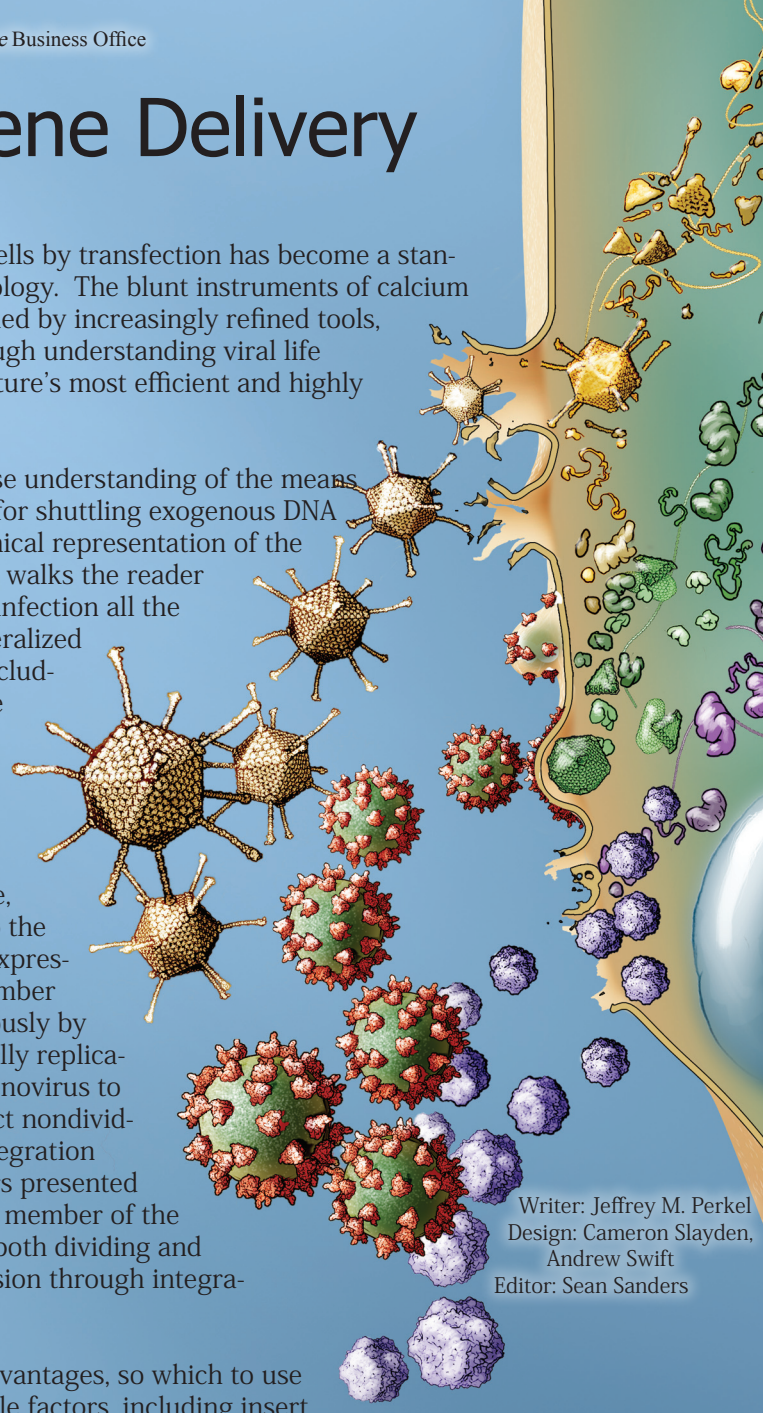
This poster brought to you by the AAAS/*Science* Business Office

Viral Mediated Gene Delivery

The introduction of foreign DNA into eukaryotic cells by transfection has become a standard and indispensable technique in molecular biology. The blunt instruments of calcium phosphate and electroporation are being superseded by increasingly refined tools, including recombinant viruses. Researchers, through understanding viral life cycles and gene function, have co-opted one of nature's most efficient and highly evolved mechanisms for infecting cells.

We have created this educational poster to increase understanding of the means and mechanisms available to you, the researcher, for shuttling exogenous DNA into your cell type of choice. The annotated graphical representation of the viral life cycles for adenovirus, AAV, and lentivirus walks the reader through the important steps in the pathway from infection all the way to replication and release. We provide a generalized protocol for transfection utilizing these viruses, including some tips and tricks that we hope will improve your success in the lab.



Representing the Adenoviridae family is the familiar adenovirus. An icosahedral, nonenveloped virus with broad tropism, adenovirus can infect both dividing and quiescent cells. This large, double-stranded DNA virus does not integrate into the genome, making it limited to transient, episomal expression. Adeno-associated virus (AAV), an ssDNA member of the family Parvoviridae, was discovered fortuitously by researchers working with adenovirus. As a naturally replication-deficient virus, it requires the presence of adenovirus to complete its life cycle. Like adenovirus, it can infect nondividing cells, but it has the additional advantage of integration competence. Rounding out the trio of virus vectors presented here is lentivirus, the newcomer to this field and a member of the Retroviridae family. An ssRNA virus, it can infect both dividing and nondividing cells, as well as provide stable expression through integration into the genome.

Writer: Jeffrey M. Perkel
Design: Cameron Slayden, Andrew Swift
Editor: Sean Sanders

Each of these viruses has its advantages and disadvantages, so which to use for a particular experiment may depend on multiple factors, including insert size, titer required, target gene expression level, type of expression desired (short- or long-term), and target cell type. Modification of viral capsids by genetic manipulation has allowed for altered tropism of the manipulated virus, an approach that has been used successfully both to overcome the broad tropism of adenovirus and AAV, and to alter the target cell type of all three viral vectors.

Delivery of foreign DNA using viral vectors has not been without its problems, particularly when attempts were made to apply the technology to gene therapy. However, through better understanding of our immune response, as well as of the viruses themselves, researchers are working toward safer, more effective viral-based treatments. In the meantime, the refinement of viral-mediated gene delivery protocols continues, providing a convenient, flexible, and reproducible system for in vitro transfection studies.

Sean Sanders, Ph.D.
Commercial Editor
AAAS/*Science*

Viral Mediated Gene Delivery

Viral Life Cycles

Generally, once bound to its receptor, a virus enters the cell via clathrin-mediated endocytosis, exits the endosome via a change in pH, and makes its way to the nucleus, for instance via actin polymerization or using molecular motors. What happens next depends on the nature of the virus.

As epichromosomal viruses, adenoviruses do not integrate their nucleic acid into their host genomes; they simply initiate a program of gene expression, using both cellular and viral proteins, to systematically produce the proteins necessary to build new virion particles. AAV and lentiviruses, in contrast, do integrate into their hosts. Upon delivery to the nucleus, AAV uses its genomic inverted terminal repeats to self-prime the synthesis, using host-cell machinery, of its complementary DNA strand. Then, via the AAV Rep protein, the virus integrates specifically into the long arm of chromosome 19; in this specificity of integration site, AAV is unique among mammalian viruses. Lentiviruses, by contrast, use viral reverse transcriptase to create DNA copies of their RNA genome, and cellular machinery to create the second strand. The resulting double-stranded molecule can integrate anywhere within the mammalian genome using a virally encoded integrase.

Construction (packaging) of a viable virion requires inclusion of the viral nucleic acid. Among the enzymes encoded by the adenovirus genome are DNA replication components (products of the E4 genes) that duplicate the viral DNA. For AAV, co- or superinfection with a helper virus, such as herpes simplex virus or adenovirus, leads to viral rescue (genomic excision), followed by virion production (unlike lentivirus, AAV integrates as a latent provirus). Lentiviruses need not be excised; provirus transcription produces the two positive-strand viral mRNA molecules that, along with the reverse transcriptase enzyme, are inserted into the membrane-bound virion.

Finally, following assembly of a complete infectious particle, the virus exits the cell, either by budding–as in the case of lentiviruses–or by lysis, to then infect another cell.

So you've cloned some interesting gene. Now you want to take it for a test drive. Your standard option: DNA transfection. But sometimes, transfection just isn't good enough. Maybe your cells are refractory to $CaCl_2$, lipofection, and electroporation. Perhaps you can get the DNA in, but expression is weak. Or maybe you want to deliver the gene in vivo. Out of luck? Not with viral transfection. A range of adenovirus, adeno-associated virus, and lentivirus based systems now exists to overcome these issues.

Adenovirus
Genome: 36kb
Tropism: Broad
Enveloped: No
Primary Receptors: Coxackievirus and adenovirus receptor
Key Coreceptors: αVβ3 and αVβ5 integrins
Nucleic Acid: dsRNA
Integration: No

Lentivirus
Genome: 9.2kb
Tropism: Helper T cells, macrophage, monocytes, intestinal epithelia, brain
Enveloped: Yes
Primary Receptor: CD4
Key Coreceptors: CCR5, CXCR4
Nucleic Acid: ssRNA
Integration: Yes

AAV
Genome: 4.7kb
Tropism: Broad (usually requires adenovirus co-infection)
Enveloped: No
Primary Receptor: Heparin sulfate proteoglycan
Key Coreceptors: FGF receptor, αVβ5 integrins, hepatocyte growth factor receptor
Nucleic Acid: ssDNA
Integration: Yes

Tip:
When optimizing your work, be sure to use the provided positive controls, as well as a vector-minus (or insert-minus) negative control. And try a range of multiplicities of infection to optimize infection efficiency.

Trick:
When amplifying viral vectors in *E. coli*, use a recA⁻ strain. When amplifying virus stocks in cell lines, use the lowest possible cell passage number you can.

Tip:
When it comes to viral work, cleanliness matters. Use the purest DNA you can make, ideally prepared via CsCl gradient centrifugation. Once you have intact virus, be sure to concentrate and purify that, too, using either CsCl banding or a commercial virus purification kit.

Tip:
Freeze stocks at -80°C. But minimize the number of freeze-thaw cycles, for instance by freezing single-use aliquots. Avoid freezing and thawing more than three times.

Basic Adenovirus Transfection Protocol

clone your insert into the multiple cloning site of your shuttle vector

Linearize

transfect linearized, purified plasmid into a recA-positive bacterial cell line, either concurrently with an adenoviral vector, or afterward

identify recombinants based on colony size (recombinants should be smaller than background colonies)

amplify plasmid in a second bacterial strain

prepare purified DNA (need approximately 5μg per transfection)

introduce linearized DNA into a packaging cell line (e.g., HEK293, which expresses adenoviral protein E1 required for virion production, lacking in some adenoviral vectors)

grow for 7 to 10 days

collect growth medium containing virions

titer virus stock by plaque assay and concentrate as necessary

infect your cells of interest

observe for morphologic changes: infected cells typically round up and detach from the plate; nuclei will become enlarged

Adenovirus:
The adenovirus genome is large and contains few restriction sites, making it difficult to build a recombinant virus directly. Most researchers use a shuttle vector, which then transfers the gene or sequence of interest via homologous recombination, either in an adenovirus packaging cell line or in bacteria.

Lentivirus:
The inserted fragment, once in the lentiviral vector, is flanked by the 5' and 3' long-terminal repeats (LTRs) and the Ψ packaging signal necessary for incorporation into an infective (but replication-incompetent) virion. Helper plasmids are often used to supply missing viral activities needed to build the virus particles (*gag*, *pol*, and *rev*).

Adeno-Associated Virus:
AAV typically requires co- or superinfection with a helper virus, such as adenovirus or herpes simplex virus; alternatively, missing viral genes (E2A, E4, VA, and E1) can be supplied by cotransfection. AAV vectors typically contain inverted terminal repeats necessary for genomic integration. Watch for yellowing of culture medium as well as rounding up of cells after about three days.

Trick:
Use multiple independent isolates to reduce the likelihood of clone-specific variations.

Trick:
Insert size and content matter. Some genes are toxic to the host or packaging cells, producing lower titers. In that case, try an inducible expression system. Other sequences may be too short or too long to be efficiently packaged. Use filler DNA to increase the insert size if necessary, or alternatively, concentrate your virus. Be aware that some inserts may be too large for your chosen system.

Useful Viral Modifications

Commercial viral transfection systems contain a number of useful modifications to limit viral replication (and thus increase user safety), broaden tropism, and make room for gene insertions. Some adenovirus-5 vectors have E1 and E3 deleted, for instance. E3 is nonessential, and its deletion enables larger sequence insertions. E1 is required, and its removal renders the virus incapable of replication; it must therefore be supplied either by a packaging cell line (such as HEK293) or a helper plasmid. Similarly, some lentiviral- and AAV-based systems have transferred structural and enzymatic genes from the viral vector to helper plasmids, both to increase room for insertions and to make the resulting virions replication-defective. Lentiviral vectors sometimes contain deletions of the 3' long terminal repeat (LTR) enhancer that has no effect on packaging, yet ensures inactivation following genome insertion – another biosafety measure. Finally, gene sequences may be altered in helper plasmids to prevent homologous recombination (either with the viral vector or endogenous sequences) and subsequent regeneration of functional virus.

If viral tropism is too narrow, it can be altered or broadened using different surface glycoproteins to direct virions at other, nonstandard cellular targets. One technique, for instance, is to replace the lentiviral coat envelope gene with one encoding the more promiscuous Vesicular Stomatitis Virus G glycoprotein.

Other modifications have been engineered to ease construction of the viral vector itself – addition of a multiple cloning site, for instance, or inclusion of recombinase-based gene transfer elements, strong promoters, polyadenylation sequences, useful restriction sites (e.g., for linearization), or purification tags.

Safety

Useful though they may be, viruses are pathogens and should be handled as such. Additionally, because modified viruses comprise recombinant nucleic acids, they should be manipulated in accordance with the National Institutes of Health's Guidelines for Research Involving Recombinant DNA Molecules.

Work with recombinant viruses should be performed under at least biosafety level-2 conditions (see *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed., from the US Centers for Disease Control and Prevention). According to the CDC, that means using a class II or better biological safety cabinet (i.e., laminar flow hood), gloves, lab coat, and possibly a face shield. These measures protect both the user and the work by ensuring that, just as the user cannot be infected, neither will the virus be modified by recombination or genetic shuffling with "wild" pathogens. Filtered pipettes and tips should be used to prevent contamination of liquid-handling instruments.

Special care should be taken when making recombinant viruses capable of expressing activated human oncogenes—these oncoviruses can potentially infect human cells and may even integrate into the host genome. This is especially true if the virus has been modified to alter its tropism to make it more broadly infective. Another risk: recombination between the viral construct and inactive cellular proviruses may create active, infective virus particles.

Science
AAAS
STRATAGENE
An Agilent Technologies Company