line typically requires transducing with a limiting multiplicity of infection (MOI) to ensure only one copy integrates, selecting by antibiotic-resistance or single cell sorting based on expression of a surface marker, subcloning, expansion, and confirmation. This procedure can take several months but should be considered in labs that will routinely be producing virus or require precise titers.

## Additional considerations

[Volume and transduction time](#) will influence lentiviral titers as will the target cell type used. Therefore, it is critical that standard conditions be used when comparing different batches of virus. In addition, some viral preps are very sensitive to freeze thaw cycles; the titer measured with a freshly collected lentiviral prep may be significantly higher than the same prep that has been stored in the freezer. If you plan to freeze a batch of virus for long term use, you may want to consider using an aliquot that has been subjected to a freeze/thaw cycle for titering, as this will better represent future experiments.

The aforementioned titering options all have their benefits and drawbacks. Labs that do not routinely produce virus may want to choose more straightforward approaches, such as the FACS-based or colony-forming unit assays, whereas labs that routinely produce virus may want to consider the more accurate proviral integration assay. In either case, properly titering your viral prep is the critical first step of a successful transduction experiment.

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# 5 tips for troubleshooting viral transductions

By Leila Haery | August 11, 2016

An [estimated 320,000 viruses can infect mammals](#). Even more abundant are the Earth's [estimated 10<sup>31</sup> bacteriophages](#) (viruses that infect bacteria), many of which are doing important work in our microbiomes. Given that viruses are everywhere and doing everything, it can be annoying when we try to use them in an experiment and they don't do anything.

Viruses are complicated and they perform complex biological tasks, both of which are factors in the technical challenges they can present. Many researchers find themselves plagued by troubleshooting a virus-based experiment at one point or another. After amplifying a viral vector, collecting virus, and transducing target cells, one may have the unfortunate experience of witnessing...nothing. For some unclear reason, cells are not getting transduced. Here, I will share five useful tips that have helped me troubleshoot some technical obstacles when working with virus:

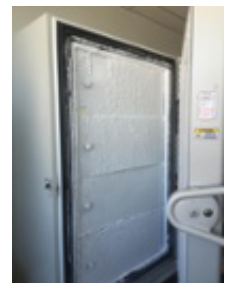
## 1. Viral vectors undergo DNA rearrangements



Depending on the composition of the viral vector and the repeat sequences present in the LTR, the vector may undergo genomic rearrangements during replication. To avoid genomic rearrangements, try amplifying the viral vector with bacteria designed to minimize rearrangements, such as [NEB Stable](#) or use a standard strain, like DH5α, grown at 30 °C instead of 37 °C. If you're unsure about the integrity of your virus, perform a diagnostic restriction enzyme digest and compare it to some reliable controls.

## 2. Viral stocks may be sensitive to freeze thaw cycles

Depending on the researcher and the type of virus, there have been varied reports of titer losses from 5% to 50% per freeze thaw cycle ([Krajden et al., 1999](#); [Ugai et al., 2002](#)). If the virus will be used in a couple of days, some researchers prefer to store freshly harvested virus at 4 °C instead of storing it briefly at -80 °C. If troubleshooting, the safest option may be to avoid freezing altogether, and instead to use the virus immediately upon harvesting. Interestingly, freeze-thawing host cells immediately prior to their transduction has been shown to increase AAV transduction efficiency by approximately 15-fold ([Chen et al., 2006](#)).



## 3. Transduction efficiency depends on viral titer

Both transduction efficiency and viral titer can be increased by concentrating a viral stock. Different titrating methods can reveal differences in physical versus functional titer. If you have low titers (which is unavoidable for some vectors) you can concentrate your virus by ultracentrifugation of the viral stock, followed by resuspension of the collected particles in a smaller volume. Always remove packaging cell debris before proceeding to ultracentrifugation to avoid contaminating your transduction with the packaging cells. Packaging cell debris can be removed either by filtration of the virus solution (through a 0.45 µm or 0.22 µm filter) or by a centrifugation step (5 minutes at 300-500 g). After removing packaging cell debris, virus can be pelleted by ultracentrifugation (75,000 - 225,000 g for 1.5 – 4 hours at 4 °C). After ultracentrifugation, virus may appear as a white pellet. The

## 5 tips for troubleshooting viral transductions (CONT'D)

pellet can then be rinsed and resuspended in cold sterile PBS.

Alternatively, to concentrate a virus solution without ultracentrifugation, you can reduce the volume of cell culture medium on the packaging cells immediately following transfection. In other words, when exchanging the medium after transfecting cells, replace the medium with half the volume typically used (e.g., use 5 mL medium on a 10 cm plate). By reducing the culture volume into which the virus is released, a more concentrated virus solution can be obtained.

### 4. Enhancing virus-cell contact

A variety of reagents can increase transduction efficiency. These reagents typically enhance the adsorption of a virus particle to a target cell by reducing the repulsive electrostatic forces between these two negatively-charged membranes ([Davis et al., 2002](#)). Polybrene is one such cationic reagent that is commonly used and has been shown to increase transduction efficiency by 10-fold (Davis et al., 2002). However, polybrene is highly sensitive to freeze-thaw cycles and should be stored in single-use aliquots. Never use a polybrene solution that has been thawed more than once. Fibronectin is another membrane-interacting protein that can be used to enhance transduction efficiency in cells that are sensitive to the toxicity of polybrene (e.g., hematopoietic or primary cells), and has been shown to increase transduction efficiency by 1.5-fold ([Stockschl der et al., 1999](#)).

### 5. Check your packaging cells for infection

If your transductions aren't working, you can check if virus is being generated by your transfected packaging cells. This check can be performed routinely and without disrupting the flow of your experiment. If your packaging cells have been successfully transfected and produced infectious virus, they may get infected with this virus, and therefore, will be selectable with the antibiotic whose resistance gene is encoded in the viral vector. This antibiotic selection can be performed approximately 72 hours after transfection and should give rise to a surviving population of approximately 20–50% of the packaging cells after 1–3 days of selection. A lower percentage of surviving cells may be the result of plasmid integration via recombination during transfection, and does not necessarily indicate the production of virus. Alternatively, if the virus encodes a [fluorescent protein](#), the packaging cells could be examined with a microscope and should appear fluorescent if they were infected. While there may be some low-level fluorescent protein or resistance marker expression following transfection, higher-level expression after approximately 72 hours is likely the result of successful viral transduction. This method may not work if the virus does not have the proper tropism to infect the packaging cells. Another method of ensuring viral production is to perform PCR against components of the viral vector using the harvested viral solution as a PCR template.

Regardless of the type of virus you're working with, there's a lot that goes into virus design, production, and transduction. Once you've harvested, it's generally a good idea to titer your virus. Getting an accurate count of the number of infectious particles in a given viral stock will give you a sense of the infectivity of your viral stock, save you time, and allow you to standardize your experiments later. [Stay tuned to the Addgene blog](#) and be sure to check out our [viral vector guide pages](#) to get up-to-date tips for your virus experiments.

# 5 tips for troubleshooting viral transductions (CONT'D)

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# CHAPTER 4

## Adenoviral vectors



# Adenoviral vector overview

By Various Addgenies

## Adenovirus components

The adenovirus genome is a linear, 36-Kb double-stranded DNA (dsDNA) molecule containing multiple, heavily spliced transcripts. At either end of the genome are inverted terminal repeats (ITRs). Genes are divided into early (E1-4) and late (L1-5) transcripts. There are 57 accepted human adenovirus types, and most adenoviral vectors are based on Ad5. Ad5-based vectors use the Coxsackie-Adenovirus Receptor (CAR) to enter cells.

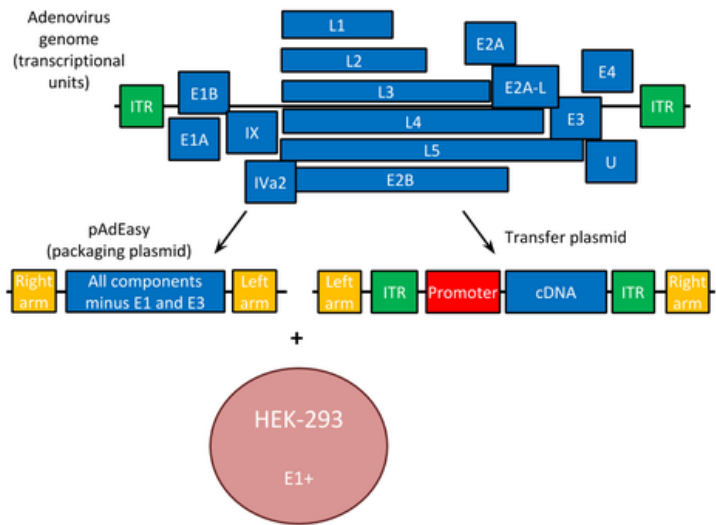
Recombinant adenovirus has two genes deleted: E1 and E3. E1 is supplied by the adenovirus packaging lines 293 or 911; its deletion from the viral vector renders the virus replication incompetent. E3 is involved in evading host immunity and is not essential for virus production. Deletion of these two components results in a transgene packaging capacity of >8 Kb. Constructs contain left and right arms to facilitate homologous recombination of the transgene into the adenoviral plasmid.

Please see our [Biosafety guide](#) for more information on working with adenovirus.

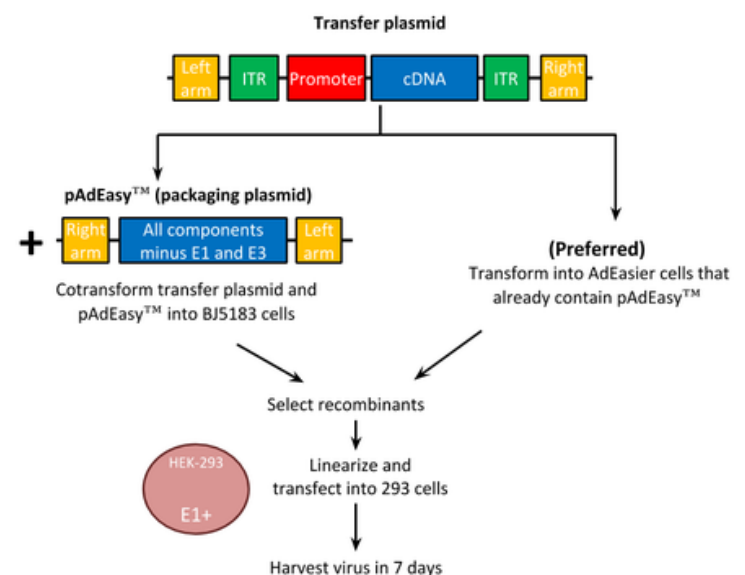
## AdEasy™ for adenoviral vector construction

AdEasy™, developed by [Bert Vogelstein](#), is by far the most popular method for creating adenoviral vector constructs. The system consists of two types of plasmids: shuttle (or transfer) vectors and adenoviral vectors. Find vectors for this system on our [adenovirus plasmid page](#).

The transgene of interest is cloned into the shuttle vector, verified, and linearized with the restriction enzyme PmeI. This construct is then transformed into [AdEasier-1 cells](#), which are BJ5183 *E. coli* cells containing pAdEasy™. pAdEasy™ is a ~33 kb adenoviral plasmid containing the adenoviral genes necessary for virus production. The shuttle vector and the adenoviral plasmid have matching left and right homology arms which facilitate homologous recombination of the transgene into the adenoviral plasmid. One can also co-transform standard BJ5183



Overview of an adenovirus plasmid system



Adenovirus packaging methods

# Adenoviral vector overview (CONT'D)

with supercoiled pAdEasy™ and the shuttle vector, but this method results in a higher background of non-recombinant adenoviral plasmids.

Recombinant adenoviral plasmids are then verified for size and proper restriction digest patterns to determine that the transgene has been inserted into the adenoviral plasmid, and that other patterns of recombination have not occurred. Once verified, the recombinant plasmid is linearized with PacI to create a linear dsDNA construct flanked by ITRs. 293 or 911 cells are transfected with the linearized construct, and virus can be harvested about 7-10 days later.

Vogelstein designed multiple shuttle vectors for different purposes. The pAdTrack series contains an IRES-GFP construct that enables co-expression of GFP with the transgene of interest. With these plasmids, one can track the infection of 293/911 cells throughout virus production. During experiments, GFP can be used to sort cells infected with adenovirus, or to verify that the infection rates are equivalent across multiple viruses.

The adenoviral backbone pAdEasy-1 is suitable for most purposes. For especially long transgenes, the use of pAdEasy-2 can increase the capacity of the adenoviral vector. pAdEasy-2 does not contain the viral gene E4, adding 2.7 Kb of packaging space. However, these constructs must be transfected into 911E4 cells for virus production, as 293 cells do not contain E4.

## Frequently asked questions about adenoviral vectors

### Are adenovirus and AAV different?

Yes! [AAV](#) is a small single-stranded DNA parvovirus and was discovered as a contaminant of adenovirus preparations, whereas adenoviruses are a class of medium-sized, non-enveloped double-stranded DNA viruses. They are not related; however, AAV requires the presence of adenoviral genes E1, E4, E2a and VA for replication.

For more information about AAV, read the [AAV chapter in this eBook](#).

### Can I make a stable cell line with adenovirus?

No, adenoviral vectors can only be used transiently.

### Are adenoviruses replication deficient?

Typically yes, because the early genes necessary for replication have been deleted from the shuttle vector. Early gene E1 is provided by the transfected cell line (either 293 or 911). To avoid creating replication competent virus, you should not serially propagate your virus as the chance of recombination events creating replication-competent virus increases with each round of amplification.

### What is RCA?

RCA stands for replication competent adenovirus. This may occur when a crossover event between your



# Adenoviral vector overview (CONT'D)

adenoviral vector and the early genes found in the packaging cell lines creates a wildtype adenovirus. The probability of a crossover event increases with each round of amplification.

## Where can I find information about using the AdEasy™ System?

The laboratory of Bert Vogelstein has published their method for generation of recombinant adenoviruses: [A Practical Guide for using the AdEasy™ System](#).

## Glossary

| Packaging vocabulary                |   |
|-------------------------------------|---|
| Term                                | Defenition  |
| pAdEasy-1                           | Adenovirus packaging plasmid that lacks E1 and E3   |
| pAdEasy-2                           | Adenovirus packaging plasmid that lacks E1, E3, and E4; Useful for very large inserts                       |
| pAdEasier-1 cells                   | BJ5183 <i>E. coli</i> cells containing the pAdEasy-1 packaging plasmid                                      |
| 911E4 cells                         | Cell line compatible with the pAdEasy-2 packaging plasmid; Supplies viral protein E4                        |
| E1                                  | Supplied in trans by 293 or 911 packaging cells; Its deletion renders the virus replication incompetent     |
| E2                                  | Non-essential gene involved in immune evasion, deleted to increase packaging capacity                       |
| E4                                  | Essential for viral transcription; Supplied by the pAdEasy-1 packaging plasmid or 911E4 packaging cell line |
| Transfer Vocabulary                 |   |
| Term                                | Defenition  |
| ITR                                 | Inverted terminal repeat; Contains elements that promote bi-directional transcription                       |
| Ad5-based vector                    | Most common type of adenoviral vector   |
| pAdTrack                            | Class of transfer vectors that contain IRES-GFP   |
| pShuttle                            | Class of transfer vectors that do not contain GFP   |
| Additional Vocabulary               |   |
| Term                                | Defenition  |
| Coxsackie-Adenovirus Receptor (CAR) | Receptor used by Ad5-based vectors to enter cells   |

# Adenoviral delivery of CRISPR/Cas9

By Kendall Morgan | September 30, 2014

Researchers have shown that it is possible to deliver RNA-guided CRISPR/Cas9 nuclease complexes to a wide range of human cells, including mesenchymal stem cells, using adenoviral vectors (AdVs). These [adenoviral CRISPR/Cas9 genome editing tools](#) developed and demonstrated by Manuel Gonçalves and his colleagues at Leiden University Medical Center are now available at Addgene along with a description of their [experimental protocol](#). The three plasmids which have been deposited to Addgene are: [pAdSh.PGK.Cas9](#), [pAdSh.U6.gRNAS1](#), [pAdSh.U6.gRNAGFP](#).

“Although AdVs are being deployed for delivering zinc-finger nucleases into human cells, we think they are still underused in the emerging field of genome editing,” Gonçalves said. “In contrast, AdVs are extensively being explored for genetic vaccination and oncolytic approaches. In genome editing, they are not used much, but we do think they have a very bright future.”

Gonçalves says that advantages of AdVs include their episomal nature and very efficient introduction of DNA into therapeutically relevant, non-transformed mammalian cells. These viral vector systems also work equally well in dividing and quiescent, post-mitotic mammalian cells.

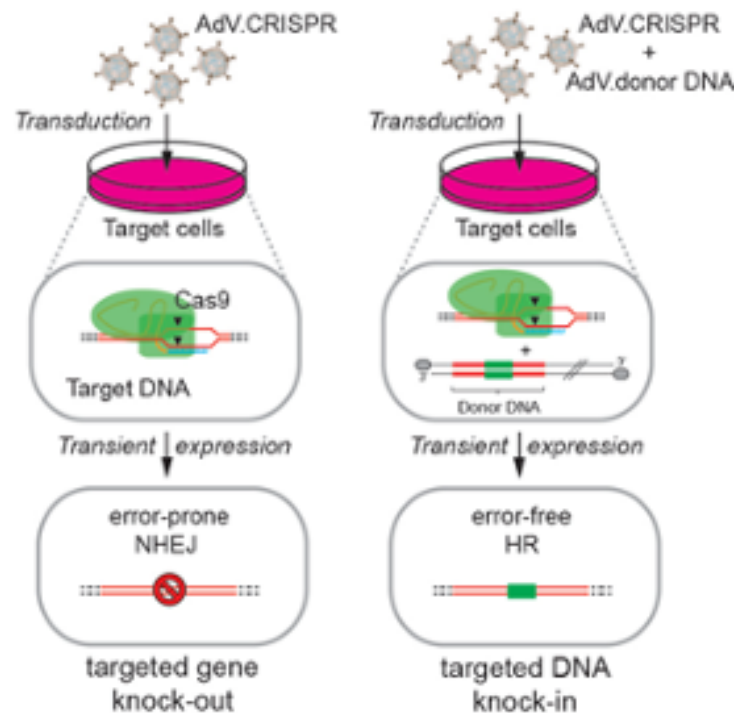


Image courtesy of Manuel Gonçalves.

## Delivering gRNA and Cas9 using adenoviral vectors

In a [Scientific Reports paper](#) introducing the delivery method in May, the researchers report that AdV-mediated transduction of gRNA:Cas9 ribonucleoprotein complexes into transformed and non-transformed cells yielded rates of targeted mutagenesis similar to those achieved by isogenic AdVs encoding TALENs targeting the same chromosomal region. The CRISPR/Cas9-derived RNA-guided nuclease-induced gene disruption frequencies in the various cell types ranged from 18% to 65%.

A second paper published online in [Nature Methods](#) in August found that delivering RNA-guided nucleases or TALENs together with AdV donor DNA leads to a vast majority of AdV-modified human cells being subjected to scarless homology-directed genome editing. Gonçalves said they attribute this phenomenon to the presence of terminal proteins capping the ends of linear double-stranded AdV genomes. Such protein-DNA structures presumably reduce the likelihood that donor DNA will interact with sporadic double-stranded chromosomal DNA breaks “that always happen naturally.”

“We think this [most recent] work gives additional rationale for investigating the usefulness of adenoviral vector technology in the context of genome editing,” he said, adding that he hopes others will now begin to make use of the new AdV delivery tools for a variety of applications.

# Adenoviral delivery of CRISPR/Cas9 (CONT'D)

“It would be rewarding if these reagents and protocol are picked up and people start to explore and test this method of introducing the CRISPR system into a broader range of cells – primary cells and cells that are not transformed – and eventually also consider *in vivo* applications.”

## Start your CRISPR/Cas9 research

To find more information about the adenoviral delivery of CRISPR/Cas9 using the Gonçalves lab's plasmids, including protocols, check out the plasmids at Addgene: [pAdSh.PGK.Cas9](#) (expresses *S. pyogenes* Cas9 from the PGK promoter) and U6 promoter-driven guide RNA constructs, [pAdSh.U6.gRNAS1](#) and [pAdSh.U6.gRNAGFP](#). Or if you're looking for a broader range of CRISPRs plasmid tools, find more plasmids, CRISPR technology guides, FAQs, and CRISPR resources on Addgene's site: <http://www.addgene.org/CRISPR/>.

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# Adenoviral vector production and troubleshooting

By Karen Guerin | June 14, 2018

[Adenoviral vectors \(AdV\)](#) are attractive vectors for research applications and gene therapy: they can be produced at high titers, can accommodate large transgenes, transduce quiescent and dividing cells, and do not integrate into the host's genome. The main challenge with using AdV is that it triggers a strong immune response after *in vivo* administration, which results in the death of transduced cells and loss of transgene expression (Interestingly, the strong immunogenicity of AdVs is what makes them ideal candidates for applications in oncolysis and vaccination!)

In the 65 years since its discovery, researchers have done significant work to improve the Adv genome's utility in research and its therapeutic potential.

**Note\*:** constructs discussed are based on Adenovirus type 5.

## Three generations of adenoviral vectors

The adenovirus virion is a non-enveloped particle of ~100 nm, consisting of a capsid surrounding an inner core which contains the adenoviral genome. The genome itself is a linear double-stranded DNA of ~36 kb, with inverted terminal repeats (ITR) present at both ends.

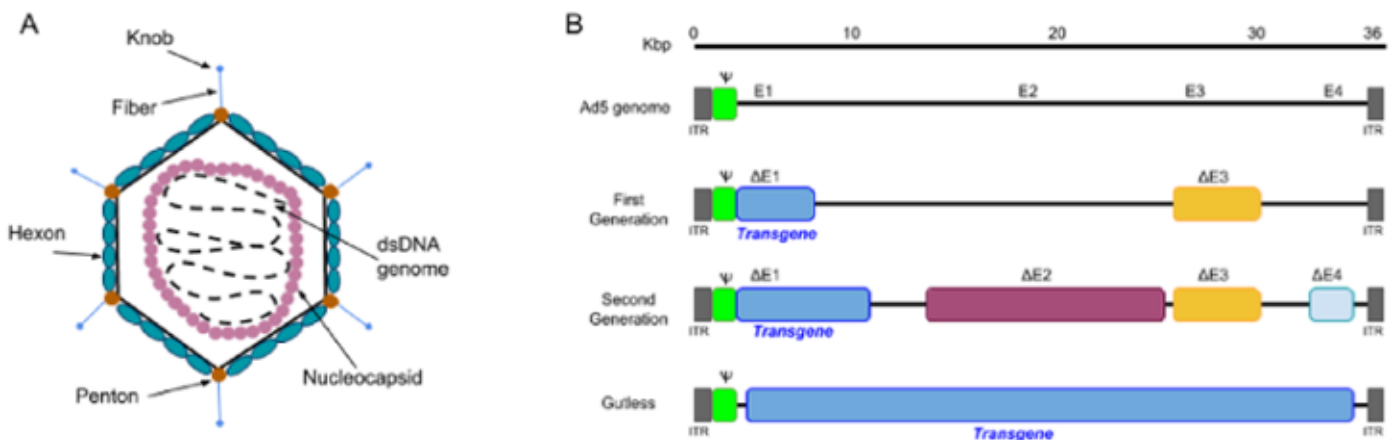


Figure 1: (A) The structure of an adenovirus - the virion is ~100 nm in size and consists of an icosahedral capsid made of 3 types of proteins: fiber, penton, and hexon proteins. The viral genome DNA is further protected by histone-like proteins forming the nucleocapsid. (B) Three generations of adenoviral vectors: the first generation vectors are deficient in the E1 and E3 regions and can accommodate up to 8.2 kb of transgene DNA. The second generation vectors are deficient in the E1, E2, E3, and E4 genes. Gutless Ad vectors (helper-dependent (HD-Ad), or high-capacity (HC-Ad)) are devoid of viral genes and therefore can accommodate up to 36 kb. Gutless Ad maintain only the ITRs and the packaging signal ( $\Psi$ ), both of which are essential for assembly of the virion. Adapted from Lee et al., Adenovirus-mediated gene delivery: Potential applications for gene and cell-based therapies in the new era of personalized medicine (2017).

**1st Gen AdVs** are stripped of regulatory genes E1 and E3. Without these genes, AdVs cannot replicate on their own but can be produced in E1-containing mammalian cell lines such as HEK293 cells. 1st generation AdV cloning capacity is limited to 8.2 kb, and *in vivo* transgene expression ceases relatively quickly due to immune response against AdV. Importantly, recombination-competent adenovirus (RCA) can be generated if the E1 gene from the packaging cell line is transferred into the AdV by recombination. It is therefore recommended to test for the presence of RCA in the viral stock by doing a [plaque formation assay](#) on A549 cells. Alternatively, antibody-

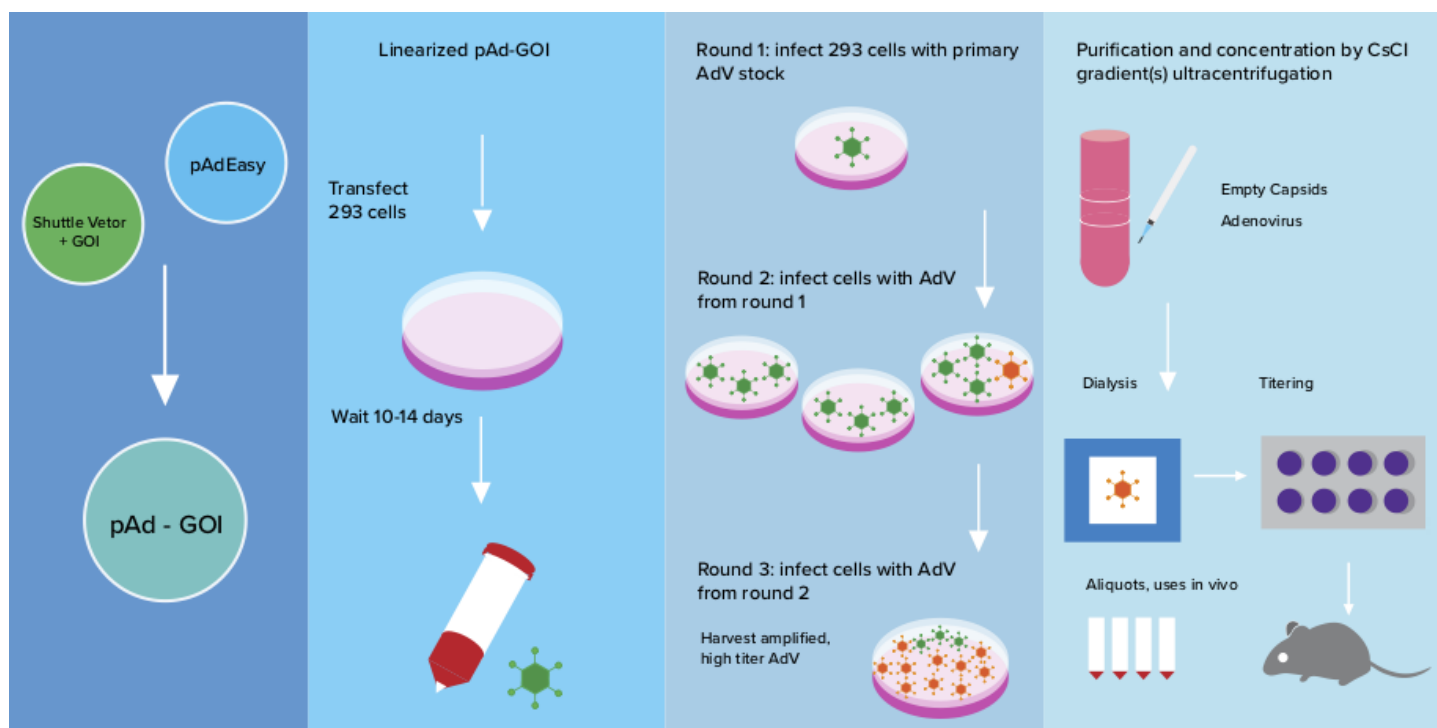
# Adenoviral vector production and troubleshooting (CONT'D)

based assays are commercially available for faster detection of RCA.

**2nd Gen AdVs** were designed to have increased cloning capacity, decreased potential for the generation of RCA and reduced immunogenicity. In addition to E1/E3, non-structural genes E2 and E4 are deleted in 2nd generation AdVs, thereby increasing cloning capacity to ~12 Kb. The ever popular [pAdEasy](#) system belongs to this category.

**3rd Gen AdV** (Gutless or high capacity AdV) are devoid of all viral coding sequences and retain only the ITRs and packaging signal in cis. Therefore, co-infection with a helper adenovirus is required to provide the viral proteins *in trans*. The Gutless AdV have generated high interest for gene therapy due their increased cloning capacity (up to 36 kb!), long-term transgene expression and negligible toxicity. However, their production is more complex and the presence of helper virus contaminants have slowed the use of this system. New and improved systems are still being developed. A detailed protocol for production of Gutless AdV can be found in reference 3.

## Production, amplification, and quality control of 2nd generation AdV



**AdV Production can be broken down into 5 key steps:**

**1. Construction of the rAdV plasmid (~1 week):** The [AdEasy™](#) system is the most popular method for creating adenoviral vector constructs. It consists of 2 plasmids: a shuttle vector (in which the transgene of interest is cloned) and pAdEasy™ which contains the adenoviral genes necessary for virus production. For detailed

# Adenoviral vector production and troubleshooting (CONT'D)

instructions on how to generate the final recombinant adenoviral plasmid construct, please see Addgene's [Adenoviral Guide webpage](#), and references [1](#), [2](#).



**\*Pro-Tip\*:** Once the correct recombinant pAdV plasmid is identified, re-transform into a bacterial strain not prone to recombination for amplification. The final purified plasmid stock should be analyzed by restriction digest or fully sequenced to confirm its integrity.

**2. Initial production (2-3 weeks):** Here you'll produce the primary recombinant adenoviral stock (rAdV-S). HEK293 cells are transfected with the AdV plasmid construct from step #1 and allowed to stay in culture for up to 20 additional days at which time the cells are scraped, and lysed by multiple freeze-thaw cycles. The resultant lysate is your primary low titer rAdV-S, and can be stored at -80C for later use or used immediately for amplification.



**\*Pro-Tip\*:** The cells will become completely confluent and the media will turn yellow. Do NOT change the media (add 2-3 mL of fresh media once a week), and do NOT harvest the cells before at least 10 days of incubation as it will result in very low titer.

**3. Amplification (1-2 weeks):** The rAdV-S is used to infect more HEK293 cells and produce more rAdV to reach higher titers. Successfully infected cells will become round and clump together ~3 days post infection. The amplified virus can be harvested once roughly 50% of the infected cells show this "cytopathic effect." Amplification can be repeated multiple times (2-4 rounds) at increasing scale over the course of 1-2 weeks. Each round of amplification should result in a 10-100-fold increase in virus.

**4. Purification (2 days):** Purification is required if rAdV is to be used *in vivo*. The standard method for purification of rAdV uses cesium chloride (CsCl) density gradients combined with ultracentrifugation to separate rAdV from other cellular debris. Purified high-titer rAdV is then dialyzed and stored at -80C. Purification and concentration kits that do not use CsCl are commercially available.

## 5. Titer calculations (1 or 10 days):

1. Physical titer (particle count, rAdV genomes/mL) can be measured by:

- Optical density: particle concentration is measured by looking at absorbance at 260 nm using UV/Vis spectrophotometry. An absorbance of 1 at 260 nm corresponds to  $1.1 \times 10^{12}$  particles/mL.
- To calculate the particle number: OD<sub>260</sub> reading x dilution factor x  $1.1 \times 10^{12}$  particles = number of particles per mL of sample.
- The purity of the sample can be estimated by looking at the ratio of DNA (260 nm) versus protein (280 nm) absorbance. The ratio for the purified virus should be ~1.3

2. Quantitative PCR: the number of viral DNA packaged in virions is determined by a standard curve of known quantity and primers specific for a viral DNA sequence.



**\*Pro-Tip\*:** Ad5 reference material should be used as an internal control (ATCC#VR-1516) until the assay is validated..

# Adenoviral vector production and troubleshooting (CONT'D)

## 3. Infectious titer: (Plaque Forming Units (PFU) or Infectious Units (IU), per mL)

- Plaque formation assay: permissive cells are infected with serial dilutions of rAdV, fixed, and then stained 10 days later. Plaques are counted and titer is calculated in terms of plaque forming units (pfu), taking initial vector dilution into account.

You're now ready to use your purified, high-titer rAdV in your experiment. Enjoy!

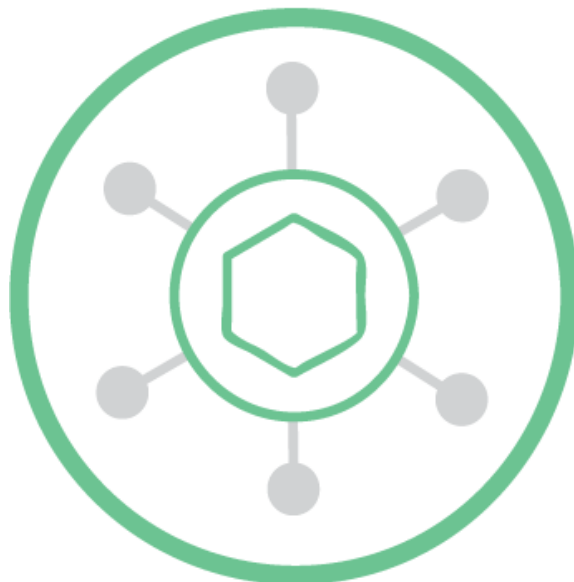
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2. He TC. et al. [A practical guide for using the AdEasy System](#).
3. Jager, Lorenz, et al. "A rapid protocol for construction and production of high-capacity adenoviral vectors." *Nature protocols* 4.4 (2009): 547. PubMed [PMID: 19373227](#).
4. Mittereder, Nanette, Keith L. March, and Bruce C. Trapnell. "Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy." *Journal of virology* 70.11 (1996): 7498-7509. PubMed PMID: 8892868. PubMed Central [PMCID: PMC190817](#).
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# CHAPTER 5

## Addgene's viral service



# Addgene's viral service - Why virus? Why now?

By Joanne Kamens | March 23, 2017

In the middle of 2016 Addgene started distributing a small but growing catalog of ready-made AAV and Lentiviral preps. This new [Viral Service](#) represents Addgene's largest new initiative since we started distributing plasmids in 2004. We've already distributed over 6,500 viral samples to scientists all over the world. Now that the service is successfully launched, I would like to thank some of the people and [organizations](#) who helped us reach this milestone.

## Viral service supporters

Addgene Silver Sponsor



Addgene Bronze Sponsor



We would like to thank the companies who supported this project with funding. [Alnylam](#) and [New England Biolabs](#) sponsored the capital construction of our new BL2+ laboratory and the infrastructure capabilities to produce and distribute virus. Addgene is a 501(c)3 nonprofit and our plasmid service was started based on donations. That seed money was used to build a completely self-supporting service so that Addgene is not dependent on grants. Aiming to operate under a similar model for our Viral Service, we solicited seed money for the new project. New England Biolabs and Alnylam see the value to the scientific community in accelerating the Viral Service. With their support we are able to provide faster access to a larger number of viral preps.

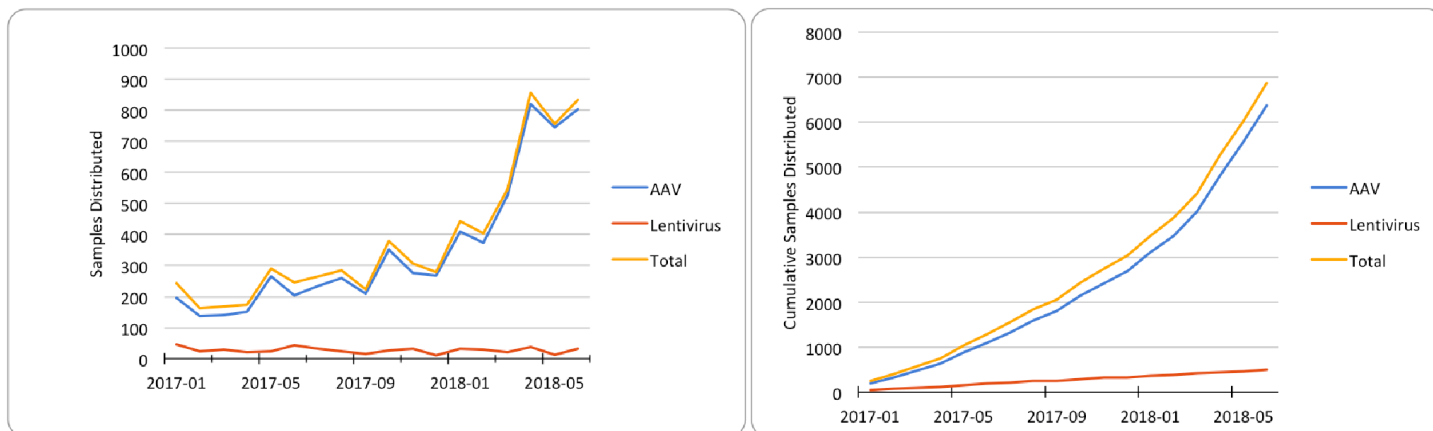
Over 3,500 laboratories are sharing plasmids through the Addgene repository (over 30 labs are currently sharing viral preps). Without these scientists and the support of their technology transfer offices, Addgene would not be able to have such an impact on scientific materials sharing.

I would also like to recognize a few scientists who have gone the extra mile for this particular project. John Doench and David Root from the Broad Institute and Alex Chavez from the Wyss Institute were instrumental in helping us establish consistent, high-quality protocols for lentiviral production. Bryan Roth's lab from the University of North Carolina, Connie Cepko's lab from Harvard Medical School, and Jonathan Ting's lab from the Allen Institute have supported the AAV initiative from the beginning and have worked closely with us to functionally test our virus. Ed Boyden's lab from MIT and Alla Karpova's lab from Janelia Research Campus have provided valuable insight into AAV production.

Finally, we are blessed to have an incredibly supportive [Board of Directors at Addgene](#). Our Board helps keep our focus on assisting scientists and accelerating research and discovery, while also pushing us to challenge assumptions, holding us to nonprofit best practices and encouraging us to explore new directions. I would like to thank them for their time, dedication, advice and encouragement.

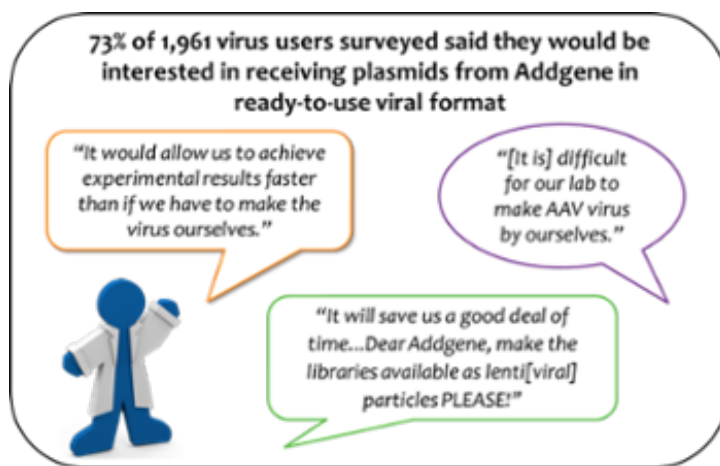
# Addgene's viral service - Why virus? Why now? (CONT'D)

Monthly and cumulative virus distribution through June 2018



## Why virus?

Why did we decide to take on this project? Addgene's mission is to *Accelerate research and discovery by improving access to useful research materials and information*. Addgene has been successful in helping thousands of sharing scientists from laboratories all over the world. With this reach and exposure in the community, we saw the opportunity to leverage our recognition, further fulfilling our mission for an even bigger impact. In early 2016 we surveyed hundreds of scientists about various ways we might expand Addgene's services. There was an overwhelmingly positive response to the option of Addgene providing plasmids in a ready-to-use viral format.



Having identified this need in the community, we approached it in the way Addgene approaches everything; we asked ourselves, "How can we do this in a way that most helps scientists and allows us to maintain Addgene's high quality standards?" Under the leadership of Addgene's co-founder, Melina Fan, we created a new team to push this project forward. The viral vector project team develops methods, performs extensive quality control, generates educational materials, and provides viral vector technical service (details about virus production protocols and quality control can be found on [Addgene's Viral Production webpage](#)).

While the new team took the lead on operationalizing viral production and distribution, this project involved every Addgene in the company. Our Finance, Business and Legal teams had to pave the way for this project with many depositors and partners. The Addgene Scientist Team has been developing expertise in answering questions on virus and helping provide educational resources on this collection. With their expertise in shipping hundreds of plasmids a day, the Lab and Office Teams develop shipping systems and protocols for viral vectors to reach scientists around the world. Our Software Engineering Team (and, added in 2018, our Product Team) continues to

# Addgene's viral service - Why virus? Why now? (CONT'D)

make improvements to our website and inventory management systems to make way in our systems for this new type of material. The Outreach Team is getting the word out about this new resource and providing fantastically useful educational content on viral vectors.

## How Addgene's viral service helps scientists

### Lowering Barriers

- Many labs lack the technical capabilities to prepare viral vectors

### Improving Reproducibility

- Addgene provides high quality, standardized reagents

### Accelerating Research

- The community saves time and money using virus prepared at scale

### Increasing Availability

- Viruses are distributed worldwide at a pace that keeps up with demand

Good science isn't the only facet of a project this big. Maintaining our excellent service and accuracy involves caring and careful Addgenies, but also a lot of good tracking. Our Software and IT Teams made major upgrades to our inventory management systems to enable efficient, tracked production and distribution of viral samples. The Office Team made sure we could ship internationally and be ready to provide extensive logistics support for export and shipping on dry ice. The Business and Finance Teams continue to facilitate the formation of partnerships to expand the service and figure out what we can afford to do and when. Addgene's Legal Team acquired permission from relevant plasmid depositors and their institutions to distribute viral preps derived from plasmids in the repository. The Outreach Team has been making sure scientists know about the new initiative and serving all the new educational content via the Addgene Blog and our expanding website resources.

I hope this gave you the impression that it took a lot of work and dedication to do this well ... because it did. So finally, a big thank you to the Addgenies who went the extra mile to create this new service. We knew the scientists were waiting. I look forward to sharing new developments on this project and on all the interesting things going on at Addgene. Sharing speeds science and we are proud to be adding viral vector services to our repertoire.

# Using Addgene's viral service for your research

By Tyler Ford | March 17, 2016

Instead of spending time and money producing virus from select vectors in the repository yourself, you can now order ready-to-use virus directly from Addgene! As part of our new [viral service](#), we're distributing [lentivirus](#) (with many CRISPR tools included among the preps that are currently available) and adeno associated virus ([AAV](#) - [chemogenetics](#), [optogenetics](#), and [more](#)). The viral preparations undergo rigorous quality control testing at Addgene meaning they come ready made to accelerate your research.

## Why distribute virus?

Plasmid distribution is booming, but, over the years, many researchers we've met with have asked if we can help them share and distribute other reagents. Virus distribution came up time and time again with many researchers lamenting the viral production process while still ordering viral vectors.

Virus production can be a frustrating process that requires a large investment of time and resources for labs that don't have experience with it. We're reducing this frustration and getting researchers to the experiments they're interested in faster by producing, titrating, and testing viral preps for them. At the same time, we're developing ourselves as a knowledge base for the processes of viral production and testing so that researchers can use our educational resources and experience to their advantage should they decide to produce virus on their own.

Harvard researcher [Connie Cepko](#) has been helping us with AAV testing and recently had this to say about the service:

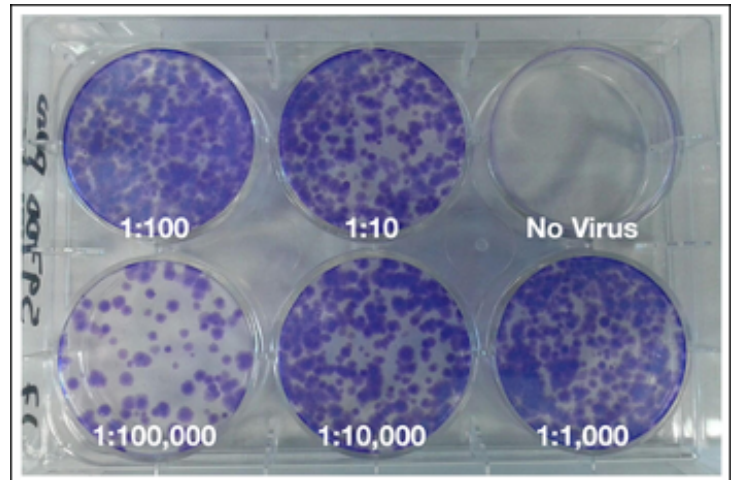
*"I'm excited that Addgene is now offering virion preparations to the scientific community. Addgene's AAV preparations have worked well in my lab, including in applications where we have delivered them in vivo to the central nervous system. This service will further enable scientists and accelerate research."*

## What viral preps are available?

There are a wide variety of tools already available from Addgene as ready-to-use viral preps and we'll be continually expanding our collection. We'll provide a brief rundown of what lentiviral and AAV preps are available below, but you can always head over to the [Viral Service page](#) to find the full list of what's available or suggest additional plasmids with the [Viral Service Suggestion Form](#).

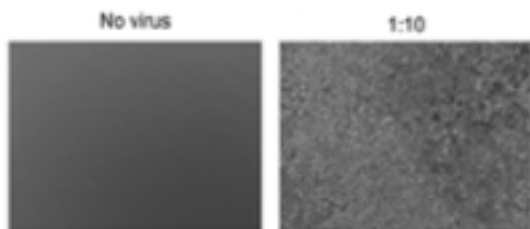
### Lentivirus

As some of the most popular lentiviral vectors in the repository are used to produce CRISPR tools, we're starting the Viral Service with lentiviral preps for the delivery of [Cas9 endonuclease](#), [Cas9 nickase](#), [Cas9 activators](#), [Cas9 repressors](#), [select gRNAs](#), and [CRISPR pooled libraries](#). Whether you'd like to knockout a gene, replace it, modulate its expression, or perform a genome-wide screen, we've got viruses to help you out.



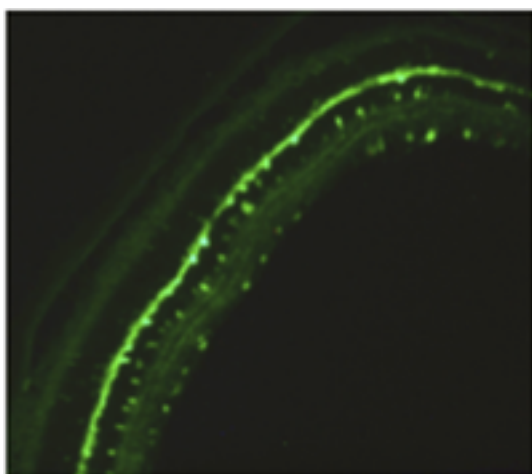


# Using Addgene's viral service for your research (CONT'D)



Colony formation assay with lentivirus.

Of course, lentiviruses are used in experiments other than just those involving CRISPR and so we also have lentivirus for [delivering control shRNAs, GFP, and rtTA from the Tet-on system](#). The first two can serve as controls in a variety of experiments, while rtTA can be used to activate expression of a gene under the control of the tet operator.



AAV infection of retinal neurons.

## AAV

AAVs are the workhorses of both chemo- and optogenetics. We're therefore beginning our AAV viral services with these tools. A variety of [chemogenetic tools](#) from Bryan Roth's lab are already available allowing you to chemically control neuronal activity while studying your phenotype of interest. [Optogenetic tools](#) for similarly controlling neuronal activity with light are also available.

We're also distributing [AAV preps with particular serotypes](#) that can be used to deliver fluorescent proteins to cells as determined by the serotype. You can use these as controls to determine if your AAVs are getting to the right cells using your particular delivery technique and conditions.

Finally, we have [retrograde AAV](#) that you can use to map neuronal connections.

## Expanding the viral vector knowledge base - you can help!

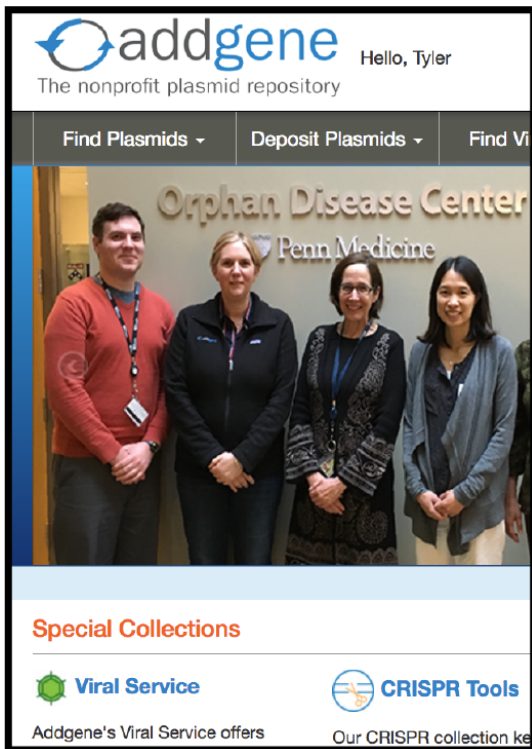
We've been expanding our viral vector educational resources as well, with both posts on our blog and pages on the Addgene website.



### [blog.addgene.org](http://blog.addgene.org)

The blog provides tips for working with viruses including advice on how to titer lentiviral preps, troubleshoot viral transductions, and overviews for beginners just starting work with virus. You can find all of our [viral vector blog posts here](#). If you have virus expertise you'd like to contribute to the blog, please [email us](#) and we'll help get you writing!

# Using Addgene's viral service for your research (CONT'D)



## www.addgene.org

On the Addgene website you'll find our updated [viral vector pages](#) as well as expanded [virus protocols](#), information on how we [produce and test virus](#), and quality control data for each of the materials we distribute. Quality control info can be found on each viral prep's specific material page and includes western blots as well as other expression data.

If you have any questions about the Viral Service or any of the materials we distribute, please send an email to [help@addgene.org](mailto:help@addgene.org). Your questions help us expand and improve our resources for future users.

We're excited to be distributing new types of materials. At the time of publication we've already fulfilled more than 6,500 orders for virus! We'll be sure to keep you updated on our progress and would love to hear your thoughts on the service as we continue to develop it. More communication and sharing will only help research advance. Remember: **Productivity is infectious!**



# Viral production at Addgene

By Various Addgenies

## Overview of viral production

Viruses are generated using standard methods that have been optimized for each specific virus in order to generate high quality preparations. After production, all virus preps are titered and subjected to quality control by Addgene before being distributed to customers. Details about our production protocols, titering methods, and quality control are described below.

## AAV

### Production

[AAV distributed by Addgene](#) has been produced either in-house by Addgene scientists or through collaboration with viral vector manufacturing facilities, such as the [University of Pennsylvania Vector Core](#). Transfections are performed using the transfer plasmid, a plasmid encoding rep and serotype-specific cap, and a plasmid encoding adenoviral helper sequences.

AAV preparations are purified by [iodixanol gradient ultracentrifugation](#) or cesium chloride gradient ultracentrifugation and concentrated to purity and titers adequate for *in vivo* studies. Preparations are then aliquoted and stored at -80°C.

### Titer

Titering is either performed by Addgene or by the University of Pennsylvania Vector Core. In general, titering is performed by the facility that produced the viral vector lot. Contact us to determine which facility produced your viral vector lot.

At Addgene, AAV particles are [titered by real-time quantitative PCR](#) using primers targeting the ITR. The amplicons are detected using SYBR green technology. Titer values are then determined by comparison to a standard curve of a plasmid sample of known concentration. The qPCR assay and corresponding titer determinations are also validated using AAV Reference Material (ATCC).

Titering by the University of Pennsylvania Vector Core (Penn Vector Core) is (as of April 2016) determined by droplet digital PCR (ddPCR). Previous experience by the Penn Vector Core suggests that titers obtained using ddPCR are generally 3 fold higher than those achieved using the standard qPCR method. However, ddPCR titers reported by the Penn Vector Core are comparable to qPCR titers reported by Addgene.

### Quality Control

Addgene ensures high quality viral vectors by optimizing and standardizing production protocols and performing rigorous quality control (QC). The specific QC experiments performed varies for each viral lot. To learn which specific QC experiments were performed on your lot, please [contact us](#).

#### *Confirmation of Transfer Plasmid*

The final AAV preparation undergoes PCR with primers targeting the transfer plasmid used in the initial transfection. Most of our primers are targeting the promoter region and/or the transgene. For DIO or FLEX

# Viral production at Addgene (CONT'D)

plasmids, we also validate the orientation of the cassette using 2 or more primer pairs. PCR products are analyzed by gel electrophoresis to ensure the correct banding pattern and sizes.

## *Full sequencing of the Viral Genome*

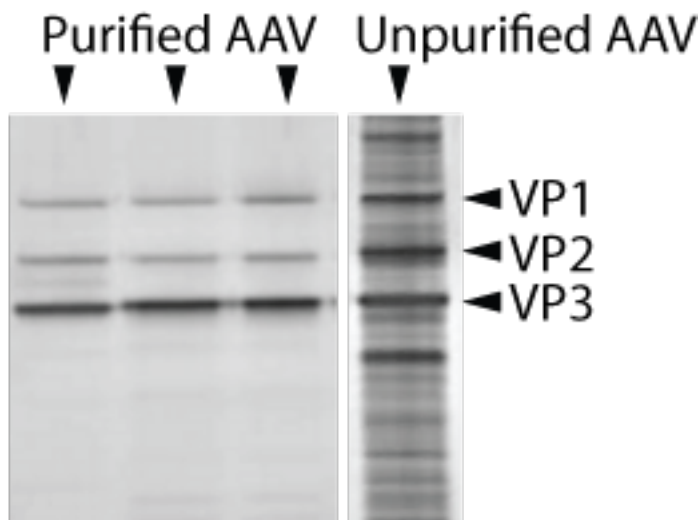
Next-generation sequencing is performed on viral genomes isolated from the final AAV preparation. Sequencing results are analyzed to confirm the identity and integrity of the viral genome and the absence of unexpected DNA contaminants.

## *Endotoxin*

Endotoxin contamination in vector preparations can alter the immunogenic properties of the final product, particularly in large animal studies. Endotoxin contamination is minimized by using an endotoxin-free plasmid purification protocol. To minimize the immunogenic properties of the final vector preparation, the quantity of gram-negative bacterial endotoxin is ensured to be less than 5 endotoxin units per mL. The endotoxin assay is carried out using the Limulus Amebocyte Lysate (LAL) gel-clot method.

## *Purity*

Purity of AAV preparations is assayed by comparing the relative stoichiometric ratios of the viral capsid proteins VP1, VP2 and VP3. Samples of viral preparations are subjected to polyacrylamide gel electrophoresis (PAGE) followed by silver staining or SYPRO Red staining and the molecular weight and relative intensity of the viral capsid proteins are analyzed. The abundance of viral capsid proteins as a fraction of total protein present in the sample is also determined and used to determine purity of the AAV preparation.



Silver staining of purified and non-purified AAV subjected to gel electrophoresis. Viral capsid proteins VP1, VP2, and VP3 are shown relative to the total protein present in the sample.

## *Sterility*

Viral vector preparations are added to cells in culture. Three to five days later, cell cultures are inspected for sterility.

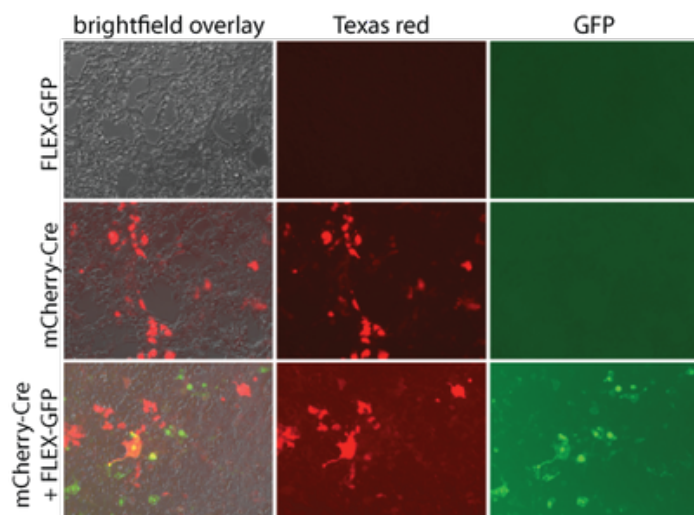
## *Transducibility*

Some viral vectors are tested *in vitro* and *in vivo* for gene expression and/or function. These data are sometimes reported or posted on the material page for the corresponding catalog item (see maps section for images).

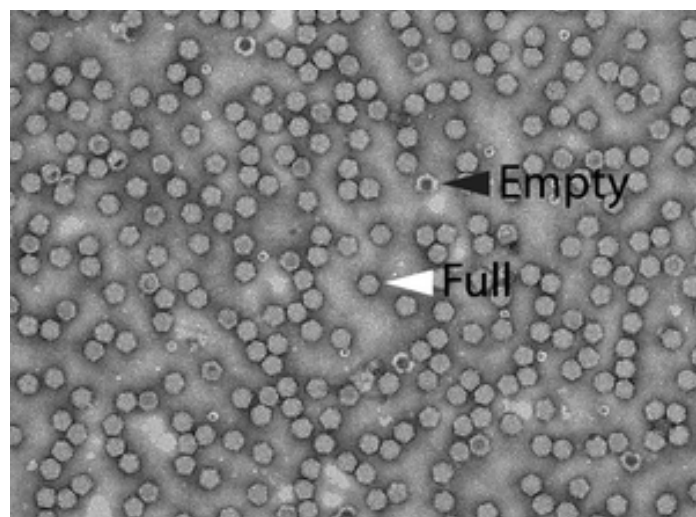
## *Electron Microscopy*

The ratio of empty to full (i.e., genome containing) AAV particles within representative vector preparations was quantified with electron microscopy after negative staining. Empty vector particles can be identified after negative staining and appear darker than full vector particles.

# Viral production at Addgene (CONT'D)



AAV Pro cells were transduced with either pAAV-Ef1a-mCherry-IRES-Cre (55634-AAVrg) alone at  $1.7 \times 10^6$  viral genomes (vg)/cell, pAAV-CAG-FLEX-rc [Jaws-KGC-GFP-ER2] (Addgene 84445-AAVrg) alone at  $1.1 \times 10^6$  vg/mL, or both. Two days later, Cre-dependent GFP expression was detected with direct fluorescence. GFP was not detected in the absence of Cre. mCherry expression alone was detected. pAAV-Ef1a-mCherry-IRES-Cre was a gift from Karl Deisseroth (Addgene viral prep # 55632-AAVrg). pAAV-CAG-FLEX-rc [Jaws-KGC-GFP-ER2] was a gift from Edward Boyden (Addgene viral prep # 84445-AAVrg).



Electron micrograph of AAV vector preparation shows that the vast majority of the vectors consist of full particles (white arrowhead) relative to empty particles (black arrowhead). Scale bar = 100 nm.

## AAV resources

- Browse [AAV preps](#) available from Addgene.
- After receiving your virus from Addgene, read our detailed [recipient instructions](#) for next steps.
- Plan an experiment using our [virus protocols](#).
- Browse our [AAV vector](#) collection.

## Lentivirus

### Production

Lentiviral preparations (-LV catalog items) are produced in the Lenti-X 293T cell line using the 2nd generation [psPAX2](#) and [pMD2.G](#) packaging system developed by the [Trono lab](#). Cell culture medium (DMEM + 10% FBS) containing lentivirus is first cleared by low speed centrifugation and then by filtration through a  $0.45 \mu\text{m}$  polyethersulfone membrane. The preparations are then aliquoted, snap frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$ . Concentrated lentiviral preparations (-LVC catalog items) are generated from lentiviral preparations (described above) that are subject to a concentration step prior to being frozen. Specifically, lentiviral particles are collected from the lentiviral preparation by precipitation in 10% polyethylene glycol (PEG) followed by centrifugation. Precipitated pellets containing viral particles are then resuspended in PBS, aliquoted, snap frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$ .

# Viral production at Addgene (CONT'D)

## Titer

All titring is performed on lentiviral preparations that have been stored at -80°C and thawed. This ensures that any loss of titer associated with the recipient's initial thaw will be accounted for in our reported titers.

Lentiviral vectors carrying selectable markers are titered using the standard [colony formation assay](#). Briefly, A549 cells are transduced with serial dilutions of a lentiviral vector, exposed to antibiotic, and resistant colonies are allowed to expand for 2 weeks. Culture dishes are then stained and macroscopic colonies are counted to determine infectious titer.

In addition to the colony formation assay, lentiviral vectors carrying puromycin and blasticidin resistance undergo a relative titring assay based on cell proliferation after antibiotic addition. Briefly, Lenti-X 293T cells are transduced with serial dilutions of a control lentiviral stock of known titer (typically [pRosetta](#)) in addition to the lentiviral stock to be titrated. After 48 hours the cells are treated with the appropriate dose of antibiotic and incubated for an additional 2-3 days. Cell proliferation is then measured using resazurin dye. Relative titers are extrapolated from a standard curve generated by the control lentiviral stock.

Lentiviral vectors carrying fluorescent markers undergo additional [titring based on expression of the fluorescent protein](#). Briefly, Lenti-X 293T cells are transduced with serial dilutions of a lentiviral vector and fluorescent cells are quantified by microscopy 96 hours post-transduction.

## Quality Control

### *Mycoplasma*

The Lenti-X 293T and A549 lines were obtained directly from Clontech and Sigma Aldrich, respectively, and are routinely tested for mycoplasma contamination using the protocols described by Uphoff and Drexler (PubMed: [PMID 23179822](#)). Lines are maintained for 15 passages before being discarded and replaced with a new vial of early passage cells. Approximately 2-3 weeks post-thaw, cell culture supernatant is tested for mycoplasma contamination. To date, Addgene has never had a case of mycoplasma contamination. In the event of contamination, all of the virus produced in the line will be taken offline and discarded.

### *Confirmation of the transfer plasmid*

Addgene uses a rigorous barcode matching system that follows the sample from the DNA tube all the way to the cleared viral preparation pool.

In addition, the final viral preparation undergoes PCR with primers targeting the transfer plasmid used in the transfection. PCR products are analyzed by gel electrophoresis to ensure the correct banding pattern and sizes.

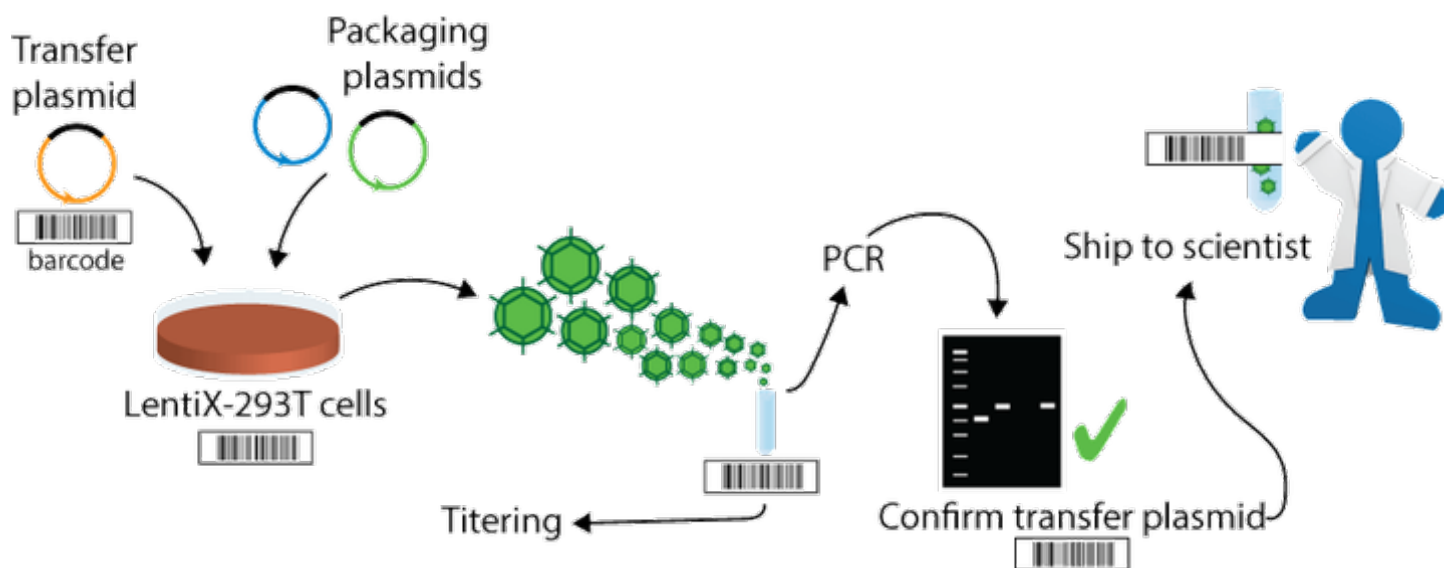
### *Sterility*

The majority of our lentiviral vectors are titered using the [colony formation assay](#). This assay involves transducing cells with serial dilutions of a lentiviral vector, and allowing antibiotic-resistant colonies to expand for 2 weeks. During this long-term cultivation, cells are routinely checked for signs of microbial contamination.

# Viral production at Addgene (CONT'D)

## Purity

When possible, all plasmids used for viral production are propagated in the endA-mutated NEB Stable strain of *E. coli*. In addition, plasmids are typically prepared using endotoxin-free plasmid purification kits.



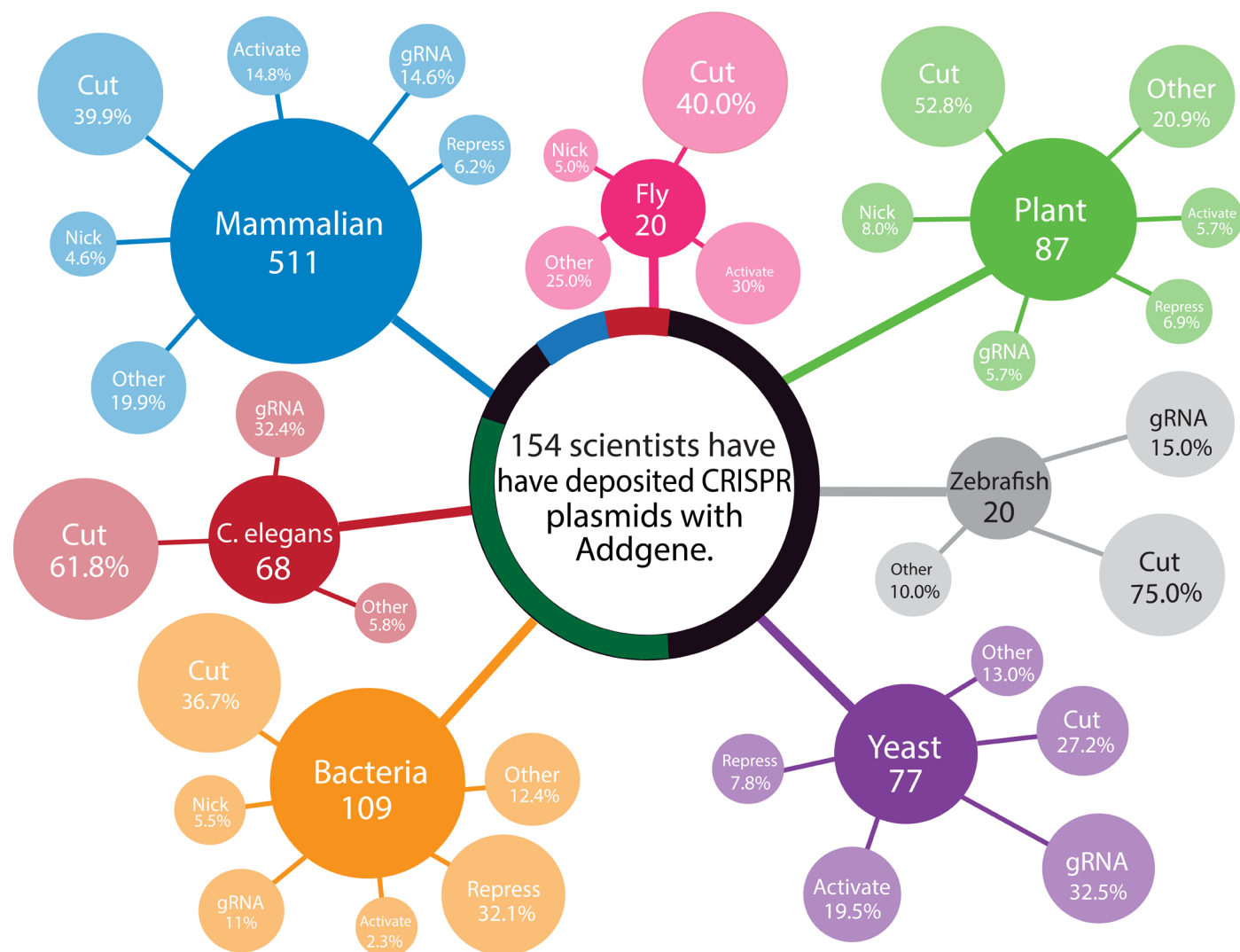
*Schematic of the barcode matching system and PCR-based confirmation of transfer plasmids used in Addgene's viral production. Barcoding allows our DNA and virus to be monitored throughout production, ensuring the identity of each sample from the DNA tube all the way to the viral preparation. After production, we confirm the identity of the transfer plasmid in the viral preparation by performing PCR against unique regions of the transfer plasmid.*

## Lentivirus resources

- Browse [lentiviral preps](#) available from Addgene.
- After receiving your virus from Addgene, read our detailed [recipient instructions](#) for next steps.
- Plan an experiment using our [virus protocols](#).
- Browse our plasmid collection of [popular lentiviral vectors](#).

# Taking CRISPR further with Addgene's viral service

By Tyler Ford | December 6, 2016



Numbers in the large colored circles are rough approximations of the total number of CRISPR plasmids for that particular organism available at Addgene. Percentages represent the fraction of that total with the indicated function.

## Why provide CRISPR viruses?



*"Like all things in science, getting a high titer Cas9 lentivirus prep takes a bit of time and effort that, for many groups, may not be worthwhile. Particularly if, in the end, all they want to do is to quickly generate a single cell line and proceed to use it for experiments."*

- Alex Chavez, researcher at the [Wyss Institute for Biologically Inspired Engineering](#) and member of the Addgene Advisory board, on the difficulty of producing CRISPR lentivirus.

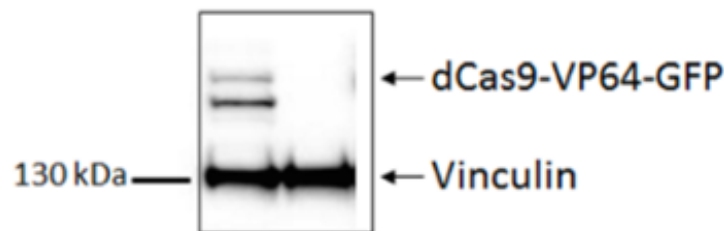


## Taking CRISPR further with Addgene's viral service (CONT'D)

One huge reason [CRISPR](#) has become such a popular genome editing tool is its developers' willingness to make their CRISPR technologies available to the academic research community. At Addgene, we've helped distribute many of these technologies in plasmid form and are proud to have facilitated their fast adoption. However, in many cases, the plasmids themselves are only the starting point for the production of viruses used to deliver CRISPR components to cells or organisms under study. In the past we've left the arduous task of virus production to individual labs. Now we're very excited to provide ready-to-use [CRISPR lentiviral preps](#) to researchers across the globe.

As we've heard from scientists, producing lentivirus is not always an easy process. The cloning capacity of [lentiviral vectors](#) (~8.5 kb of insert) and the reduced titer associated with large inserts can make lentivirus production frustrating - a researcher can complete an entire lentiviral production protocol only to find that the low titer of her prep makes it unuseable. With the large size of the SpCas9 nuclease (~4.2 kb) this is a particularly prominent problem in CRISPR genome editing experiments. We aim to alleviate this frustration by [producing, titrating, testing, and providing lentiviral preps](#) of select lentiviral vectors from the repository for you. We perform test infections with all of our viral preps and host our expression data (western blots or fluorescent microscopy) on the material pages associated with each item.

There are multiple CRISPR tools available as lentivirus and we'll be adding more as the need arises. For example, we've teamed up with the Broad Institute to provide lentiviruses for delivery of [SpCas9 and Cas9 nickase](#) for genome editing. You can also enhance expression of your gene of interest with a variety of [Cas9 activators](#) available in lentiviral format. Finally, while we can't currently produce custom gRNA-containing lentivirus, we are providing [lentiviral preps containing highly requested gRNAs](#) including those targeting EGFP, BRAF, and MAP3K4. The gRNAs targeting EGFP can be used as positive controls for gene editing or gene activation, while the BRAF and MAP3K4 gRNAs allow you to target these widely studied signalling pathways.



Sample western blot showing dCas9-VP64-GFP expression after lentiviral transduction.

## Pooled libraries are now available in lentiviral format



Another popular set of tools in the repository are [CRISPR pooled gRNA libraries](#) that can be used to screen for genes involved in a wide variety of cellular processes. While these are widely requested and incredibly useful tools, they require extensive background work from the requesting scientist for their proper use; after receiving a pooled gRNA library in plasmid format, a researcher must amplify the library, use next-generation sequencing to ensure that the library has retained gRNA representation, produce high titer lentivirus from the library, ensure gRNA representation in the viral prep, infect target cells, and, finally, conduct the screen.

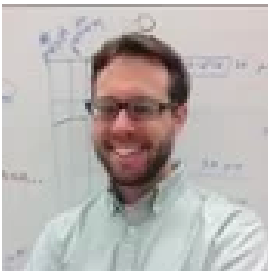
To help alleviate some of this labor for our users, we now provide the [a variety of pooled libraries](#) from the labs of John Doench and David Root as ready-to-use lentiviral preps. Each library uses nearly 80,000 gRNAs to



## Taking CRISPR further with Addgene's viral service (CONT'D)

target around 20,000 individual genes. We provide enough lentivirus for you to both perform an optimization-scale infection and at least one screening-scale experiment achieving the gRNA representation recommended in the publications associated with these libraries. Please note, the pooled libraries we distribute in lentivirus format only deliver gRNA and, therefore, must be used to infect a cell line already expressing Cas9.

As [John Doench](#) (Broad Institute and member of the Addgene Advisory Board) says:



*“When you’re setting up to do a genetic screen, you need to focus on building a really good model that reflects the underlying biology you’re interested in studying. You need to make sure your assay is scalable and robust. What you don’t need to do is spend your time troubleshooting reagent production, because that’s a distraction from your primary goal. Getting these quality-controlled reagents from Addgene can greatly expedite a researcher’s ability to make discoveries.”*

If you ever run out of ready-to-use virus and want to make new lentiviral preps on your own, fear not - anytime you order ready-to-use lentivirus from Addgene, you’ll also receive transfer plasmid DNA that you can use along with lentiviral packaging plasmids to create your own lentivirus stocks. Our research scientists have provided their standard [lentivirus production protocol](#) here and you can find more tips and protocols for working with lentivirus on our [blog](#) and [protocol pages](#).

We’re excited to provide this new service for researchers wanting an easier way to use CRISPR and would love to hear from you if you have used or are thinking about using our viral services. Please send any questions or comments to [help@addgene.org](mailto:help@addgene.org).

*Finally we’d like to thank all those who are allowing us to distribute their CRISPR tools as ready-to-use virus and, in particular, the [Church lab](#) at the Wyss Institute for Biologically Inspired Engineering and the [Zhang](#) and [Doench/Root](#) labs from the Broad Institute for providing us with advice and expertise as we’ve gotten these services up and running. Both Alex Chavez and John Doench are members of the Addgene Advisory Board.*

# ACKNOWLEDGEMENTS AND FINAL WORDS

## Many thanks to our contributors!



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Katrina Armstrong is a Neurophysiology Msc Student at the University of Manitoba. Her project is examining the use of DREADDs (Designer Receptors Exclusively Activated By Designer Drugs) to determine the role of serotonin in movement. Follow her on twitter [@katrinabboards](https://twitter.com/katrinabboards).



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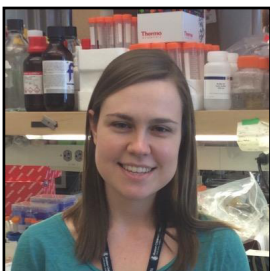
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Harshana S De Silva Feelixge is a researcher whose work has focused on gene therapy for viral infections. She is particularly passionate about HIV cure research and science communication.



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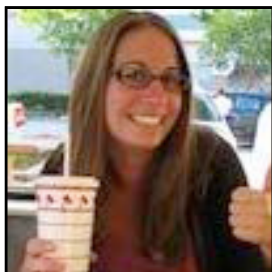
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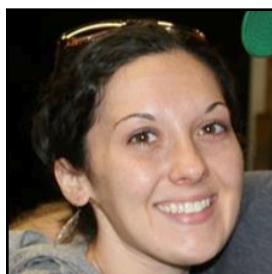
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# ACKNOWLEDGEMENTS AND FINAL WORDS

If you have any questions, comments, or suggestions about how Addgene can improve its educational content, please [contact us](#).

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