REFERENCES AND ABSTRACTS OF PAPERS PUBLISHED ON METHODS FOR PURIFYING, QUANTIFYING AND ANALYSIS OF rAAV VECTORS

Since 1999 at least 800 papers have been published that have described the use of iodixanol gradients for the purification and concentration of rAAV. This selection of papers has concentrated principally on the methods used for production, purification and analysis of rAAV. The references are listed according to year of publication.

Zolotukhin, S., Byrne, B.J., Mason, E., Zolotukhin, I., Potter, M., Chesnut, K., Summerford, C., Samulski, R.J. and Muzyczka, N. (1999) *Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield* Gene Ther., **6,** 973-985
Conventional methods for rAAV purification that are based on cesium chloride ultracentrifugation have often produced vector preparations of variable quality and resulted in significant loss of particle infectivity. We report here several novel purification strategies that involve the use of non-ionic iodixanol gradients followed by ion exchange or heparin affinity chromatography by either conventional or HPLC columns. These methods result in more than 50% recovery of rAAV from a crude lysate and routinely produce vector that is more than 99% pure. More importantly, the new purification procedures consistently produce rAAV stocks with particle-to-infectivity ratios of less than 100, which is significantly better than conventional methods. The new protocol increases the overall yield of infectious rAAV by at least 10-fold and allows for the complete purification of rAAV in 1 working day. Several of these methods should also be useful for large-scale production.

Summerford, C. and Samulski, R.J. (1999) *Viral receptors and vector purification: New approaches for generating clinical-grade reagents* Nat. Med., **5**, 587-588
Extract of text: Two independent laboratories have developed protocols in which heparin, an analog of the natural receptor for AAV, is used as the affinity matrix for AAV vector purification. In each protocol, a commercially available heparin affinity column is used (POROS HE/M heparin, Boehringer Mannheim, Germany). Given that heparan sulfate proteoglycan is known to serve as viral receptor for AAV, the success of heparin columns in rAAV purification and their superiority over cellulose sulfate or ion-exchange columns is not surprising. A potential problem with the use of this affinity approach is that many cellular proteins are also known to associate physically with heparin. Thus, use of a heparin column requires the incorporation of a specific strategy to remove contaminating heparin-binding proteins. Each laboratory has taken a different successful approach to address this problem. In one study, Zolotukhin et al. semi-purified virus from a 'freeze/thaw' cell lysate by centrifugation in a density step-gradient of non-ionic media (iodixanol). The semi-pure preparation was then applied to an HPLC heparin column (POROS HE/M heparin) for further purification. The procedure takes less than 1 day and is superior to traditional CsCl centrifugation in that it results in a higher yield of virus (greater than tenfold) that is more infectious (lower total particle-to-infectious particle ratio). The particle-to-infectivity ratios are consistently less than 100:1, which is considerably better than the 1,000:1 ratio often resulting from traditional purification strategies. The reduced particle-to-infectious particle ratio is presumably due to the use of more benign conditions, as well as the use of a molecule for purification that is essentially analogous to the natural receptor of the virus. The iodixanol/heparin procedure for rAAV purification is fast (completion in I working day), convenient (uses a commercially available column), reproducible and results in 50-70% recovery of virus (yield) that is greater than 99% pure.

Monahan, P.E. and Samulski, R.J. (2000) *AAV vectors: is clinical success on the horizon?* Gene Ther. **7**, 24-30
Potential applications and impact of the adeno-associated virus (AAV) as a gene transfer vector have expanded rapidly in the last decade. Recent advances in the production of high-titer purified rAAV vector stocks have made the transition to human clinical trials a reality in the last moments of the millenium. Production improvements will be complemented in the coming years with understanding of and innovations in the targeting and packaging of rAAV, the design of transgene cassettes, and the host immune response to the vectors. These expected areas of progress are discussed, with special attention to clinical applications for which rAAV vectors may help close the gap towards successful gene therapy.

Pfeifer, A. and Verma, I.M. (2001) *Gene therapy: Promises and problems* Annu. Rev. Genom. Hum. Genet., **2**, 177-211
Gene therapy can be broadly defined as the transfer of genetic material to cure a disease or at least to improve the clinical status of a patient. One of the basic concepts of gene therapy is to transform viruses into genetic shuttles, which will deliver the gene of interest into the target cells. Based on the nature of the viral genome, these gene therapy vectors can be divided into RNA and DNA viral vectors. The majority of RNA virus-based vectors have been derived from simple retroviruses like murine leukemia virus. A major shortcoming of these vectors is that

they are not able to transduce nondividing cells. This problem may be overcome by the use of novel retroviral vectors derived from lentivirus, such as human immunodeficiency virus (HIV). The most commonly used DNA virus vectors are based on adenoviruses and adeno-associated viruses. Although the available vector systems are able to deliver genes in vivo into cells, the ideal delivery vehicle has not been found. Thus, the present viral vectors should be used only with great caution in human beings and further progress in vector development is necessary.

Rohr, U-P., Wulf, M-A., Stahn, S., Steidl, U., Haas, R. and Kronenwett, R. (2002) *Fast and reliable titration of recombinant adeno-associated virus type-2 using quantitative real-time PCR* J. Virol. Methods, **106**, 81-88
In this study, a quantitative real-time PCR (qPCR) was developed to determine genomic rAAV-2 titers using the Light-Cycler technology. Since the CMV promoter is the most commonly used promoter in gene therapeutic approaches, primers were designed which hybridize with the human CMV promoter sequence. PCR products were detected by the addition of SYBR green. qPCR of a 5 log spanning serial dilution of the vector plasmid containing one CMV promoter per plasmid molecule yielded a high amplification efficiency of 1.99 per cycle. To quantify the copy number of viral genomes, the qPCR curves of adeno-associated virus type 2 (AAV-2) samples were related to a standard curve assessed by the 5 log spanning serial vector plasmid dilution (0.01-100 pg DNA). For validation of the method, rAAV-2 preparations were analyzed by a standard method and qPCR in parallel. As standard method, flow cytometry was used for titration of infectious viral particles on HeLa cells using the Enhanced Green Fluorescent Protein as a marker. A significant correlation was found between the results obtained by flow cytometry and the results from the qPCR over a 5 log range ($r=0.85$, $P<0.0001$). The mean ratio between infectious rAAV-2 particles titrated via flow cytometry and genomic copies of rAAV-2 measured by qPCR of the same sample was 1:253. The higher titers found by qPCR might be due to multiple transduction of a single cell or to non-infectious particles generated during rAAV-2 preparation. In conclusion, qPCR is a fast and reliable method for determination of rAAV-2 titers and might be a powerful tool for standardization of rAAV-2 preparations particularly in the context of clinical studies.

De, B., Mendez, B., Hackett, N.R., Kaminsky, S.M. and Crystal, R.G. (2002) *Adaptation of laboratory grade recombinant AAV production to manufacture of vector for human administration* 5th. Annu. Meeting Am. Soc. Gene Ther., Abstr. 147
Gene transfer using AAV2 vectors is applicable to the treatment of human genetic disease due to the persistent expression of the transgene in many organs. However, laboratory grade AAV2 production uses a number of protocols that are not practical to scale to large batch size for vector in quantities relevant to human applications, and the process is difficult to adapt to Good Manufacturing Practice. The objective of this work is the transition of laboratory grade AAV2 vector production to GMP production including the identification and control of possible sources of contaminants, identification of critical control parameters in the production process and demonstrating that the overall process is robust. The simplest AAV2 production method was deemed to be the two plasmid cotransfection method in which the recombinant AAV2 plasmid consisting of the expression cassette for the therapeutic gene between the AAV2 inverted terminal repeats is contransfected with a helper plasmid providing the AAV2 rep and cap proteins driven by the mouse mammary tumor virus (MMTV) promoter and the Ad E2, E4 and VA genes driven by their own promoter. Two different GMP certified 293 derived cell lines were assessed and shown to produce similar yields of recombinant AAV2 vector (by anti-capsid ELISA) after cotransfection by either the $CaPO_4$ or Polyfect (QIAGEN) method. The rAAV yield was limited by the amount and purity of the plasmids used for cotransfection and yield increased in linear manner with plasmid quantity from 0.4 to 16 mg per 15 cm plate of cells. The optimal purification method consisted of making a crude viral lysate by freeze thaw followed by a discontinuous iodixanol gradient. Isolated rAAV is affinity purified using a salt gradient on a heparin agarose column and desalted by spin gel filtration. The discontinuous iodixanol gradient was optimized for separation of infectious rAAV (assessed by gene transduction) relative to empty AAV2 capsids. Excluding deoxycholate from the purification process, to avoid testing for residual deoxycholate in the product, was assessed, but high purity product was not obtained. On that basis, several batches of rAAV expressing a variety of different reporter genes and therapeutic genes have been produced. Purity assessed by SDS-PAGE was >95% and average yield (n=34 preparations) was $3.9x10^3$ particle units by ELISA per transfected cell. Preparations were also assessed for genome content by TaqMan realtime quantitative PCR using primers and probes specific to the promoter and therefore applicable to a number of different vectors. Electron microscopy provides an independent measure of the relative abundance of empty capsid particles and a general measure of the quality of the lot. These data show the existence of a robust process for rAAV production in which critical production parameters have been identified, raw materials identified and a batch record written based on an extensive series of laboratory grade preparations.

Zolotukhin, S., Potter, M., Zolotukhin, I., Sakai, Y., Loiler, S., Fraites, T.J., Chiodo, V.A., Phillipsberg, T., Muzyczka, N., Hauswirth, W.W., Flotte, T.R., Byrne, B., and Snyder, R.O. (2002) *Production and purification of serotype 1,2, and 5 recombinant adeno-associated viral vectors* Methods, **28**, 158-167

Recombinant adeno-associated viral (rAAV) vectors based on serotype 2 are currently being evaluated most extensively in animals and human clinical trials. rAAV vectors constructed from other AAV serotypes (serotypes 1, 3, 4, 5, and 6) can transduce certain tissues more efficiently and with different specificity than rAAV2 vectors in animal models. Here, we describe reagents and methods for the production and purification of AAV2 inverted terminal repeat-containing vectors pseudotyped with AAV1 or AAV5 capsids. To facilitate pseudotyping, AAV2*rep*/AAV1*cap* and AAV2*rep*/AAV5*cap* helper plasmids were constructed in an adenoviral plasmid backbone. The resultant plasmids, pXYZ1 and pXYZ5, were used to produce rAAV1 and rAAV5 vectors, respectively, by transient transfection. Since neither AAV5 nor AAV1 binds to the heparin affinity chromatography resin used to purify rAAV2 vectors, purification protocols were developed based on anion-exchange chromatography. The purified vector stocks are 99% pure with titers of 1×10^{12} to 1×10^{13} vector genomes/ml.

K. R. Clark (2002) *Recent advances in recombinant adeno-associated virus vector production* Kidney Int., **61**, Sympos. 1, S9-S15

Adeno-associated virus (AAV) is a replication-defective parvovirus that is being developed as a vector for human gene transfer. Recombinant AAV (rAAV) vectors are being proposed as a gene transfer vehicle for an array of human diseases. The recent interest in rAAV has been driven by the unexpected finding that these simple vectors can efficiently transduce a variety of postmitotic cells, resulting in long-lived, robust gene expression. However, a major obstacle to commonplace usage of rAAV vectors was the production in sufficient quantities for preclinical and human trials. Fortunately, several recent technological advances in vector production, purification, and titration have resulted in significant increases (>10-fold) in production capacity. Thus, there are several methods for the production of rAAV in excess of 10^4 particles/cell, levels that should permit widespread use of this technology for clinical applications.

Mistry, A.R., De Alwis, M., Feudner, E., Ali, R.R. and Thrasher, A.J. (2002) *High-titer stocks of adeno-associated virus from replicating amplicons and herpes vectors* Methods Mol. Med., **69**, 445-460

The adeno-associated virus (AAV) is a nonpathogenic member of the Parvoviridae family (for review, see ref. 1) Recently this virus has gained considerable interest and has been developed as a gene delivery vector (2). Six primate AAV serotypes (designated AAV types 1–6) have so far been identified and characterized in the literature (3,4). The most extensively studied of these isolates is AAV type 2. The vast majority of the transduction studies have been carried out using recombinant vectors (rAAV) based on serotype 2. These studies have shown that rAAV2 has the ability to transduce a wide range of both dividing and nondividing cells, achieving efficient long-term gene expression in vivo in a variety of tissues including retina (5), muscle (6), central nervous system (7), and liver (8). The range of tissues transduced by recombinant AAV vectors based on other serotypes is currently being investigated by several laboratories (9). It is hoped that rAAV vectors produced from these serotypes may prove to be useful for the transduction of tissues that are poorly infected by AAV2. A major problem associated with the use of rAAV has been the difficulty in producing large quantities of high-titer stock (10,11). This has become an important issue as vectors based on rAAV2 have now reached the stage at which they are starting to be used in human clinical trials (12). This chapter describes the use of the herpes simplex virus type 1 (HSV-1) amplicons that have been developed in our laboratory to attain high-titer stocks of rAAV2 (13). A brief description of the background and basis of the system is given in the first section.

Grimm, D., Kay, M.A. and Kleinschmidt, J.A. (2003) *Helper virus-free, optically controllable and two-plasmid-based production of adeno-associated virus vectors of serotypes 1 to 6* Mol. Ther., **7**, 839-850

We present a simple and safe strategy for producing high-titer adeno-associated virus (AAV) vectors derived from six different AAV serotypes (AAV-1 to AAV-6). The method, referred to as “HOT,” is helper virus free, optically controllable, and based on transfection of only two plasmids, i.e., an AAV vector construct and one of six novel AAV helper plasmids. The latter were engineered to carry AAV serotype *rep* and *cap* genes together with adenoviral helper functions, as well as unique fluorescent protein expression cassettes, allowing confirmation of successful transfection and identification of the transfected plasmid. Cross-packaging of vector DNA derived from AAV-2, -3, or -6 was up to 10-fold more efficient using our novel plasmids, compared to a conservative adenovirus-dependent method. We also identified a variety of useful antibodies, allowing detection of Rep or VP proteins, or assembled capsids, of all six AAV serotypes. Finally, we describe unique cell tropisms and kinetics of transgene expression for AAV serotype vectors in primary or transformed cells from four different species. In sum, the HOT strategy and the antibodies presented here, together with the reported findings, should facilitate and support the further development of AAV serotype vectors as powerful new tools for human gene therapy.

Lee, K., Kim, Y-G., Jo, E-C. (2003) *Shuttle PCR-based cloning of the infectious adeno-associated virus type 5 genome* J. Virol. Methods, **111**, 75-84

Adeno-associated virus type 5 (AAV5), which is distinct from the other serotypes of AAV, has attracted considerable interest as a premier gene delivery vector. As do the other serotypes, AAV5 contains its 4.7 kb-sized, single-stranded genome flanked with inverted terminal repeats (ITRs) in a hairpin conformation, which serves frequently as pause and arrest sites for DNA polymerases during PCR. To amplify the full-length of the AAV5 genome in single step, we established a shuttled, long and accurate PCR (LAPCR) procedure in the present study. Furthermore, helper oligonucleotides, which hybridize with the palindromic sequence elements in ITR, were designed and employed in PCR to prevent the formation of hairpin structures by highly GC-rich ITRs. Consequently, a 4.7 kb-sized PCR product was amplified successfully, and cloned into a pBluescript® II KS(+) plasmid. Six plasmids, harboring the full-length AAV5 genome, rescued wild type AAV5 viruses on transfection to HeLa and HEK 293 cells, which were co-infected with helper adenoviruses. Western and Southern blot analyses supported further the fact that the pAAV5 plasmids harbored the full-length AAV5 genome. The PCR method described in this study is applicable for the cloning of genomes containing variable palindromic structures, in addition to AAV genomes of other serotypes.

Harris, J.D., Beattie, S.G. and Dickson, J.G. (2003) *Novel tools for production and purification of recombinant adeno-associated viral vectors* Methods Mol. Med., **76**, 255-267

Extract from Introduction: This novel procedure for rAAV production has yielded high titer stocks with improved infectivity-to-particle ratios compared to previous methodologies. In the development of this method by Zolotukhin et al. 2 x 10^{12} virus particles were purified from 3 x 10^{8} 293 cells with a particle to infectivity ratio of 56: 1. Virus particle titer was determined by a quantitative competitive polymerase chain reaction (PCR) assay and infectious titer determined by the infectious center assay. Hermens et al. investigated the reproducibility and quality of virus stocks purified by iodixanol gradient ultracentrifugation and purified 9.6 x 10^{11}, 3.6 x 10^{11} and 6 x 10^{11} virus particles titered by DNA dot-blot hybridization, with particle to infectivity ratios of 385: 1, 600: 1, and 500: 1, respectively. In this study infectious particle titer was also determined by the infectious center assay. In our studies, we have routinely recovered between 5 x 10^{11} to 2 x 10^{12} viral particles from a total of 2.7 x 10^{8} 293-T cells.

Fraser Wright, J., Chunlin Tang, G.Q. and Sommer, J.M (2003) *Recombinant adeno.associated virus: formulation challenges and strategies for a gene therapy vector* Curr. Opin. Drug Discov. Devel., **6**, 174-178

Recombinant adeno-associated virus (AAV)-based vectors capable of expressing therapeutic gene products in vivo have shown significant promise for human gene therapy. One challenge facing the field is the development of vector formulations to achieve optimal vector safety, stability and efficacy. Formulation challenges for AAV vectors can be divided into those relating to maintaining vector activity during purification and storage, and those relating to efficient target tissue transduction in vivo. AAV vectors are potentially susceptible to loss of activity through aggregation, proteolysis and oxidation, as well as through non-specific binding to product contact materials used for vector purification and storage. These deleterious changes need to be thoroughly characterized, and the conditions and excipients to prevent them need to be identified. For in vivo administration, major vector formulation challenges include optimization of efficiency and specificity of target tissue transduction, and the ability to overcome host immune responses.

Weitzman, M., Young Jr., S.M., Cathhomen, T. and Samulski, T.J. (2003) *Targeted integration by adeno-associated virus* Methods Mol. Med., **76**, 201-219

The integration of foreign DNA into the genomes of host cells is of fundamental importance for viral oncology, evolution, transgenic organisms, and gene therapy applications. Expression of foreign genes in eukaryotic cells is highly dependent upon the effi ciency of integration events and the site of insertion. Integration can some-times have detrimental effects on the host cell, such as insertional mutagenesis or activation of proto-oncogenes. For gene therapy, it would therefore be attractive to target insertion to innocuous chromosomal sites. Under-standing the mechanisms for integration of foreign DNA and target site selection is crucial for approaches where long-term expression from delivered transgenes is required. One of the most promising viral vector systems is based on the adenoassociated virus (AAV). AAV vectors consist of a transgene cassette flanked by viral inverted terminal repeats (ITRs). AAV is unique in that the wild-type virus can preferentially integrate its genome in a site-specific manner into an integration locus (AAVS1) on human chromosome 19. Understanding the requirements and mechanism for site-specific integration will provide insights into how targeting can be incorporated into gene therapy vectors. Targeted vectors will avoid the potential hazards of insertional mutagenesis and will remove the positional effects on gene expression from the integrated provirus. This chapter describes what is known about targeted integration by AAV and assays that have been developed to study this process, in order to harness it for transgene integration.

Faust, S.M., Bowles, D.E., Cunningham, S.E., Rabinowitz, J.E. and Samulski, R.J. (2004) *Universal purification of AAV serotypes 1-5 modified to contain a heparin binding epitope* Mol. Ther., **9** Suppl. 1 S36
To directly evaluate the utility of distinct serotypes of adeno-associated virus (AAV) for gene therapy applications, it will be necessary to purify each in a similar manner. The methodology to obtain highly purified rAAV2 for use in clinical trials has been established using heparin column affinity binding in conjunction with iodixanol gradients. With the exception of AAV3, the other serotypes of AAV do not bind heparin. Recently the amino acids responsible for the ability of AAV2 to bind to heparin were identified (Kern et al 2003, J Virol 77:11072–81) and tested in the context of rAAV5 (Opie et al 2003, J Virol 77:6995–7006). To assess whether all serotypes could be modified to bind heparin, the AAV2 amino acids R585 R588 and A590 were substituted into the homologous positions in serotypes 1, 3, 4, and 5 to generate the pxr1RRA, pxr3RRA, pxr4RRA, and pxr5RRA plasmids. These helper vectors were then used to produce recombinant eGFP virus which were initially purified by either cesium chloride or iodixanol gradients followed by dialysis. 1 × 1010 viral genome-containing particles from serotypes 1–5 and the analogous RRA-containing serotypes were applied to three types of commercially available heparin agarose (Sigma: Heparin type I (cat # H6508), II-S (cat # H3025), and III-S (cat # H1277)). As expected, rAAV2 and rAAV3 bound all three types of heparin with rAAV2 binding type III-S and rAAV3 binding type I heparin most efficiently. As expected AAV1, 4, and 5 did not bind to most types of heparin; however, there were some exceptions. Most notably, approximately 70% of rAAV4 bound type III-S heparin while approximately 40% of AAV1, 4, and 5 bound to and eluted from type II-S heparin. These results suggest that other types of heparin should be considered in the optimal purification of AAV serotypes. AAV serotypes modified to contain the RRA epitope bound and eluted from all types of heparin agarose tested in a profile similar to rAAV2. The binding affinity of rAAV5 RRA was the least efficient with approximately 60% of total virus eluting from the columns. rAAV RRA 1, 3, and 4 bound with greater efficiency (between 80–85%). The ability to purify these AAV serotypes in similar manners will allow more accurate comparisons to be made regarding tissue tropisms. In addition, since every purification method utilized for clinical trials must undergo its own certification, a universal purification scheme for all AAV serotypes would eliminate this need.

Rohr, U-P., Heyd, F., Neukirchen, J., Wulf, M-A., Quietsch, I., Kreoner,-Lux, G., Steidl, U., Fenk, R. Haas, R. and Kronenwett, R. (2005) *Quantitative real-time PCR for titration of infectious recombinant AAV-2 particles* J. Virol. Methods, **127**, 40-45
In this report, we present a fast, reliable and easy to perform method to quantify infectious titers of recombinant AAV-2 (rAAV-2) particles using the LightCycler technology, which is independent from the therapeutic transgene and without the presence of a marker gene. The method is based on the life cycle of AAV-2: after infection of the host cell, the single stranded (ss) AAV-2 genome is converted into a double stranded (ds) form. Following infection with rAAV-2, HeLa cells were lysed and ssDNA of transcriptionally inactive particles were efficiently removed by ssDNA-specific S1 nuclease digestion. The remaining viral dsDNA can be quantified by quantitative real-time PCR (qPCR). For validation of the new method, rAAV-2 preparations were analyzed by two other standard methods for titration of infectious particles in parallel, i.e. the infectious center assay (ICA) as well as flow cytometry using GFP as a marker. Comparing the infectious titers of 40 different AAV-2 fractions assessed by qPCR with the titers determined by FACS analysis a significant correlation ($r = 0.87$, $p < 0.001$) with a mean ratio of the titers assessed by qPCR and FACS of 1.92 (S.D. ± 1.59) was found. Further, the titers of seven rAAV-2 fractions using qPCR and ICA covering 5 log ranges were compared and a significant correlation was found between the results ($r = 0.80$, $p < 0.001$) with a mean ratio of 3.38 (S.D. ± 1.79), respectively.

Zolotukhin, S. (2005) *Production of recombinant adeno-associated virus vectors* Hum. Gene Ther., **16**, 551-557
Recombinant adeno-associated virus (rAAV) is a prototypical gene therapy vector characterized by excellent safety profiles, wide host range, and the ability to transduce differentiated cells. Numerous rAAV-based vectors providing efficient and sustained expression of transgenes in target tissues have been developed for preclinical studies. Interest in rAAV has been driven by advances in production methods originally developed for rAAV serotype 2 vectors and expanded to include alternative serotypes. The transition to clinical trials is dependent on the development of scalable production methods of Good Manufacturing Practice-grade vectors described in this review.

Burova, E. and Ioffe, E. (2005) *Chromatograpahic purification of recombinant adenoviral and adeno-associated viral vectors: methods and implications* Gene Ther., **12**, S5-S17
In recent years, recombinant adenoviral and adeno-associated viral (AAV) vectors have been exploited in a number of gene delivery approaches. The use of these vectors in clinical gene transfer has increased the demand for their characterization, production and purification. Although the classical method of adenovirus or AAV purification by density gradient centrifugation is effective on a small scale, chromatographic separation is the most versatile and powerful method for large-scale production of recombinant adenovirus or AAV. This review describes different chromatographic modes for adenovirus or AAV purification and process development, as well as the utility of different purification steps for virus production. Advances in the development of viral vectors for

gene therapy, such as the discovery of new AAV serotypes, adenoviral and AAV retargeting and improved production of helper-dependent adenoviral vectors, require further development of efficient purification methods.

Duffy, A.M., O'Doherty, A.M., O'Brien, T. and Strappe, P.M. (2005) *Purification of adenovirus and adeno-associated virus: comparison of novel membrane-based technology to conventional techniques* Gene Ther., **12**, S62-S72
Adenovirus (Ad) and Adeno-associated virus (AAV) are efficient gene delivery systems; manipulation of the wild-type genome allows their use as vectors for the overexpression of desirable transgenes. Generation and purification of such viral vectors can be labour intensive, costly and require specialized equipment, but a new generation of membrane-mediated ion exchange kits for purification of recombinant virus may facilitate this process. Here, we examine the yields, transgene expression and purity of preparations of Ad and AAV purified using commercially available kits in comparison to other established techniques for purification of recombinant viral vectors. We demonstrate comparable results for Ad and AAV respectively in all parameters investigated, with a substantial reduction in purification time for the kit-based technology. Such approaches are attractive methods for small-scale purification of recombinant Ad and AAV viral vectors.

Morenweiser, R. (2005) *Downstream processing of viral vectors and vaccines* Gene Ther., **12**, S103-S110
Viral vectors and viral vaccines more and more play an important role in current medical approaches. Gene vectors like adenoviruses, adeno-associated viruses or retroviruses are the vehicles being developed for delivering genetic material to the target cell in gene therapy. Viral vaccines, like attenuated or inactivated rabies virus, influenza virus or hepatitis virus vaccines, are powerful tools to limit the number of serious viral infections and pandemics. Higher safety demands, that is, reduction of side effects, by regulatory authorities like Food and Drug Administration (FDA) and European Agency for the Evaluation of Medicinal Products (EMEA), nowadays force developers as well as manufacturers to improve their production and purification processes for viral vectors and vaccines. Like for influenza viral vaccines, manufacturers begin to switch from egg cultivation to mammalian cell culture systems. Also within the purification procedure, a clear trend from classical purification methods like sucrose gradient centrifugation towards more sophisticated techniques like tangential flow filtration and liquid chromatography can be observed.

Merten, O-W., Geny-Fiamma, C. and Douar, A.M. (2005) *Current issues in adeno-assocaited viral vector production* Gene Ther., **12**, S51-S61
Adeno-associated virus (AAV) is currently one of the most promising systems for human gene therapy. Numerous preclinical studies have documented the excellent safety profile of these vectors along with their impressive performances in their favored target, consisting of highly differentiated postmitotic tissues such as muscle, central nervous system and liver. Clinical trials have been conducted confirming these data, but also emphasizing the requirement of further high-tech developments of the production and purification procedures that would allow both scaling-up and improvement of vector batch quality, necessary to human application. The scope of this review will be the state of the art in the various production methods of recombinant AAV (rAAV), delimiting their respective perimeter of application and also their main advantages and drawbacks, and thereby shedding light on the main challenges to take in the near future to bring AAV vectors more widely into the clinics.

Kohlbrenner, E., Aslanidi, G., Nash, K., Shklyaev, S., Campbell-Thompson, M., Byrne, B.J., Snyder, R.O., Muzyczka, N., Warrington, K.H. and Zolotukhin, S. (2005) *Successful production of pseudotyped rAAV vectors using a modified Baculovirus expression system* Mol. Ther., **12**, 1217-1225
Scalable production of rAAV vectors remains a major obstacle to the clinical application of this prototypical gene therapy vector. A recently developed baculovirus-based production protocol (M. Urabe *et al.*, 2002, *Hum. Gene Ther.* 13, 1935–1943) found limited applications due to the system's design. Here we report a detailed analysis of the stability of the original baculovirus system components BacRep, BacVP, and transgene cassette-containing BacGFP. All of the baculovirus helpers analyzed were prone to passage-dependent loss-of-function deletions resulting in considerable decreases in rAAV titers. To alleviate the instability and to extend the baculovirus platform to other rAAV serotypes, we have modified both Rep- and Cap-encoding components of the original system. The modifications include a parvoviral phospholipase A2 domain swap allowing production of infectious rAAV8 vectors *in vivo.* Alternatively, an infectious rAAV8 (or rAAV5) vector incorporating the AAV2 VP1 capsid protein in a mosaic vector particle with AAV8 capsid proteins was produced using a novel baculovirus vector. In this vector, the level of AAV2 VP1 expression is controlled with a "riboswitch," a self-cleaving ribozyme controlled by toyocamycin in the "ON" mode. The redesigned baculovirus system improves our capacity for rAAV manufacturing by making this production platform more applicable to other existing serotypes.

Lane, M.D., Nam, H-J., Padron, E., Gurda-Whitaker, B., Kohlbrenner, E., Aslanidi, G., Byrne, B., McKenna, R., Muzycka, N., Zolotukhin, S. and Agbandje-McKenna, M. (2005) *Production, purification, crystallization and preliminary X-ray analysis of adeno-associated virus serotype 8* Acta Cryst., **F61**, 558-561
Adeno-associated viruses (AAVs) are actively being developed for clinical gene-therapy applications and the efficiencies of the vectors could be significantly improved by a detailed understanding of their viral capsid structures and the structural determinants of their tissue-transduction interactions. AAV8 is ~80% identical to the more widely studied AAV2, but its liver-transduction efficiency is significantly greater than that of AAV2 and other serotypes. The production, purification, crystallization and preliminary X-ray crystallographic analysis of AAV8 viral capsids are reported. The crystals diffract X-rays to 3.0 Å resolution using synchrotron radiation and belong to the hexagonal space group P6322, with unit-cell parameters a = 257.5, c = 443.5 Å. The unit cell contains two viral particles, with ten capsid viral protein monomers per crystallographic asymmetric unit.

Blits, B. and Bunge, M.B. (2006) *Direct gene therapy for repair of the spinal cord* J. Neurotrauma, **23**, 508-520
For regrowth of injured nerve fibers following spinal cord injury (SCI), the environment must be favorable for axonal growth. The delivery of a therapeutic gene, beneficial for axonal growth, into the central nervous system for repair can be accomplished in many ways. Perhaps the most simple and elegant strategy is the so-called direct gene therapy approach that uses a single injection for delivery of a gene therapy vehicle. Among the vectors that have been used to transduce neural tissue *in vivo* are non-viral, herpes simplex viral, adeno-associated viral, adenoviral, and lentiviral vectors, each with their own merits and limitations. Many studies have been undertaken using direct gene therapy, ranging from strategies for neuroprotection to axonal growth promotion at the injury site, dorsal root injury repair, and initiation of a growth-supporting genetic program. The limitations and successes of direct gene transfer for spinal cord repair are discussed in this review.

Peng, H.H., Wu, S., Davis, J.J., Wang, L., Roth, J.A., Marini III, F.C. and Fang, B. (2006) *A rapid and efficient method for purification of recombinant adenovirus with arginine–glycine–aspartic acid-modified fibers* Anal. Biochem., **354**, 140-147
Recombinant adenoviral vectors (adenovectors) have been subject to various genetic modifications to improve their transduction efficiency and targeting capacity. Production and purification of adenovectors with modified capsid proteins can be problematic using conventional two-cycle CsCl gradient ultracentrifugation. We have developed a new method for purifying recombinant adenovectors in two steps: iodixanol discontinuous density gradient ultracentrifugation and size exclusion column chromatography. The purity and infectious activity of adenovectors isolated by the two methods were comparable. The new method yielded three to four times more adenovectors with arginine–glycine–aspartic acid (RGD)-modified fiber proteins than did the conventional CsCl method. For other fiber-modified and wild-type adenovectors, the yields of the two methods were comparable. Thus, the iodixanol-based method can be used not only to improve the production of RGD-modified adenovectors but also to purify adenovectors with or without fiber modifications. Moreover, the whole procedure can be completed in 3 h. Therefore, this method is rapid and efficient for production of recombination adenovectors, especially those with RGD-modified fibers.

Arnold, G.S., Sasser, A.K., Stachler, M.D. and Bartlett, J.S. (2006) *Metabolic biotinylation provides a unique platform for the purification and targeting of multiple AAV vector serotypes* Mol. Ther., **14**, 97-106
The development of rationally designed targeted gene delivery vectors is an important focus for gene therapy. While genetic modification of AAV can produce vectors with modified tropism, incorporation of targeting peptides into the structural context of the AAV virion often results in loss of function or loss of virion integrity. To address this issue, we have developed a targeting system using metabolically biotinylated AAV. We generated serotype 1, 2, 3, 4, and 5 AAV capsids with small peptide insertions that are metabolically biotinylated in packaging cells during vector production by coexpression of the *Escherichia coli* BirA, biotin ligase, gene. Biotin moieties are exposed on the surface of assembled AAV particles and can interact with avidin. Metabolically biotinylated AAV vectors produced in this manner maintained endogenous titer and tissue tropism, could be purified on monomeric avidin resin, and could be retargeted to cells engineered to express an artificial avidin–biotin receptor. This technology provides not only a single platform for the purification of multiple AAV vector serotypes, but also a means for the development of multiple targeted AAV vectors utilizing a single capsid modification via straightforward avidin–biotin ligand coupling.

Wu, Z., Asokan, A. and Samulski, R.J. (2006) *Adeno-associated virus serotypes: vector toolkit for human gene therapy* Mol. Ther., **14**, 316-327
Recombinant adeno-associated viral (AAV) vectors have rapidly advanced to the forefront of gene therapy in the past decade. The exponential progress of AAV-based vectors has been made possible by the isolation of several naturally occurring AAV serotypes and over 100 AAV variants from different animal species. These isolates are ideally suited to development into human gene therapy vectors due to their diverse tissue tropisms and potential to evade preexisting neutralizing antibodies against the common human AAV serotype 2. Despite their prolific

application in several animal models of disease, the mechanisms underlying selective tropisms of AAV serotypes remain largely unknown. Efforts to understand cell surface receptor usage and intracellular trafficking pathways exploited by AAV continue to provide significant insight into the biology of AAV vectors. Such unique traits are thought to arise from differences in surface topology of the capsids of AAV serotypes and variants. In addition to the aforementioned naturally evolved AAV isolates, several strategies to engineer hybrid AAV serotype vectors have been formulated in recent years. The generation of mosaic or chimeric vectors through the transcapsidation or marker-rescue/domain-swapping approach, respectively, is notable in this regard. More recently, combinatorial strategies for engineering AAV vectors using error-prone PCR, DNA shuffling, and other molecular cloning techniques have been established. The latter library-based approaches can serve as powerful tools in the generation of low-immunogenic and cell/tissue type-specific AAV vectors for gene delivery. This review is focused on recent developments in the isolation of novel AAV serotypes and isolates, their production and purification, diverse tissue tropisms, mechanisms of cellular entry/trafficking, and capsid structure. Strategies for engineering hybrid AAV vectors derived from AAV serotypes and potential implications of the rapidly expanding AAV vector toolkit are discussed.

Koerber, J.T., Maheshri, N., Kaspar, B.K. and Schaffer, D.V. (2006) *Construction of diverse adeno-associated viral libraries for directed evolution of enhanced gene delivery vehicles* Nat. Protoc., **1**, 701-706
Rational design of improved gene delivery vehicles is a challenging and potentially time-consuming process. As an alternative approach, directed evolution can provide a rapid and efficient means for identifying novel proteins with improved function. Here we describe a methodology for generating very large, random adeno-associated viral (AAV) libraries that can be selected for a desired function. First, the AAV2 *cap* gene is amplified in an error-prone PCR reaction and further diversified through a staggered extension process. The resulting PCR product is then cloned into pSub2 to generate a diverse ($>10^6$) AAV2 plasmid library. Finally, the AAV2 plasmid library is used to package a diverse pool of mutant AAV2 virions, such that particles are composed of a mutant AAV genome surrounded by the capsid proteins encoded in that genome, which can be used for functional screening and evolution. This procedure can be performed in approximately 2 weeks.

Grieger, J.C., Choi, V.W. and Samulski, R.J. (2006) *Production and characterization of adeno-associated viral vectors* Nat. Protoc., **1**, 1412-1428
The adeno-associated virus (AAV) is one of the most promising viral vectors for human gene therapy. As with any potential therapeutic system, a thorough understanding of it at the *in vitro* and *in vivo* levels is required. Over the years, numerous methods have been developed to better characterize AAV vectors. These methods have paved the way to a better understanding of the vector and, ultimately, its use in clinical applications. This review provides an up-to-date, detailed description of essential methods such as production, purification and titering and their application to characterize current AAV vectors for preclinical and clinical use.

Waterkamp, D.A., Müller, O.J., Ying, Y., Trepel, M. and Kleinschmidt, J.A. (2006) *Isolation of targeted AAV2 vectors from novel virus display libraries* J. Gene Med., **8**, 1307-1319
Random peptide ligands displayed on viral capsids are emerging tools for selection of targeted gene transfer vectors even without prior knowledge of the potential target cell receptor. We have previously introduced adeno-associated viral (AAV)-displayed peptide libraries that ensure encoding of displayed peptides by the packaged AAV genome. A major limitation of these libraries is their contamination with wild-type (wt) AAV. Here we describe a novel and improved library production system that reliably avoids generation of wt AAV by use of a synthetic cap gene. Selection of targeted AAV vectors from wt-containing and the novel wt-free libraries on cell types with different permissivity for wt AAV2 replication suggested the superiority of the wt-free library. However, from both libraries highly specific peptide sequence motifs were selected which improved transduction of cells with moderate or low permissivity for AAV2 replication. Strong reduction of HeLa cell transduction compared to wt AAV2 and only low level transduction of non-target cells by some selected clones showed that not only the efficiency but also the specificity of gene transfer was improved. In conclusion, our study validates and improves the unique potential of virus display libraries for the development of targeted gene transfer vectors.

Koerber, J.T., Jang, J-Y., Yu, J.H., Kane, R.S. and Schaffer, D.V. (2007) *Engineering adeno-associated virus for one-step purification via immobilized metal affinity chromatography* Hum. Gene Ther., **18**, 367-378
Adeno-associated virus (AAV) is a promising vehicle for gene therapy, which will rely on the generation of high-titer, high-purity recombinant vectors. However, numerous purification protocols can involve challenging optimization or scalability issues, and most AAV serotypes do not bind heparin or sialic acid, used for AAV2/3 or AAV4/5 purification, requiring the development of new chromatography strategies. Immobilized metal affinity chromatography (IMAC) allows for robust protein purification via affinity tags such as the hexahistidine (His6) sequence. Through the combination of a diverse AAV2 library and rational peptide insertions, we have located an optimal His6 tag insertion site within the viral capsid. This mutant and a related AAV8 variant can be purified from clarified cell lysate in a single gravity column step at infectious particle yields exceeding 90%. Furthermore,

injection of IMAC-purified vector into the brain demonstrates that it mediates high-efficiency gene delivery *in vivo*, equivalent to that of wild-type capsid, with minimal immune cell activation. This affinity chromatography method may offer advantages in ease of purification, final vector purity, and process scalability. Moreover, a combined rational design and high-throughput library selection approach can aid in the design of enhanced viral gene delivery vectors.

Leonard, J.N., Ferstl, P., Delgado, A. and Schaffer, D.V. (2007) *Enhanced preparation of adeno-associated viral vectors by using high hydrostatic pressure to selectively inactivate helper adenovirus* Biotechnol. Bioeng., **97**, 1170-1179
Gene delivery vectors based on adeno-associated virus (AAV) have significant therapeutic potential, but much room for improvement remains in the areas of vector engineering and production. AAV production requires complementation with either helper virus, such as adenovirus, or plasmids containing helper genes, and helper virus-based approaches have distinct advantages in the use of bioreactors to produce large quantities of AAV vectors for clinical applications. However, helper viruses must eventually be inactivated and removed from AAV preparations to ensure safety. The current practice of thermally inactivating adenovirus is problematic as it can also inactivate AAV. Here, we report a novel method using high hydrostatic pressure (HHP) to selectively and completely inactivate helper adenovirus without any detectable loss of functional AAV vectors. The pressure inactivation kinetics of human adenovirus serotype 5 and the high-pressure stabilities of AAV serotypes 2 and 5 (AAV2, AAV5), which were previously unknown, were characterized. Adenovirus was inactivated beyond detection at 260 MPa or higher, whereas AAV2 was stable up to 450 MPa, and surprisingly, AAV5 was stable up to at least 700 MPa. The viral genomic DNA of pressure-inactivated AAV2 was made sensitive to DNAse I digestion, suggesting that gross changes in particle structure had occurred, and this hypothesis was further supported by transmission electron microscopy. This approach should be useful in the laboratory- and clinical-scale production of AAV gene delivery vectors. Moreover, HHP provides a tool for probing the biophysical properties of AAV, which may facilitate understanding and improving the functions of this important virus.

Büning, H., Perabo, L., Coutelle, O., Quadt-Humme, S. and Hallek, M. (2008) *Recent developments in adeno-associated virus vector technology* J. Gene Med., **10**, 717-733
Adeno-associated virus (AAV), a single-stranded DNA parvovirus, is emerging as one of the leading gene therapy vectors owing to its nonpathogenicity and low immunogenicity, stability and the potential to integrate site-specifically without known side-effects. A portfolio of recombinant AAV vector types has been developed with the aim of optimizing efficiency, specificity and thereby also the safety of in vitro and in vivo gene transfer. More and more information is now becoming available about the mechanism of AAV/host cell interaction improving the efficacy of recombinant AAV vector (rAAV) mediated gene delivery. This review summarizes the current knowledge of the infectious biology of AAV, provides an overview of the latest developments in the field of AAV vector technology and discusses remaining challenges.

Clément, N., Knop, D.R. and Byrne, B.J. (2009) *Large-scale adeno-associated viral vector production using a herpesvirus-based system enables manufacturing for clinical studies* Hum. Gene. Ther., **20**, 796-806
The ability of recombinant adeno-associated viral (rAAV) vectors to exhibit minimal immunogenicity and little to no toxicity or inflammation while eliciting robust, multiyear gene expression in vivo are only a few of the salient features that make them ideally suited for many gene therapy applications. A major hurdle for the use of rAAV in sizeable research and clinical applications is the lack of efficient and versatile large-scale production systems. Continued progression toward flexible, scalable production techniques is a prerequisite to support human clinical evaluation of these novel biotherapeutics. This review examines the current state of large-scale production methods that employ the herpes simplex virus type 1 (HSV) platform to produce rAAV vectors for gene delivery. Improvements have substantially advanced the HSV/AAV hybrid method for large-scale rAAV manufacture, facilitating the generation of highly potent, clinical-grade purity rAAV vector stocks. At least one human clinical trial employing rAAV generated via rHSV helper-assisted replication is poised to commence, highlighting the advances and relevance of this production method.

Toelen, J., Lock, M., Vandenberghe, L., Carlon, M., Wilson, J. and Debyser, Z. (2009) *Novel and scalable approach to research grade AAV vector manufacturing and separation of distinct AAV serotypes* Hum. Gene Ther., ESGCT, DGGT, GSZ, and ISCT 2009 Poster Presentations, # 52
Background: AAV vector manufacturing protocols of research grade vector for which several combinations of serotype and genome may be required, still involve labor-intensive processes. Several desired outcomes of the downstream process, e.g. purification of multiple serotypes or separation of empty from full particles, are difficult to obtain and must be individually tailored for each serotype. Our observations that AAV is found in the culture supernatant during production of many serotypes, suggested that the supernatant represents a relatively pure source of vector in comparison with cell-derived material. **Methods**: Here we describe a serum-free AAV production system based upon PEI-mediated transfection of HEK 293 cells in 10-layer Hyperflasks. The

supernatant is collected after 5 days, followed by a 50 fold concentration using tangential flow filtration (TFF) and loaded to an optimized iodixanol step-gradient. After ultracentrifugation the gradient fractions containing vector are identified, pooled and subjected to further concentration and buffer exchange. **Results**: Process conditions have been optimized such that final yields of several vector serotypes approach 70% with >90% capsid protein purity from a single gradient as assessed with EM and PAGE analysis. AAV serotypes 1, 2, 5, 6, 7, 8 and 9 have been produced using this protocol and used for in vivo application in the murine brain and lung. **Conclusions:** We present a fast and broadly applicable AAV production protocol based on the isolation of AAV from the supernatant and a purification using a single iodixanol gradient. This process had the capacity for upscaling since TFF enables a >100 fold concentration. AAV vectors produced with this method are successfully used in small animal models.

Lock, M., Alvira, M., Vandenberghe, L.H., Samanta, A., Toelen, J., Debyser, Z. and Wilson, J.M. (2010) *Rapid, simple, and versatile manufacturing of recombinant adeno-associated viral vectors at scale* Hum. Gene Ther., **21**, 1259–1271

Adeno-associated viral (AAV) manufacturing at scale continues to hinder the application of AAV technology togene therapy studies. Although scalable systems based on AAV–adenovirus, AAV–herpesvirus, and AAV–baculovirus hybrids hold promise for clinical applications, they require time-consuming generation of reagents and are not highly suited to intermediate-scale preclinical studies in large animals, in which several combinations of serotype and genome may need to be tested. We observed that during production of many AAV serotypes, large amounts of vector are found in the culture supernatant, a relatively pure source of vector in comparison with cell-derived material. Here we describe a high-yielding, recombinant AAV production process based on polyethylenimine (PEI)-mediated transfection of HEK293 cells and iodixanol gradient centrifugation of concentrated culture supernatant. The entire process can be completed in 1 week and the steps involved are universal for a number of different AAV serotypes. Process conditions have been optimized such that final purified yields are routinely greater than $1x10^{14}$ genome copies per run, with capsid protein purity exceeding 90%. Initial experiments with vectors produced by the new process demonstrate equivalent or better transduction both *in vitro* and *in vivo* when compared with small-scale, CsCl gradient-purified vectors. In addition, the iodixanol gradient purification process described effectively separates infectious particles from empty capsids, a desirable property for reducing toxicity and unwanted immune responses during preclinical studies.

Khan, I.F., Hirata, R.K. and Russell, D.W. (2011) *AAV-mediated gene targeting methods for human cells* Nat. Protoc., **6**, 482-501

Gene targeting with adeno-associated virus (AAV) vectors has been demonstrated in multiple human cell types, with targeting frequencies ranging from 10 − 5 to 10 − 2 per infected cell. These targeting frequencies are 1–4 logs higher than those obtained by conventional transfection or electroporation approaches. Awide variety of different types of mutations can be introduced into chromosomal loci with high fidelity and without genotoxicity. Here we provide a detailed protocol for gene targeting in human cells with AAV vectors. We describe methods for vector design, stock preparation and titration. Optimized transduction protocols are provided for human pluripotent stem cells, mesenchymal stem cells, fibroblasts and transformed cell lines, as well as a method for identifying targeted clones by Southern blots. This protocol (from vector design through a single round of targeting and screening) can be completed in ~10 weeks; each subsequent round of targeting and screening should take an additional 7 weeks.

Kotin, R.M. (2011) *Large-scale recombinant adeno-associated virus production* Hum. Mol. Genet., **20**, R2-R6

Since recombinant adeno-associated virus (rAAV) was first described as a potential mammalian cell transducing system, frequent reports purportedly solving the problems of scalable production have appeared. Yet few of these processes have enabled the development of robust and economical rAAV production. Two production platforms have emerged that have gained broad support for producing both research and clinical grade vectors. These processes differ fundamentally in several aspects. One approach is based on adherent mammalian cells and uses optimized chemical transient transfection for introducing the essential genetic components into the cells. The other approach utilizes suspension cultures of nvertebrate cells. Baculovirus expression vectors are used for introducing the AAV genes into the cells. In addition, the baculovirus provides the helper functions necessary for efficient AAV DNA replication. The use of suspension cell culture provides an intrinsically more scalable platform system than using adherent cells. The upstream processes for suspension cultures are amenable for automation and are easily monitored and regulated to maintain optimum conditions that produce consistent yields of rAAV. Issues relating to developing new and improving existing rAAV production methods are discussed.

Segura, M.M., Kamen, A.A. and Garnier, A. (2011) *Overview of current scalable methods for purification of viral vectors* In, Viral Vectors for Gene Therapy: Methods and Protocols, Methods in Molecular Biology, **737** (eds. Merten O.W. and Al-Rubeai, M.) Springer Science+Business Media, pp 89-116
As a result of the growing interest in the use of viruses for gene therapy and vaccines, many virus-based products are being developed. The manufacturing of viruses poses new challenges for process developers and regulating authorities that need to be addressed to ensure quality, efficacy, and safety of the final product. The design of suitable purification strategies will depend on a multitude of variables including the vector production system and the nature of the virus. In this chapter, we provide an overview of the most commonly used purification methods for viral gene therapy vectors. Current chromatography options available for large-scale purification of g-retrovirus, lentivirus, adenovirus, adeno-associated virus, herpes simplex virus, baculovirus, and poxvirus vectors are presented.

De Backer, M.W.A., Garner, K.M., Luijendijk, M.C.M. and Adan, R.A.H. (2011) *Recombinant adeno-associated viral vectors* In Methods Mol. Biol., 789, Neuropeptides: Methods and Protocols (ed. Merighi, A.), Springer Science+Business Media, pp 357-376
Recombinant adeno-associated viral (rAAV) vectors can be used to locally or systemically enhance or silence gene expression. They are relatively non-immunogenic and can transduce dividing and non-dividing cells, and different rAAV serotypes may transduce diverse cell types. Therefore, rAAV vectors are excellent tools to study the function of neuropeptides in local brain areas. In this chapter, we describe a protocol to produce high-titer, in vivo grade, rAAV vector stocks. The protocol includes an Iodixanol gradient, an anion exchange column and a desalting/concentration step and can be used for every serotype. In addition, a short protocol for rAAV injections into the brain and directions on how to detect and localize
transduced cells are given.

Weitzman, M.D. and Linden, R.M. (2011) *Adeno-associated virus biology* In Adeno-Associated Virus: Methods and Protocols, Methods Mol. Biol., **807** (ed. Snyder, R.O. and Moullier, P.), Springer Science+Business Media, pp 1-23
Adeno-associated virus (AAV) was first discovered as a contaminant of adenovirus stocks in the 1960s.
The development of recombinant AAV vectors (rAAV) was facilitated by early studies that generated infectious molecular clones, determined the sequence of the genome, and defined the genetic elements of the virus. The refi nement of methods and protocols for the production and application of rAAV vectors has come from years of studies that explored the basic biology of this virus and its interaction with host cells. Interest in improving vector performance has in turn driven studies that have provided tremendous insights into the basic biology of the AAV lifecycle. In this chapter, we review the background on AAV biology and its exploitation for vectors and gene delivery.

Wang, L., Blouin, V., Brument, N., Bello-Roufai, M. and Francois, A. (2011) *Production and purification of recombinant adeno-associated vectors* In Adeno-Associated Virus: Methods and Protocols, Methods Mol. Biol., **807** (ed. Snyder, R.O. and Moullier, P.), Springer Science+Business Media, pp 361-404
The use of recombinant adeno-associated virus (rAAV) vectors in gene therapy for preclinical studies in animal models and human clinical trials is increasing, as these vectors have been shown to be safe and to mediate persistent transgene expression in vivo. Constant improvement in rAAV manufacturing processes (upstream production and downstream purifi cation) has paralleled this evolution to meet the needs for larger vector batches, higher vector titer, and improved vector quality and safety. This chapter provides an overview of existing production and purification systems used for adeno-associated virus (AAV) vectors, and the advantages and disadvantages of each system are outlined. Regulatory guidelines that apply to the use of these systems for clinical trials are also presented. The methods described are examples of protocols that have been utilized for establishing rAAV packaging cell lines, production of rAAV vectors using recombinant HSV infection, and for chromatographic purification of various AAV vector serotypes. A protocol for the production of clinical-grade rAAV type 2 vectors using transient transfection and centrifugation based purification is also described.

Lock, M., Alvira, M.R. and Wilson, J.M. (2012) *Analysis of particle content of recombinant adeno-associated virus serotype 8 vectors by ion-exchange chromatography* Hum. Gene Ther. Methods, Part B **23**, 56-64
Advances in adeno-associated virus (AAV)-mediated gene therapy have brought the possibility of commercial manufacturing of AAV vectors one step closer. To realize this prospect, a parallel effort with the goal of everincreasing sophistication for AAV vector production technology and supporting assays will be required. Among the important release assays for a clinical gene therapy product, those monitoring potentially hazardous contaminants are most critical for patient safety. A prominent contaminant in many AAV vector preparations is vector particles lacking a genome, which can substantially increase the dose of AAV capsid proteins and lead to possible unwanted immunological consequences. Current methods to determine empty particle content suffer from inconsistency, are adversely affected by contaminants, or are not applicable to all serotypes. Here we describe the

development of an ion-exchange chromatography- based assay that permits the rapid separation and relative quantification of AAV8 empty and full vector particles through the application of shallow gradients and a strong anion-exchange monolith chromato-graphy medium.

Kohlbrenner, E., Henckaerts, E., Rapti, K., Gordon, R.E., Linden, M., Hajjar, R.J. and Weber, T. (2012) *Quantification of AAV particle titers by infrared fluorescence scanning of Coomassie-stained sodium dodecyl sulfate–polyacrylamide* gels Hum. Gene Ther. Methods, **23**, 198–203

Adeno-associated virus (AAV)-based vectors have gained increasing attention as gene delivery vehicles in basic and preclinical studies as well as in human gene therapy trials. Especially for the latter two—for both safety and therapeutic efficacy reasons—a detailed characterization of all relevant parameters of the vector preparation is essential. Two important parameters that are routinely used to analyze recombinant AAV vectors are (1) the titer of viral particles containing a (recombinant) viral genome and (2) the purity of the vector preparation, most commonly assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by silver staining. An important, third parameter, the titer of total viral particles, that is, the combined titer of both genome-containing and empty viral capsids, is rarely determined. Here, we describe a simple and inexpensive method that allows the simultaneous assessment of both vector purity and the determination of the total viral particle titer. This method, which was validated by comparison with established methods to determine viral particle titers, is based on the fact that Coomassie Brilliant Blue, when bound to proteins, fluoresces in the infrared spectrum. Viral samples are separated by SDS–PAGE followed by Coomassie Brilliant Blue staining and gel analysis with an infrared laser-scanning device. In combination with a protein standard, our method allows the rapid and accurate determination of viral particle titers simultaneously with the assessment of vector purity.

Huang, X., Hartley, A-V., Yin, Y., Herskowitz, J.H., Lah, J.L., Ressler, K.J. (2013) *AAV2 production with optimized N/P ratio and PEI-mediatedtransfection results in low toxicity and high titer for in vitro andin vivo applications* J. Virol. Methods, **193**, 270– 277

The adeno-associated virus (AAV) is one of the most useful viral vectors for gene delivery for both *in vivo* and *in vitro* applications. A variety of methods have been established to produce and characterize recom-binant AAV (rAAV) vectors; however most methods are quite cumbersome and obtaining consistently high titer can be problematic. This protocol describes a triple-plasmid co-transfection approach with 25 kDa linear polyethylenimine (PEI) in 293T cells for the production of AAV serotype 2. Seventy-two hours post-transfection, supernatant and cells were harvested and purified by a discontinuous iodixanol density gradient ultracentrifugation, then dialyzed and concentrated with an Amicon 15 100,000 MWCO concentration unit. To optimize the protocol for AAV2 production using PEI, various N/P ratios and DNA amounts were compared. We found that an N/P ratio of 40 coupled with 1.05 μg DNA per ml of media (21 μg DNA/15 cm dish) was found to produce the highest yields for viral replication and assembly measured multiple ways. The infectious units, as determined by serial dilution, were between 1 x 10^8 and 2 x 10^9 IU/ml. The genomic titer of the viral stock was determined by qPCR and ranged from 2 x 10^{12} to 6 x 10^{13} VG/ml. These viral vectors showed high expression both *in vivo* within the brain and *in vitro* incell culture. The use of linear 25 kDa polyethylenamine PEI as a transfection reagent is a simple, morecost-effective, and stable means of high-throughput production of high-titer AAV serotype 2. The use of PEI also eliminates the need to change cell medium post-transfection, lowering cost and workload, while producing high-titer, efficacious AAV2 vectors for routine gene transfer.

Chahal, P.S., Schulze, E., Tran, R., Montes, J. and Kamen, A.A. (2014) *Production of adeno-associated virus (AAV) serotypes by transient transfection of HEK293 cell suspension cultures for gene delivery* J. Virol. Methods, **196**, 163– 173

Adeno-associated virus (AAV) is being used successfully in gene therapy. Different serotypes of AAV tar-get specific organs and tissues with high efficiency. There exists an increasing demand to manufacture various AAV serotypes in large quantities for pre-clinical and clinical trials. A generic and scalable method has been described in this study to efficiently produce AAV serotypes (AAV1-9) by transfection of a fully characterized cGMP HEK293SF cell line grown in suspension and serum-free medium. First, the production parameters were evaluated using AAV2 as a model serotype. Second, all nine AAV serotypes were produced successfully with yields of 10^{13}Vg/L cell culture. Subsequently, AAV2 and AAV6 serotypes were produced in 3-L controlled bioreactors where productions yielded up to 1013Vg/L similar to the yields obtained in shake-flasks. For example, for AAV2 10^{13}Vg/L cell culture (6.8×10^{11}IVP/L) were measured between 48 and 64 h post transfection (hpt). During this period, the average cell specific AAV2 yields of 6800 Vg per cell and 460 IVP per cell were obtained with a Vg to IVP ratio of less than 20. Successful operations in bioreactors demonstrated the potential for scale-up and industrialization of this generic process for manufacturing AAV serotypes efficiently.

Lock, M., Alvira, M.R., Chen, S-J. and Wilson, J.M. (2014) *Absolute determination of single-stranded and self-complementary adeno-associated viral vector genome titers by droplet digital PCR* Hum. Gene Ther. Methods **25**, 115–125

Accurate titration of adeno-associated viral (AAV) vector genome copies is critical for ensuring correct and reproducible dosing in both preclinical and clinical settings. Quantitative PCR (qPCR) is the current method of choice for titrating AAV genomes because of the simplicity, accuracy, and robustness of the assay. However, issues with qPCR-based determination of self-complementary AAV vector genome titers, due to primer–probe exclusion through genome self-annealing or through packaging of prematurely terminated defective interfering (DI) genomes, have been reported. Alternative qPCR, gel-based, or Southern blotting titering methods have been designed to overcome these issues but may represent a backward step from standard qPCR methods in terms of simplicity, robustness, and precision. Droplet digital PCR (ddPCR) is a new PCR technique that directly quantifies DNA copies with an unparalleled degree of precision and without the need for a standard curve or for a high degree of amplification efficiency; all properties that lend themselves to the accurate quantification of both single-stranded and self-complementary AAV genomes. Here we compare a ddPCRbased AAV genome titer assay with a standard and an optimized qPCR assay for the titration of both single-stranded and self-complementary AAV genomes. We demonstrate absolute quantification of single-stranded AAV vector genomes by ddPCR with up to 4-fold increases in titer over a standard qPCR titration but with equivalent readout to an optimized qPCR assay. In the case of self-complementary vectors, ddPCR titers were on average 5-, 1.9-, and 2.3-fold higher than those determined by standard qPCR, optimized qPCR, and agarose gel assays, respectively. Droplet digital PCR-based genome titering was superior to qPCR in terms of both intra and interassay precision and is more resistant to PCR inhibitors, a desirable feature for in-process monitoring of early-stage vector production and for vector genome biodistribution analysis in inhibitory tissues.

Doerfler, P.A., Byrne, B.J. and Clément, N. (2014) *Copackaging of multiple adeno-associated viral vectors in a single production step* Hum. Gene Ther. Methods, **25**, 269–276

Limiting factors in large preclinical and clinical studies utilizing adeno-associated virus (AAV) for gene therapy are focused on the restrictive packaging capacity, the overall yields, and the versatility of the production methods for single AAV vector production. Furthermore, applications where multiple vectors are needed to provide long expression cassettes, whether because of long cDNA sequences or the need of different regulatory elements, require that each vector be packaged and characterized separately, directly affecting labor and cost associated with such manufacturing strategies. To overcome these limitations, we propose a novel method of vector production that allows for the packaging of multiple expression cassettes in a single transfection step. Here we combined two expression cassettes in predetermined ratios before transfection and empirically demonstrate that the output vector recapitulates the predicted ratios. Titration by quantitative polymerase chain reaction of AAV vector genome copies using shared or unique genetic elements allowed for delineation of the individual vector contribution to the total preparation that showed the predicted differential packaging outcomes. By copackaging green fluorescent protein (GFP) and mCherry constructs, we demonstrate that both vector genome and infectious titers reiterated the ratios utilized to produce the constructs by transfection. Copackaged therapeutic constructs that only differ in transcriptional elements produced a heterogeneous vector population of both constructs in the predefined ratios. This study shows feasibility and reproducibility of a method that allows for two constructs, differing in either transgene or transcription elements, to be efficiently copackaged and characterized simultaneously, reducing cost of manufacturing and release testing.

Piedra, J., Ontiveros, M., Miravet, S., Penalva, C., Monfar, M. and Chillon, M. (2015) *Development of a rapid, robust, and universal PicoGreen-based method to titer adeno-associated vectors* Hum. Gene Ther. Meth., **26**, 35–42

Recombinant adeno-associated viruses (rAAVs) are promising vectors in preclinical and clinical assays for the treatment of diseases with gene therapy strategies. Recent technological advances in amplification and purification have allowed the production of highly purified rAAV vector preparations. Although quantitative polymerase chain reaction (qPCR) is the current method of choice for titrating rAAV genomes, it shows high variability. In this work, we report a rapid and robust rAAV titration method based on the quantitation of encapsidated DNA with the fluorescent dye PicoGreen_. This method allows detection from 3 x 10^{10} viral genome/ml up to 2.4 x 10^{13} viral genome/ml in a linear range. Contrasted with dot blot or qPCR, the PicoGreenbased assay has less intra- and interassay variability. Moreover, quantitation is rapid, does not require specific primers or probes, and is independent of the rAAV pseudotype analyzed. In summary, development of this universal rAAV-titering method may have substantive implications in rAAV technology.

Qu, W., Wang, M., Wu, Y. and Xu, R. (2015) *Scalable downstream strategies for purification of recombinant adeno-associated virus vectors in light of the properties* Curr. Pharm. Biotechnol., **16**, 684-695

Recombinant adeno-associated virus (rAAV) vector is one of the promising delivery tools for gene therapy. Currently, hundreds of clinical trials are performed but the major barrier for clinical application is the absence of

any ideal large scale production technique to obtain sufficient and highly pure rAAV vector. The large scale production technique includes upstream and downstream processing. The upstream processing is a vector package step and the downstream processing is a vector purification step. For large scale downstream processing, the scientists need to recover rAAV from dozens of liters of cell lysate or medium, and a variety of purification strategies have been developed but not comprehensively compared till now. Consequently, this review will evaluate the scalable downstream purification strategies systematically, especially those based on the physicochemical properties of AAV virus, and attempt to find better scalable downstream strategies for rAAV vectors.

Florencio, G.D., Precigout, G., Beley, C., Buclez, P-O., Garcia, L. and Benchaouir, R. (2015) *Simple downstream process based on detergent treatment improves yield and in vivo transduction efficacy of adeno-associated virus vectors* Mol. Ther. Meth. Clin. Devel., 2: 15024
Recombinant adeno-associated viruses (rAAV) are promising candidates for gene therapy approaches. The last two decades were particularly fruitful in terms of processes applied in the production and purification of this type of gene transfer vectors. This rapid technological evolution led to better yields and higher levels of vector purity. Recently, some reports showed that rAAV produced by transient tri-transfection method in adherent human embryonic kidney 293 cells can be harvested directly from supernatant, leading to easier and faster purification compared to classical virus extraction from cell pellets. Here, we compare these approaches with new vector recovery method using small quantity of detergent at the initial clarification step to treat the whole transfected cell culture. Coupled with tangential flow filtration and iodixanol-based isopycnic density gradient, this new method significantly increases rAAV yields and conserves high vector purity. Moreover, this approach leads to the reduction of the total process duration. Finally, the vectors maintain their functionality, showing unexpected higher *in vitro* and *in vivo* transduction efficacies. This new development in rAAV downstream process once more demonstrates the great capacity of these vectors to easily accommodate to large panel of methods, able to furthermore ameliorate their safety, functionality, and scalability.

Heindorf, M. and Hasan, M.T. (2015) *Fluorescent calcium indicator protein expression in the mouse brain using recombinant adeno-associated viruses* Cold Spring Harb Protoc; doi: 10.1101/pdb.prot087635
One method for gene delivery and long-term fluorescent calcium indicator protein (FCIP) expression in mammalian neurons in vivo involves the introduction of FCIPs via recombinant adeno-associated virus (rAAV) vectors using constitutive and cell type-specific promoters. This protocol describes the use of rAAVs to express FCIPs in the brain for imaging. Human embryonic kidney 293 cells are first transfected using calcium phosphate. rAAV is then prepared using either an iodixanol gradient or a heparin column. After the virus is purified, its quality is assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, estimation of genomic and functional virus titers by quantitative polymerase chain reaction, and expression in dissociated neurons. Mice are injected with rAAV using a stereotactic instrument and can be imaged ~3 wk later.