

Recent advances in recombinant adeno-associated virus vector production

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Recent advances in recombinant adeno-associated virus vector production. 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.

Interest in recombinant adeno-associated virus vectors (rAAV) has steadily increased because of the ability of this vector to mediate long-term, robust *in vivo* gene expression in numerous nondividing cell types (i.e., hepatocytes, neurons, and skeletal myocytes). Efficient *in vivo* gene transfer via rAAV transduction requires reasonably high multiplicities of infection, estimated between 10^3 to 10^5 DNA-containing particles per cell, depending on the cell type targeted. Moreover, based on large animal studies, a clinical dose in humans will require 10^{12} to 10^{14} rAAV vector particles, depending on the level of therapeutic protein expression needed for treatment efficacy [1]. Therefore, the ability to produce high-titer rAAV is critical for clinical applications and has historically been the primary impediment to the widespread use of this vector system. This review discusses the recent advances in vector production technology that have resulted in the routine isolation of $>10^4$ recombinant viral particles per cell. The vast majority of rAAV is produced in one of two ways. The first method, referred to as

transient transfection production, relies on the use of plasmid DNA transfection to introduce required AAV production components into human cell lines. The second approach focuses on the use of stable rAAV producer cell lines for the large-scale production of rAAV. Transient transfection is amenable to higher throughput, allowing the investigator more flexibility to generate and test multiple rAAV vector constructs. It is hampered by the requirement for the transfection of large numbers of mammalian cells in culture. Conversely, although stable cell line approaches are readily scaleable, they are less flexible with regards to the number of vectors that can be rapidly generated and assayed. Both production methods require three essential components for rAAV production. The first element is the rAAV vector, which is a transgene expression cassette flanked by AAV inverted terminal repeats (ITRs). The AAV ITRs are located at the ends of the AAV genome and contain all of the *cis* sequence information necessary for vector DNA replication and encapsidation [2, 3]. Thus, rAAV vectors contain only a very small proportion (6%) of the wild-type AAV genome and lack all viral coding sequences. Second, the two AAV genes (*rep* and *cap*), which encode replication and AAV structural proteins, are expressed *in trans*. The physical separation of these genes and the ITR packaging signal is critical for avoiding the formation of wild-type AAV. The *rep* gene possesses two promoters (*p5* and *p19*) from which 4 Rep proteins are expressed (Rep78, Rep68, Rep52, and Rep40). The *p5* derived Rep proteins (Rep78 or Rep68) are essential for rAAV replication. Three capsid proteins (VP1, VP2, and VP3) are synthesized following transcription from the *rep*-inducible *p40 cap* gene promoter. Finally, because AAV and recombinant derivatives are replication defective, these viruses require helper virus gene products from adenovirus or herpesvirus for efficient viral production. The helper virus (or specific helper virus genes) provides a favorable intracellular milieu for robust rAAV replication and viral synthesis. A schematic of both production strategies is shown in Figure 1.

Key words: rAAV production, AAV, stable cell lines.

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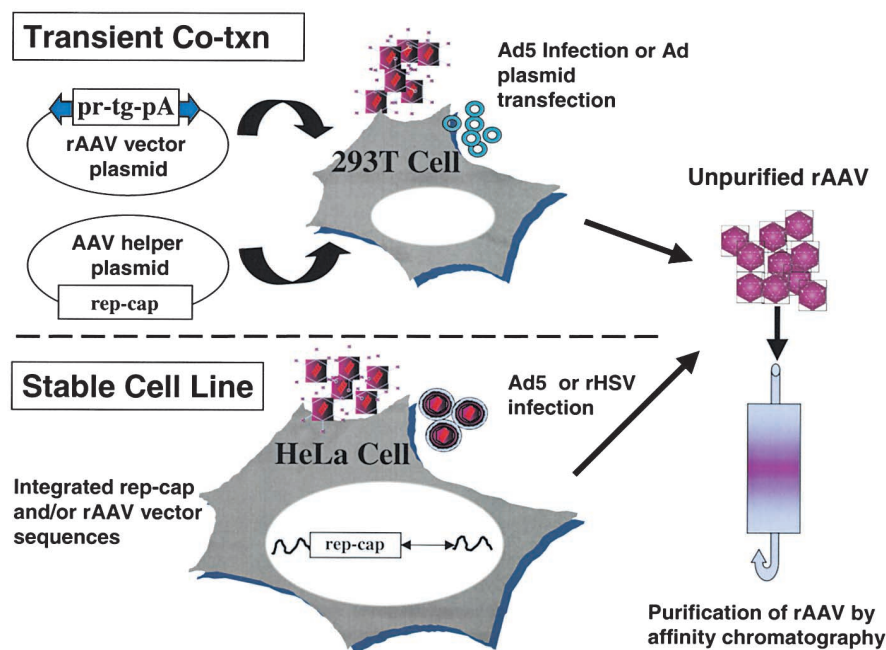


Fig. 1. rAAV production schematic. The top half of the figure depicts rAAV production via transient transfection. The process involves the cotransfection of a rep-cap-containing plasmid and an rAAV vector plasmid containing the AAV ITRs (arrowheads) flanking a gene expression cassette [promoter (pr), desired transgene (tg), and polyadenylation signal (pA)]. Helper functions for efficient rAAV synthesis are supplied by adenovirus infection or an adenovirus helper plasmid containing the VA, E2A, E4 genes. The lower portion of the figure depicts rAAV production using stable cell line approaches. Generally, HeLa cells containing integrated rep-cap and/or rAAV vector sequences are infected with either adenovirus or recombinant HSV to provide the needed helper functions. rAAV is then rapidly purified from crude cellular lysates using affinity chromatography (antibody or heparin sulfate).

ADVANCES IN TRANSIENT TRANSFECTION PRODUCTION

rAAV is most commonly generated in cell culture by co-transfecting 293 or 293T cells with a rAAV vector plasmid and an AAV rep-cap plasmid followed by helper adenovirus infection. Yields of between 10^2 to 10^3 viral particles per cell are typical using an optimized calcium phosphate coprecipitation method. For purposes of clarity, all references to rAAV particles, unless otherwise indicated, assume that these are DNA-containing particles. Unfortunately, this method of production is inherently inefficient because of the requirement for three separate “hits” on a cell to produce rAAV (cotransfection of two plasmids plus infection with adenovirus) and is difficult to perform reproducibly on a large scale. However, three recent observations have resulted in ≥ 10 -fold increases in vector yields. The first was the recognition that below a threshold level, AAV-2 capsid protein levels can limit rAAV production [4]. Second, downmodulation of Rep78 protein expression results in increased capsid protein synthesis and rAAV production [4–7]. Finally, by introducing the necessary adenovirus helper genes into cells via plasmid DNA transfection rather than by standard adenovirus infection, moderate increases in rAAV yields are possible [7–12]. Importantly, the resulting rAAV preparations were free of contaminating infectious adenovirus and most adenovirus structural proteins. Vincent et al observed that transient transfection vector yields could be increased approximately 10-fold if AAV capsid expression levels were increased using a strong constitutive promoter [human immediate

early CMV promoter/enhancer (CMV IE)] in place of the endogenous p40 cap gene promoter [4]. They also observed that overexpression of the Rep78 protein (using the RSV promoter) resulted in reduced capsid protein production. The authors suggested that Rep78 exerts its negative effect on cap expression by translational repression of capsid mRNA. Moreover, the finding that a minimum threshold level of capsid protein is necessary for efficient intranuclear AAV-2 virion formation added further support to the conclusion that capsid protein levels can be a limiting element in rAAV production [12–14]. Li et al also observed that overexpression of Rep78 protein correlated with decreased capsid protein levels, reduced rAAV genome replication, and rAAV titers [6]. Conversely, when the authors downmodulated Rep78/68 expression (by using a nonconsensus ACG start codon), they observed increased capsid protein production and rAAV yields by approximately 10-fold. Using a similar strategy, Grimm et al achieved low level Rep expression by using the mouse mammary tumor virus LTR promoter (under non-induced conditions) in place of the native p5 rep promoter [7]. These authors also observed increased capsid and rAAV production (by a factor of 10-fold). How high-level Rep78 expression acts to reduce capsid expression and vector yields in the transient production schema is not completely understood, but because the AAV Rep proteins have pleiotropic effects on cellular and AAV gene expression, tightly regulated Rep expression appears necessary for optimal rAAV titers. For example, Rep78/68 has been shown to inhibit cap mRNA translation, but these same proteins are required for efficient cap mRNA transcription from

the native p40 promoter. Additionally, elevated Rep78/68 expression can repress adenovirus replication and inhibit adenovirus helper functions, resulting in reduced rAAV production capacity. Thus, although clearly needed for AAV replication and transcriptional activation, excess Rep78 levels appear deleterious to efficient rAAV formation. Regardless of the molecular mechanism, the use of these downmodulated rep expression plasmids has resulted in increased rAAV production. The third significant advancement in transient rAAV vector production was the development of helper virus-free packaging systems that use a DNA plasmid to supply all helper adenovirus functions but is incapable of generating infectious adenovirus. Earlier work defined five adenovirus gene regions that were required for complete helper functions for wild-type and rAAV synthesis (E1, E2, VA I/II RNA, and E4). Subsequent research refined the minimal set to E1a, E1b, VA I/II RNA, E2A, and E4 ORF6 [11]. Although this approach added a third plasmid to the transfection mixture, rAAV yields were equal to or higher than vector stocks produced by standard adenovirus infection. Among the numerous variations, Xiao et al reported the generation of a miniadenovirus genome plasmid termed pXX6, which deleted several adenovirus structural genes and the MLP promoter [9]. When cotransfected into 293T cells, the authors reported an approximately threefold increase in vector yield over adenovirus infection. Similarly, Matsushita et al also found that adenovirus gene transfection resulted in vector particle yields that were four times higher than adenovirus infection [11]. Two groups have combined the rep-cap and adenovirus helper genes on a single plasmid and reported 10-fold increases in rAAV yields [7, 10]. Elevated rAAV yields may be due to the absence of competition between two concurrent viral infections (rAAV and adenovirus) for the host cell macromolecular synthesis machinery. Clearly, the major benefit of this approach is the complete removal of contaminating adenovirus (and most structural proteins) that could deleteriously affect rAAV-mediated transgene expression through host immune activation. An important question is whether all five gene products are necessary for high-level rAAV production. When using the native rep-cap promoters for rAAV production, it appears that all five genes contribute to optimal rAAV yields [11]. Interestingly, when the rep and cap gene products were expressed by constitutive promoters (the mouse metallothionein gene 1 and CMV promoters, respectively), only the E4 ORF6 adenovirus protein appeared necessary for high-level rAAV production in 293 cells [15]. Thus, under the appropriate rep and cap conditions, it appears that only the E1 region and the E4 ORF6 protein are required for rAAV production. A summary of the various rAAV production methods is shown in Table 1.

STABLE CELL LINE PRODUCTION

In addition to transient transfection strategies, there are several methods currently in use for the large-scale production of rAAV using packaging cell lines [16–21]. In 1990, Vincent et al demonstrated that a cell line expressing rep and cap could be used to package rAAV; however, the method still relied on transfection of the rAAV genome into the cell line, and reported titers were low [22]. Recently, we and others have developed a simplified method for generating rAAV based on stable HeLa cell lines containing integrated copies of the AAV rep-cap genes and a rAAV vector [16, 17]. To generate rAAV, the cells are simply infected with wild-type adenovirus, which results in the induction of the p5 rep promoter. rAAV producer cell lines are initially isolated by first cloning the desired cDNA sequence into a rAAV vector plasmid. Also present on the same plasmid (or a separate plasmid) is a selectable marker gene and the native rep-cap helper genes [16, 17]. Following HeLa cell transfection, stable cell lines are screened for viral production by adenovirus infection and yields quantitated by polymerase chain reaction (PCR) or dot blot hybridization [23]. Depending on the producer cell line, yields between 4×10^3 to 2×10^4 particles/cell are typical. Importantly, recombinant virus generated in this manner appears free of replication competent wild-type-like AAV (<1 IU of wild-type-like AAV/ 10^{11} rAAV particles). A variation on this basic approach is to infect a stable rep-cap expressing HeLa cell line (C12 or B50) with a recombinant adenovirus harboring a rAAV vector genome in the adenovirus E1 region [18, 20, 24, 25]. Co-infection with wild-type adenovirus is also required to supply the missing E1 helper gene products. Upon co-infection, the rAAV vector is excised from the adenovirus genome, replicated, and packaged into infectious virions. The primary advantage of this strategy is that repeated stable cell line selection is not necessary and reported rAAV yields were between 10^4 to 10^5 rAAV particles/cell [20]. Another potential strategy would be to generate a second recombinant adenovirus containing the AAV rep-cap genes; fortunately, such a vector has been isolated recently (**Note added in proof**). Interestingly, human herpes virus type 6 contains a functional rep gene homologue [26], and HSV amplicon vectors that carry and express the AAV Rep proteins in a stable manner have been reported [27, 28]. This has led to the development of a novel stable cell line production system based on a recombinant herpes simplex virus type I vector (rHSV-1) carrying the AAV rep-cap genes (d27.1-rc) [21]. In this production scheme, rHSV d27.1-rc provides complete helper functions for rAAV production. Moreover, d27.1-rc is itself replication defective because it does not produce a required herpes protein (ICP27) and, therefore, can only be grown in the appropriate comple-

menting cell line. Infection of a stable cell line containing an integrated rAAV genome with d27.1-rc resulted in vector yields of $>10^4$ particles per cell. It is notable that to date all stable cell line production methods rely on viral infection to supply helper gene products, and therefore, the potential for contamination of rAAV vector stocks with helper virus or associated viral proteins remains a cause for concern. Clearly, improved purification methods (discussed later here) have alleviated many of these concerns, but the development of stable packaging cells that supply all rAAV helper functions remains an important goal for future production strategies. As with all stable cell line methods, the process is scaleable. Currently, several groups are using microcarrier beads, spinner cells, or adherent cell bioreactors (Corning Cell Cube, Acton, MA, USA; or Nunc Cell Factory, Rochester, PA, USA) for the growth of $>10^{10}$ to 10^{11} producer cells. Assuming rAAV particle per cell yields of 10^4 , stable cell line approaches can yield between 10^{14} to 10^{15} rAAV particles per large-scale preparation.

RAAV PRODUCTION USING REP-CAP GENE AMPLIFICATION

The wild-type AAV genome (rep-cap genes) is replicated to $\geq 10^6$ copies per cell, of which a significant proportion is in a double-stranded, transcriptionally active conformation. Thus, a logical goal for rAAV production systems is to mimic as closely as possible wild-type AAV-2 rep-cap gene amplification as it occurs during a productive infection. Accordingly, it has been documented that the transfection of additional rep-cap plasmid copies relative to the rAAV vector plasmid augments rAAV yields proportionally [28]. Several production systems have been reported that allow for varying levels of rep-cap gene amplification, although no system to date approaches wild-type levels. The initial report placed a simian virus 40 (SV40) origin of replication on an AAV rep-cap plasmid. This helper plasmid was electroporated into COS cells (SV40 T-antigen expressing African green monkey cells) in an attempt to achieve episomal rep-cap gene amplification via SV40 ori/T-antigen interaction. However, low viral yields were reported (1000 particles per cell), and it was unclear whether rep-cap amplification was actually achieved in this system [29]. Limited rep-cap gene amplification was also reported using an HSV amplicon system [30]. In this production system, the AAV rep-cap genes and rAAV vector sequences were incorporated into HSV amplicon plasmids that contained the HSV origin of replication and packaging signal (oriS-pac). Upon transfection, the plasmids were amplified by infection with a replication defective HSV helper virus (glycoprotein H deleted). This virus supplied both helper rAAV functions and replicative ability to the HSV amplicon

plasmids. Vector yields of $>10^4$ rAAV particles per cell were reported. A rep-cap amplification strategy was also developed using a stable HeLa cell line system, which permitted inducible amplification (4-fold) of integrated rep-cap sequences from a nearby SV40 origin of replication using a doxycycline activated SV40 T antigen gene cassette [31]. Viral yields of 10^4 particles per cell were also reported. Recently, adenovirus inducible rep-cap gene amplification was also documented in stable, HeLa rAAV producer cell lines by two groups [32, 33]. Quantitative PCR documented episomal rep-cap gene amplification of approximately 100-fold in response to adenovirus infection, which corresponded to rep-cap copy numbers of approximately 400 to 500 per cell. This observation provides a plausible mechanism by which stable HeLa producer cell lines produce high levels of rAAV even though the initial AAV rep-cap copy number is low (typically 5 to 10 copies per cell). Curiously, adenovirus-inducible rep-cap gene amplification was not observed when the rep-cap-containing plasmid was transiently transfected into HeLa cells, indicating that a dramatic difference exists in the amplification potential of transfected rep-cap sequences versus stably integrated sequences. In conclusion, there are multiple, viable methods for the production of rAAV in excess of 10^4 particles per cell. These production levels should permit the widespread use of this technology for clinical applications (Table 1).

VECTOR PURIFICATION

Another significant advance in rAAV vector technology has been the recent development of several refined chromatography-based purification methods that render traditional isopycnic CsCl gradient purification methods obsolete [7, 23, 34]. Importantly, vector purity, biological potency, and process throughput are all increased using these chromatographic methods. All three methods allow for one-day vector purification from clarified cell lysates in volumes in excess of 1 L. One method uses an immunoaffinity approach, whereby a monoclonal antibody (A20) that recognizes a conformational epitope on the intact viral particle is coupled to a sepharose resin. Viral purification is achieved by a single pass of a crude cell lysate containing rAAV, followed by washing and viral elution with 2.5 mol/L $MgCl_2$. Percent recoveries of 70% were reported, and vector preparations contained low levels ($\leq 20\%$) of contaminating cellular proteins [7]. The other two approaches use heparin sulfate (a recently identified co-receptor for AAV-2) as an affinity ligand for viral purification. Clark et al reported a single-pass, crude lysate purification procedure using a commercial heparin sulfate resin (HE POROS; Applied Biosystems, Foster City, CA, USA) and column elution with a linear salt gradient [23]. Recoveries were on average $\geq 70\%$

Table 1. Estimated rAAV DNase resistant particle per cell yields of several production methods

	Producer cell	Particles/cell ^a	Helper function	Rep-cap element	Features	Ref.
Transient transfection						
CaPO ₄ co-txn	293/293T	1,000	wt Ad infection	native rep-cap genes		[3]
pDG	293	15,000 ^b	Ad mini-plasmid	MMLVp rep gene	rep downmodulation, wt AAV free	[7]
MTrep & CMVcap	293	10,000	Ad 5 E4 ORF6	mouse metallothionein rep gene	rep downmodulations, split rep-cap helper, wt AAV free	[15]
pXX2	293T	100,000	Ad mini-plasmid	ACG rep start codon	rep downmodulation, dual p5 promoter element	[9]
pVAE2AE4-5	293	13,300 ^c	Ad mini-plasmid	native rep-cap genes		[11]
pSH3/pSH5	293	13,600	Ad mini-plasmid	native rep-cap genes	combined Ad and rep-cap genes on a single plasmid	[10]
HSV amplicons	BHK	28,000 ^c	HSV-1 (gH-/TK-)	native rep-cap genes	rep-cap gene amplification	[30]
pspRC	293	12,500 ^d	Ad plasmid	native rep-cap genes		[12]
Cell lines						
HeLa (B50)	HeLa (B50)	55,000	rAd (E1 deleted) and Ad coinfection	~5 copies of native rep-cap genes integrated	order of infection critical for high titer production	[20]
B50/rAd						
pTp	HeLa	40,000	wt Ad infection	~5–20 copies of rAAV vector and rep-cap genes	rep-cap gene amplification	[23]
d27.1-rc	293	24,000 ^f	rHSV (ICP27 deleted)	native rep-cap genes	rHSV provides <i>all</i> rAAV helper functions	[21]
TtetA2	HeLa	29,000	wt Ad infection	native rep-cap genes with SV40 ori	SV40ori/Tantigen mediated rep-cap gene amplification	[19]

^arAAV DNase resistant particle/cell values were calculated based on the total cell number used to prepare the rAAV stock

^bAuthors reported 150 replication units per cell; above value was estimated by using a conservative conversion factor of 100 DNase resistant particles per replication unit

^cAuthors reported ~100 lacZ functional units per cell using plasmid pVAE2AE4-5, and a particle to functional unit ratio of 133

^dAuthors reported a maximal yield of 5×10^{12} rAAV particles from 25×15 cm dishes (calculation assumes 1.6×10^7 cells per dish $\times 25 = 4 \times 10^8$ 293 cells total)

^eAuthors reported a maximal yield of 280 GFP transducing units per cell and a ratio of 1:100 for GFP transducing units:DNase resistant particles

^fAuthors reported a maximal yield of 480 C12 replication units per cell (calculation assumes a conversion factor of 1 C12 RU:50 DNase resistant particles)

with purity in excess of 95%. Column capacity (1.4 mL bed volume) was $>10^{14}$ DNA-containing particles. A second method also used the HE POROS resin after partial rAAV purification using the iso-osmotic gradient reagent Iodixanol [34] and reported viral recoveries and purity similar to Clark et al. All three methods appear to produce lower rAAV particle:infectious particle ratios ($\leq 100:1$) than that observed using CsCl purification. Importantly, all three methods greatly decrease the level of contaminating cellular and helper virus proteins and result in a product that is of sufficient purity for clinical testing.

VECTOR TITRATION

The lack of a single method for determining the titer of rAAV stocks has led to some confusion in the field. Essentially, rAAV can be measured in four ways: (1) A physical particle titer enumerates the total number of viral particles (whether containing DNA or not) present in a rAAV preparation. A particle titer can be determined by EM, a commercial A20 antibody-based enzyme-linked immunosorbent assay (ELISA) [35] or can be calculated using the known molecular mass of AAV-2 and the protein concentration of a highly purified vector stock (1.5×10^{11} particles per 1 μ g of AAV-2 protein). Grimm et al and we have independently estimated that the majority ($\geq 80\%$) of vector particles in a rAAV prep-

aration are devoid of DNA [23, 35]. Accordingly, this may imply that the efficiency of rAAV genome encapsidation may be a rate-limiting step in infectious rAAV formation. (2) The second method determines the number of DNase-resistant vector genomes (encapsidated viral genomes) present in the rAAV vector stock. Briefly, to derive this value, a rAAV vector preparation is treated with DNase to degrade residual nonencapsidated vector genomes. The DNase is inactivated by proteinase K treatment, and the remaining viral nucleic acid quantitated by PCR or dot blot hybridization and compared with a known plasmid DNA standard curve. The advantage to this particle titer determination is that using quantitative PCR, titers can be rapidly generated in four hours [23]. Moreover, comparable DNase-resistant genome titers can be derived for all vector preparations, regardless of the transgene or promoter used in the construct. (3) A third method, also applicable to all vector stocks, determines the proportion of viral particles that can infect and *replicate* in a target cell line. This method, commonly referred to as a replication center assay, determines the number of infectious units in a rAAV preparation by intracellular amplification of the rAAV genome in the presence of wild-type AAV and adenovirus [36]. Detection of rAAV replication foci is accomplished by filter hybridization of infected cell monolayers. Recently, this approach has been refined using stable rep expressing cell lines and real time PCR detection of the amplified

vector genomes [12, 23, 37]. (4) Finally, a transduction (expression) titer measures the number of virus particles competent to infect and express the transgene in the target cell. Although a common method, widely varying titers (over three orders of magnitude) can be generated from the same vector stock by altering the target cell line or the intracellular environment (e.g., concurrent helper adenovirus infection or cell confluency). Therefore, comparisons of rAAV efficacy in different target cells or between vectors with different promoters become highly suspect. How do these various titers relate to each other? Although each vector preparation will possess a specific particle to infectivity ratio, an approximate ratio for heparin purified particles can be roughly given as 2500 total particles: 500 Dnase-resistant vector genome containing particles: 10 replication units: 1 transduction unit. Ideally, for each rAAV production method, the viral yields should be expressed in the form of infectious units per cell and Dnase-resistant vector genome particles per cell.

VECTOR QUALITY CONTROL

Prior to in vivo use, all rAAV vector stocks should be characterized with respect to four basic properties: (1) sterility, (2) replication competent AAV, (3) replication competent helper virus, and (4) purity. The effect that stock impurities have on rAAV transgene expression has been well documented [38, 39]. For example, replication competent adenovirus can dramatically increase rAAV-mediated transgene expression in vitro and can activate host immune responses in vivo. Furthermore, crude vector preparations containing transgene protein can lead to artifactual gene expression through a process termed pseudotransduction [38]. Therefore, each rAAV vector preparation should be characterized with respect to these four properties prior to use. Purified rAAV vector stocks can be sterile filtered through 0.45 μm membranes with little loss in vector titer (typically $\leq 20\%$) and subsequently assayed for sterility using thioglycolate media. The presence of replication competent adenovirus can be readily assayed using 1% of the rAAV preparation and a 293-plaque assay over a five-day period. Purity can be qualitatively monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and a more thorough estimate can be calculated by comparing the preparation's total protein content and the total particle yield (using the A20 particle ELISA) [35]. Contamination of vector stocks by replication competent AAV has been a persistent problem when using the transient transfection production method to produce rAAV. Wild-type-like AAV can be formed as a result of nonhomologous recombination between the rep-cap helper plasmid and the rAAV vector plasmid and can result in contamination levels of 0.1 to 10% [40, 41]. To address this problem, split packaging rep-cap plasmids that do not contain any

sequence homology between the rAAV vector and the AAV helper plasmid were created [40, 41], and this has greatly reduced or eliminated contaminating wild-type AAV from rAAV vector stocks. Fortunately, stable cell line production approaches do not appear to produce detectable wild-type-like AAV [16, 20, 21]. To test vector stocks for replication competent AAV, 1% of the rAAV stock should be used in a serial passage assay (typically on adenovirus-infected 293 or HeLa cells) to confirm the absence of wild-type AAV replication intermediates (monomeric and dimeric forms) in DNA isolated from the P2 cell population [16].

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