ISSN : 1225-9918
Journal of Life Science **2012** Vol. 22. No. 6. 703~712 DOI : http://dx.doi.org/10.5352/JLS.2012.22.6.703

Improved Production Efficiencies of Various Adeno-Associated Virus (AAV) Serotypes and a Novel Universal AAV Titration Method

Young-Hwa Cho[1†], Yejin Choi[2†], Jung-Hee Yun[1,4†], Nam Hee Kim[2], Mira Choi[2], Young-Kook Choi[1], Kyung-Hee Kim[1], Young-Ill Lee[3], Beom Jun Lee[4] and Keerang Park[1]*

[1]*Juseong Gene Therapy R&D Center, Juseong University, Chung-Buk 363-794, Korea*
[2]*Biologics Research Division, National Institute of Food and Drug Safety Evaluation, KFDA, Seoul 122-704, Korea*
[3]*School of Engineering, University of Suwon, Gyeonggi-Do 445-743, Korea*
[4]*College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Chung-Buk 361-763, Korea*

Received March 20, 2012 / Revised April 23, 2012 / Accepted May 15, 2012

Adeno-associated virus (AAV) has been considered to be a very safe and efficient gene delivery system. However, the major obstacles to therapeutic usage of AAV have been to achieve highly efficient and reproducible production processes, and also to develop a reliable quantifying method of various serotypes with a simple protocol. We compared the efficiency of the conventional production protocol of AAV2 and adenovirus (Ad) co-infection to that of a new method containing AAV2 infection followed by pHelper transfection. We tested HEK293 and 293T, and further examined the time-dependent changes of AAV2 production. The new method of AAV2 and pHelper DNA gave about ten times higher production efficiency than that of the conventional protocol. The highest production efficiency in 293T was achieved as 1.61 x 10^5 virus genomes (v.g.)/cell by the new method of 10 MOI of AAV2 infection and 5 days post-infection. This protocol of the highest efficiency was then applied to produce various AAV serotypes and showed the efficiencies higher than 10^5 v.g./cell. Next, we designed the universal PCR primers of highly conserved regions for various AAV serotypes to develop a simple and reliable titration method. The universal primers could amplify all the tested AAV serotypes with similar sensitivities by ten molecular copies. Therefore, this pair of universal primers can be further utilized to detect AAV contaminants in therapeutic adenoviral vectors.

Key words : Adeno-associated virus, AAV, gene therapy, scale-up production, virus titration

Introduction

Gene therapy has offered highly possible promises for treatment of cancers, vascular diseases, threatening infectious diseases and monogenic diseases using various gene delivery vector systems. Therefore, viral and non-viral gene delivery systems have been intensively developed to establish an ideal gene delivery vector for a target disease. Among many gene delivery vectors currently developed, AAV has been evaluated as one of the most efficient and the closest ideal vectors for serious diseases due to a number of advantages such as their ability to infect diving and no dividing cells of various tissue origins, the lack of pathogenicity and toxicity, the absence of significant host immune response and persistent transgene expression. Therefore, human clinical trials using AAV vector systems have been rapidly increased up to about 86 cases worldwide, which require significant increase in production capacity and efficiency [16].

It has been reported the range of 10^{14} to 10^{15} v.g. is needed for large animal studies and human clinical trials [14,28]. To maximize production efficiency, various packing cell lines containing *rep-cap* genes and producer stable cell lines derived from HeLa, HEK293 and BHK have been made [4,8,20,28]. The cells were previously transfected with plasmid DNAs for a transgene expression cassette flanked by ITRs and a packing *rep-cap* gene followed by a helper virus infection. Later, the helper functions from viruses were replaced by an adenovirus helper plasmid containing the E2A, E4 and VA RNA genes [9], which eliminated the additional infection step and facilitated the following purification process. Many different kinds of transfection reagents such as calcium phosphate precipitation, linear or branched polyethylenimine (PEI), various liposomes and other reagents were developed to enhance production efficiencies [10,13,23]. Efficient, cost-effective scale-up production processes have been developed by adaptation techniques of ad-

†These three authors contributed equally.
*Corresponding author
Tel : +82-43-210-8462, Fax : +82-43-210-8465
E-mail : krpark@jsu.ac.kr

herent cell lines to suspension cultures in bioreactors or by employing the baculovirus/insect cell system [23,28].

Since AAV was originally discovered as contaminants in adenovirus preparations and has been emerged as one of the most versatile gene delivery vectors, more than 11 AAV serotypes have been discovered and characterized [5,22]. However, there are few reliable standard protocols for measuring AAV, so there are increasing needs to establish standard reliable quantifying methods of various AAV serotypes, furthermore to develop a simple titration protocol of various serotypes with a universal primer set. All AAV serotypes contain a single stranded DNA genome consisting of approximately 4,700 nucleotides which encodes non-structural proteins (Rep78, Rep68, Rep52, Rep40) and capsid proteins (VP1, VP2 and VP3) [22]. To quantify AAV virus particles or virus genomes, the quantitative technologies using a southern blot hybridization, a quantitative PCR, an enzyme-linked immunosorbent assay (ELISA), an analytical high performance liquid chromatography (HPLC), electron microscopy and spectro-photometry have been developed [11,18,21,25-27,29,30]. Although each quantifying protocol needs to be further improved, a quantitative PCR has been evaluated as a more reliable, robust and accurate titration way of AAV genomes and has also been regarded as a quicker way to assay AAV vectors. Recently, ELISA titration kits for several serotypes of AAV2, 4, 5, and 6 have been developed and utilized. However, none of the titration protocols described elsewhere can quantify various AAV serotypes with a simple, reliable and universal method [2].

In this communication, we enhanced the production efficiencies of several AAV serotypes using the new method of AAV infection followed by pHelper plasmid DNA transfection in 293T, which can be utilized for highly efficient scale-up production. We also established a simple, reliable titration protocol to quantify various AAV serotypes using a pair of universal PCR primers developed by aligning all nucleotides of 10 different AAV serotypes each other and locating the mostly conserved region among 10 serotypes. Our universal titration protocol can be further utilized as a detection method of AAV contaminants in therapeutic adenovirus vectors.

Materials and Methods

Cell lines

HEK293 and 293T were purchased from the American Type Culture Collection (ATCC, VA, USA) and maintained in a complete media containing Dulbeco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin at 37°C in a 5% CO_2 incubator. All materials for tissue cultures were purchased from Invitrogen (CA, USA).

AAV vector production

AAV1-5 and adenoviruses as helper viruses were purchased from the ATCC (AAV-1, VR-645; AAV-2, VR-680; AAV-3, VR-681; AAV-4, VR-646; AAV-5, VR-1523; Adenovirus 2, VR-846; SV-15, VR-1449; Adenovirus 7a, VR-848). For AAV vector production, HEK293 or 293T cells were seeded at 1 x 10^5 cells per well in a 12-well plate or 3 x 10^6 cells per 100 mm plate, were allowed to grow overnight and became approximately 70% confluency prior to infection. The cells were treated with two different production protocols as follows;

First, the cells were infected with both of AAV at various MOI and the proper adenovirus serotype of helper virus (Ad7a for AAV1; Ad2 for AAV2, AAV3, and AAV5; SV-15 for AAV4) at 10 MOI for 24 hours, were replaced with fresh DMEM media containing 2% FBS and maintained for another 24 hours before harvest. Second, the cells were infected with AAV at various MOI, which were then followed by transfection of pHelper plasmid DNA for 24 hours using a calcium-phosphate precipitation method [6]. The treated cells were replaced with a fresh DMEM media containing 2% FBS for another 24 hours prior to harvest. Helper viruses for AAV vector amplification were heat-inactivated at 56℃ for 30 min.

After the cells were treated for 48 hours, they were harvested, disrupted by repeated freezing-thawing cycles and subjected to CsCl gradient centrifugations for AAV purification [7]. The purified AAV were stored at 4℃ or -70℃ for analyses and a scale-up AAV production. All productions under different conditions were repeated more than three times and the most representative results were summarized in Tables.

Quantification of AAV vectors

The titers of AAV vectors were estimated by a quantitative real-time PCR using a Fast Real-time PCR System 7900HT and TaqMan Universal PCR Master Mix (Applied Biosystems, CA, USA). As shown in Fig. 1B, a pair of universal primer set of the conserved *rep* gene regions for various

AAV serotypes (AAV1-8, 10 and 11) was designed as follows;

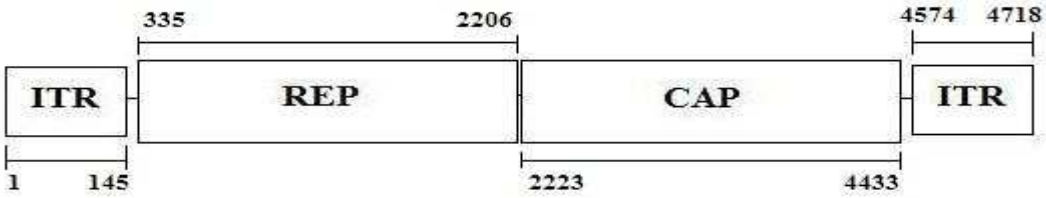
AAV Universal forward primer (AAV-uF), 5′- ggg tgg ccg aga agg aat - 3′,

AAV Universal reverse primer (AAV-uR), 5′ - cct ccg ggg cct tac tca - 3′,

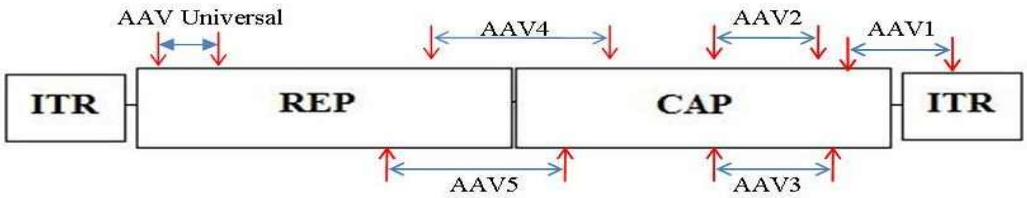
AAV probe for TaqMan PCR, 5′ - [FAM]cag gca ccc ctg acc[MGBNFQ] - 3′

The PCR reaction mixtures containing the diluted virus sample of template, 10 µl of 2 x TaqMan Universal PCR Master Mix, 2 µl of each probe and 250 nM primers were prepared to give 20 µl total volume. The virus samples were amplified by the following PCR conditions; 5 min at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C, completed by a final extension for 10 min at 72°C. The size of the amplified products is expected to be 141-bp for AAV1 to AAV4, but 138-bp for AAV5. The fluorescence of the amplified PCR products was measured by an ABI PRISM 7700. All virus samples were titrated in triplicate, the titers were averaged and the same experiments were re-

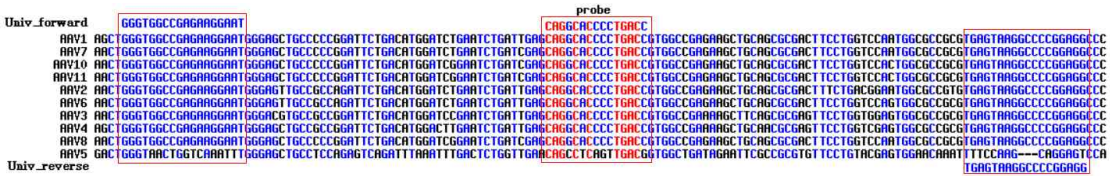
A.



B.



C.



D.

	AAV-uF	AAV-uR	Taqman probe	PCR product size	*rep* gene	*cap* gene
AAV1	420-437	560-543	482-496	141-bp	335-2206	2223-4433
AAV2	406-423	546-529	468-482	141-bp	321-2252	2203-4410
AAV3	403-420	543-526	465-479	141-bp	318-2192	2209-4419
AAV4	457-474	597-580	519-533	141-bp	372-2243	2260-4464
AAV5	444-461	581-567	506-520	138-bp	35-2191	2207-4381
AAV6	405-422	545-528	467-481	141-bp	320-2191	2208-4418
AAV7	419-436	559-542	481-495	141-bp	334-2205	2222-4435
AAV8	312-329	452-435	374-388	141-bp	227-2104	2121-4337
AAV10	86-103	226-209	148-162	141-bp	1-1869	1886-4102
AAV11	86-103	226-209	148-162	141-bp	1-1869	1886-4087

Fig. 1. A. AAV1 genome organization. The genome is composed of two encoding regions of the nonstructural Rep proteins and the structural Cap proteins flanked by the short inverted terminal repeats (ITR). B. Location of the pair of universal titration primers and five different serotype-specific primer sets as described in Materials and Methods. C. Alignments of 10 different AAV serotype genes and location of the mostly conserved region among 10 serotypes marked in the boxes. D. Summary of the location of the universal primers (AAV-uF and AAV-uR) and Taqman probe, the amplified product sizes by these primers and the *rep* and *cap* genes for various AAV serotypes.

peated three times.

The serotype-specific primers for AAV1 to AAV5 were designed as follows; for AAV1, forward primer of 5′- ttc agc tac aaa gtt tgc t -3′ and reverse primer of 5′- acc cga tga cgt aag tct tt -3′, the product size of 368-bp; for AAV2, forward primer of 5′- aga aca aac act cca agt gg -3′ and reverse primer of 5′- cca cgg gat tgg ttg tcc -3′, the product size of 375-bp; for AAV3, forward primer of 5′- cgc aag gaa caa cct ctg g -3′ and reverse primer of 5′- cag ttc cag tcg tgg gag c -3′, the product size of 443-bp; for AAV4, forward primer of 5′- ctt gga tga ctg tga cat g -3′ and reverse primer of 5′- tag gtg ggc aag acc cag gtt ct -3′, the product size of 780-bp; for AAV5, forward primer of 5′- aac att tct aac aaa tgt -3′ and reverse primer of 5′- agt cct ctt cgg tcc gag cct tct t -3′, the product size of 657-bp. The virus samples of the isolated AAV genomic DNAs were amplified using the serotype-specific primers in 20 μl of PCR reaction mixture containing 2 μl of 10 x PCR buffer, 1 μl of 50 mM $MgCl_2$, 250 nM dNTP, 5 pmoles of forward and reverse primers, and 1 unit of Taq polymerase (Invitrogen, CA, USA) in a MyCycler (BioRad, USA). DNA size maker of 100-bp ladder was purchased from Invitrogen (USA, Cat# 15628-019).

Isolation of AAV genome DNA

AAV genome DNA was isolated essentially by the procedure described elsewhere [19]. Briefly, 100 μl of each AAV suspension was mixed with 100 μl of a viral DNA extraction buffer (100 mM Tris-HCl, 150 mM NaCl, 12.5 mM EDTA, 2% SDS, pH 7.4), which was then digested with 5 μl of proteinase K (20 mg/ml) at 50℃ for 30 min. Equal volume of phenol-chloroform was added to the digestion mixture, vortexed for 2 min and then centrifuged at 4℃ for 10 min. The top aqueous phase of viral DNA was transferred to a new tube, extracted two more times with phenol/chloroform, and precipitated with 3 M sodium acetate (pH 5.2) and ice-cold absolute ethanol. After centrifugation, the viral DNA pellet was washed with 80% ethanol, air-dried, and resuspended in a 20 μl distilled water.

Results

Due to many beneficial properties of AAV as a safe, highly efficient gene delivery vector, clinical trials using AAV have been greatly increased up to about 86 cases. However, most of AAV production protocols have employed the adherent cell culture systems and have not shown repeatable production efficiencies, so the efficient scale-up processes showing consistent production efficiencies need to be developed. First, we examined several AAV production protocols to establish a highly efficient production system for AAV1 through AAV5. According to the ATCC information, each serotype of AAV has been produced by co-infection of AAV and the corresponding Ad helper virus as follows; AAV1 was amplified by co-infection of AAV1 and adenovirus type 7a (Ad7a), AAV2 was produced by co-infection of AAV2 and adenovirus type 2 (Ad2), AAV3 was amplified by co-infection of AAV3 and Ad7a, AAV4 was produced by co-infection of AAV4 and simian adenovirus 3 (SV-15), and AAV5 can be amplified by co-infection of AAV5 and Ad2 [1,3,12,15,24]. We compared the production efficiency of the conventional co-infection of AAV2 and Ad2 to that of the new production method of AAV2 infection followed by pHelper plasmid DNA transfection. As summarized in Table 1, the production efficiencies of the method using AAV2 infection followed by pHelper plasmid DNA transfection were about 10 times higher than those of the previous method using AAV2 and Ad2 co-infection under any co-infection conditions. We then tested various MOIs for AAV2 infections to measure changes of AAV2 production efficiencies, and also examined time-dependent changes of AAV2 production. As shown in Table 2, the highest AAV2 production efficiency was achieved by AAV2 infection of MOI 10 and on day 5 post infection. We tested two different producer cell lines of HEK293 and 293T, and demonstrated that the production efficiencies in 293T were about 25 times higher than those in HEK293 under any production conditions (see Table 3). Lastly, we applied the production protocol of the highest efficiency to amplifications of AAV1 through AAV5 vectors. As shown in Table 4, all AAVs except AAV4 yielded higher efficiencies than 1.0 x 10^5 v.g./cell. The efficiencies of AAV4 productions could not be reached to 1.0 x 10^5 v.g./cell, although the recommended producer cell line, LLC-MK2 (ATCC CCL-7.1) was also tested (data not shown). The AAV4 production efficiency using 293T was about 10 times higher than that using LLC-MK2 (data not shown).

Next, we designed the universal primer set of the conserved region to develop a simple, reliable and sensitive quantitative method for various AAV serotypes. As shown in Fig. 1A of AAV1, the genome structure of any known AAV is composed of the *rep* and *cap* genes flanked by ITRs.

Table 1. Comparison of AAV2 production efficiencies of AAV2 and Ad co-infection method to those using AAV2 infection followed by pHelper plasmid DNA transfection

Producer cell line	Total amount of AAV2, Ad2 and pHelper plasmid			Production Efficiencies (v.g./cell)
	AAV2 (M.O.I)	Ad2 (M.O.I)	pHelper plasmid DNA (μg)	
HEK293	1000	100 (live Ad2)	-	3.92×10^{2}
	1000	100 (heat inactivated Ad2)	-	8.32×10^{1}
	1000	1 heat inactivated Ad2)	-	2.56×10^{2}
	1000	**-**	**15**	**2.16×10^{3}**

The experiments under the same production conditions were repeated three times and the data of the representative experiment were summarized. AAV2 titers were quantified in triplicate by a real-time PCR using the universal primers. The production condition of the highest efficiency was underlined in bold.

Table 2. Time-dependent AAV2 production efficiencies under various MOIs of AAV2 infection followed by pHelper transfection

Producer cell line	Total amount of AAV2 and pHelper plasmid DNA		Production efficiencies (v.g./cell)			
			Incubation time post infection (days)			
	AAV2 (M.O.I)	pHelper DNA (μg)	Day 2	Day 3	Day 4	Day 5
HEK293	1000	15	2.18×10^{2}	8.47×10^{2}	4.14×10^{3}	2.81×10^{3}
	100	15	1.27×10^{3}	9.31×10^{2}	3.40×10^{3}	2.36×10^{3}
	10	**15**	**2.34×10^{2}**	**1.77×10^{3}**	**4.10×10^{3}**	**6.29×10^{3}**
	1	15	3.90×10^{1}	2.36×10^{3}	3.55×10^{3}	1.55×10^{3}
	0.1	15	8.21×10^{1}	7.00×10^{1}	3.14×10^{3}	9.62×10^{2}

The experiments under the same production conditions were repeated three times and the data of the representative experiment were summarized. AAV2 titers were quantified in triplicate by a real-time PCR using the universal primers. The production condition of the highest efficiency was underlined in bold.

Table 3. Comparison of AAV2 production efficiencies in HEK293 to those in 293T

Total amount of AAV2 and pHelper plasmid DNA		Production efficiencies (v.g./cell) On Day 5	
		Producer cell line	
AAV2 (M.O.I)	pHelper DNA (μg)	HEK293	**293T**
1000	15	2.81×10^{3}	2.85×10^{4}
100	15	2.36×10^{3}	7.43×10^{4}
10	**15**	**6.29×10^{3}**	**1.61×10^{5}**

The experiments under the same production conditions were repeated three times and the data of the representative experiment were summarized. AAV2 titers were quantified in triplicate by a real-time PCR using the universal primers. The production condition of the highest efficiency was underlined in bold.

Table 4. Production efficiencies of AAV1 through AAV5 using the production condition of the highest efficiency

Producer cell line	Total amount of AAV and pHelper plasmid DNA		Production efficiencies (v.g./cell) On Day 5				
			Production of each serotype AAV				
	AAV (M.O.I)	pHelper DNA (μg)	AAV1	AAV2	AAV3	AAV4	AAV5
293T cell	1000	15	3.65×10^{4}	4.98×10^{3}	1.28×10^{5}	**3.29×10^{4}**	1.89×10^{5}
	100	15	1.30×10^{5}	4.57×10^{4}	2.18×10^{5}	3.93×10^{3}	1.91×10^{5}
	10	15	**1.21×10^{5}**	**1.78×10^{5}**	**2.89×10^{5}**	**1.35×10^{3}**	**1.41×10^{5}**
	1	15	**2.15×10^{5}**	**2.52×10^{5}**	**9.25×10^{4}**	**N. D.***	**2.59×10^{5}**

* N. D., not determined

The experiments under the same production conditions were repeated three times and the data of the representative experiment were summarized. AAV titers were quantified in triplicate by a real-time PCR using the universal primers. The production conditions of the higher efficiency were underlined in bold.

Among the fully sequenced serotypes of AAV1 through AAV8, AAV10 and AAV11, we were able to locate highly conserved regions of a potential universal forward and reverse primer set within the *rep* genes as depicted in Fig. 1B and aligned in the marked boxes of Fig. 1C. The sequences of AAV5 were able to be aligned to the conserved regions of the other AAVs, but the homology of AAV5 to the others was somewhat low, whereas the conserved regions of the other AAVs showed 100% identity each other. The locations of the universal primers (AAV-uF and AAV-uR), Taqman probe, PCR product sizes, and the *rep* and *cap* genes in 10 AAV serotypes were summarized in Fig. 1D. For a control amplification of each AAV serotype, we also designed a pair of serotype-specific primers as shown in Fig. 1B and described in Materials and Methods. The universal primer set was evaluated using five different AAV virus stocks (AAV1 to AAV5) purchased from ATCC to determine how efficiently each AAV serotype could be amplified with great reliability and similar sensitivity. As shown in Fig. 2, AAV1 through AAV5 were efficiently amplified to generate the expected size of 141-bp for AAV1 through AAV4 or 138-bp for AAV5, whereas the negative control containing no template did not show any amplified DNA fragment. Serotype-specific amplifications were verified using a pair

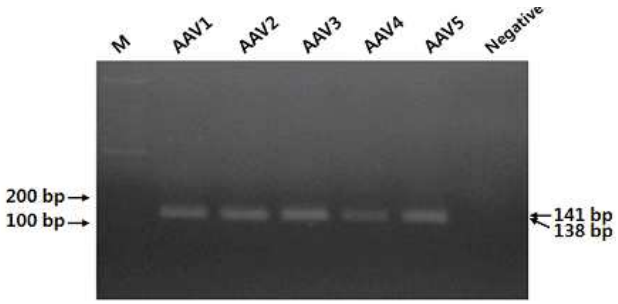


Fig. 2. The amplified PCR products of AAV1 through AAV5 by the pair of universal primers. A pair of universal titration PCR primers was designed and synthesized using the highly conserved region of the *rep* genes among 10 AAV serotypes as depicted in Fig. 1B. AAV virus stocks of AAV1 through AAV5 were purchased from ATCC and 10^4 virus particles of each AAV virus stock were amplified using the pair of universal primers. The amplified PCR products for AAV1 through AAV5 were analyzed by an agarose/TBE gel electrophoresis as shown in lanes AAV1 through AAV5, whereas no amplified PCR product of the negative control containing no template in a PCR mixture was generated (lane Negative). DNA size maker of 100-bp ladder (lane M) was purchased from Invitrogen.

of serotype-specific primers and genomic DNAs extracted from the corresponding AAV serotype stocks as shown in Fig. 3. Each serotype genomic DNA was amplified to generate the expected PCR product of 368-bp, 375-bp, 443-bp, 780-bp and 657-bp, respectively from AAV1 through AAV5 using the corresponding pair of primers. To verify further serotype-specific amplifications of the corresponding AAV genomic DNA, we amplified five different AAV genomic DNAs using each pair of serotype-specific primers. As shown in Fig. 3, each panel of PCR amplification for each AAV serotype demonstrated only serotype-specific amplification, whereas there was no amplification for the control PCRs containing the other four serotype genomic DNAs and for the negative control of no template. We also tested sensitivities of amplification for each serotype using the universal primer set and demonstrated that all the tested serotypes of AAV were efficiently amplified by about 10 molecules (Table 5).

Discussion

AAV has been emerged as one of promising gene delivery vectors due to beneficial features such as safety, robust and persistent expression and very low immune response. As the clinical trials using AAV-based therapeutic vectors greatly increased, efficient scale-up processes have been intensively developed. However, a few production processes barely meet the production efficiencies for large animal studies and human clinical trials. Therefore, we tested several production protocols using either AAV and Ad co-infection or AAV infection followed by pHelper plasmid DNA transfection. When we replaced the adenovirus's helper functions with pHelper plasmid DNA containing E2A, E4 and VA RNA genes, the production efficiencies of all the tested AAV serotypes were greatly increased by about 10 times. This new production process has two major benefits for AAV scale-up process as follows; one is a marked increase in production efficiency and the other is a highly convenient purification step after production due to the absence of helper virus. Here we tested various production conditions to raise the efficiencies, but we only employed the adherent culture systems. To establish efficient scalable production, it has been reported that the suspension culture systems are more feasible and reproducible to scale-up the production volume as much as required than the adherent culture systems [23]. Therefore, our suspension culture systems

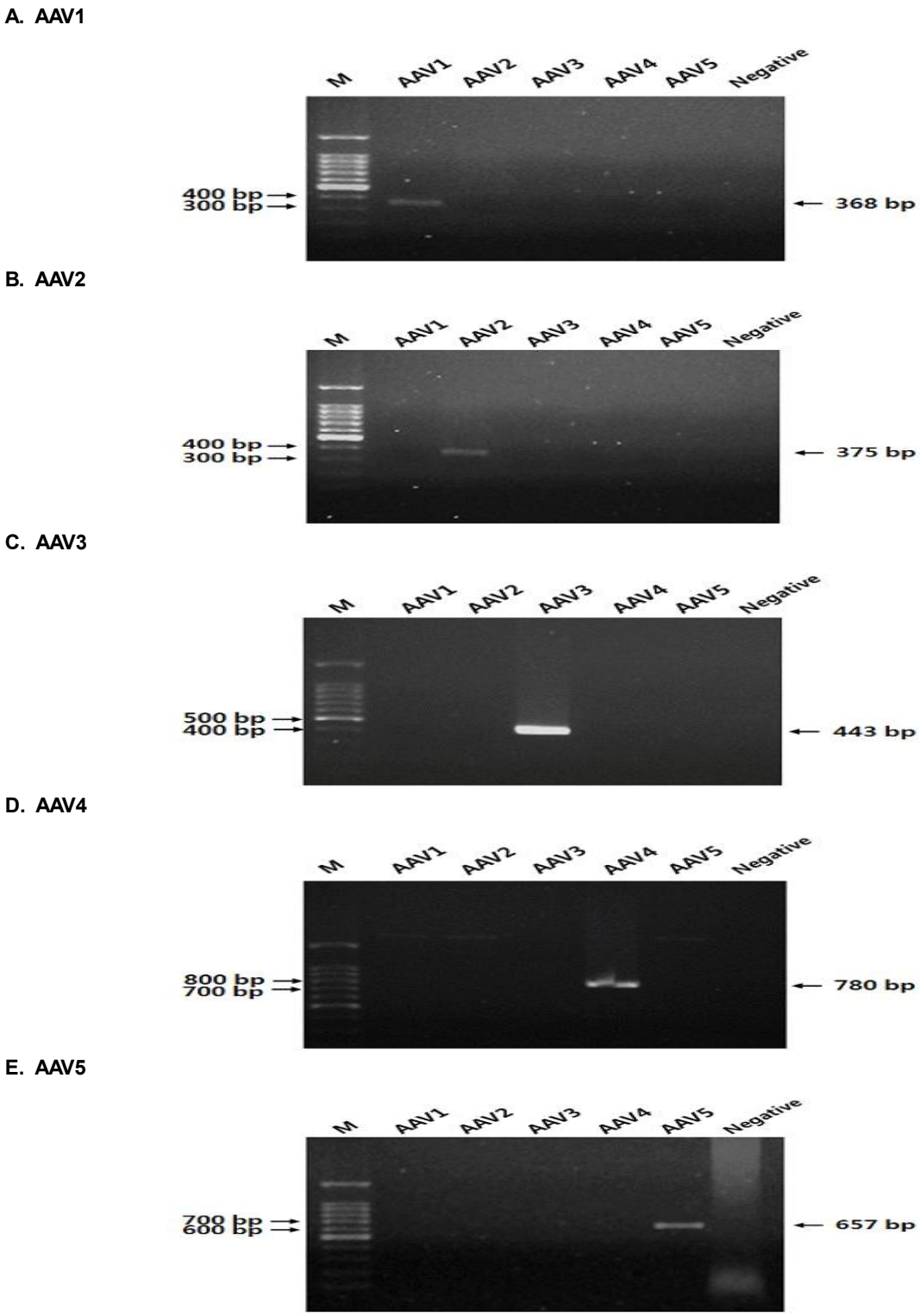


Fig. 3. Serotype-specific amplifications of AAV genomic DNAs using serotype-specific primers. Five different AAV genomic DNAs isolated from AAV virus stocks of AAV1 through AAV5 were amplified using each serotype-specific primers. Five genomic DNAs of AAV1 through AAV5 (lanes AAV1 through AAV5) were amplified by AAV1-specific primers (panel A), AAV2-specific primers (panel B), AAV3-specific primers (panel C), AAV4-specific primers (panel D) or AAV5-specific primers (panel E). For each serotype-specific amplification, a negative control was also included using a PCR mixture containing no template (negative lane). DNA size maker of 100-bp ladder (lane M) was purchased from Invitrogen.

Table 5. Sensitivity of various AAV detections by approximately 10 genome copies

AAV1		AAV2		AAV3		AAV4		AAV5	
C_t	Genome copies	C_t	Genome copies	C_t	Genome copies	C_t	Genome copies	C_t	Genome copies
31.077	479.221	31.782	303.628	31.010	556.743	30.215	891.878	31.010	556.743
32.980	156.108	33.677	89.403	33.144	142.039	32.172	236.233	33.754	96.289
35.792	24.135	35.698	23.391	35.804	23.112	35.816	25.354	35.932	21.517
36.607	12.409	36.481	15.162	36.282	14.680	36.806	11.767	36.628	13.718
37.355	7.845	37.247	10.661	37.763	7.688	37.573	7.336	37.426	7.434

The experiments under the same production conditions were repeated three times and the data of the representative experiment were summarized. C_t stands for the cycle threshold.

are being intensively established using the production process of the highest efficiency that we developed in this study. Since HEK293 generated by transformation of human embryonic kidney cells with shearing Ad5 DNA has been a useful producer cell line for AAV vectors and 293T containing the SV40 Large T-antigen has also been the most important variant of HEK293 due to highly efficient amplification of transfected plasmids, we examined the AAV production efficiencies in both of HEK293 and 293T. As reported elsewhere and as expected, the present study demonstrated that production efficiency in 293T was greatly higher than that in HEK293. When we tested various MOI of AAV infections, production efficiencies using low MOI of 10 or even 1 were much better than those using high MOI of 100 or even 1000. This production condition using low MOI of AAV infection can be immediately utilized for mass production of wild type AAV serotypes as the standard reference materials due to decreased manufacturing cost. The various wild type AAV serotype vectors are particularly required for validation process of adenovirus-based clinical materials to demonstrate the absence of AAV as adventitious viruses.

To establish an accurate, reliable and simple quantitative method to assay AAV vector, various quantifying protocols have been developed as described in Introduction. However, each titration method has pros and cons, and more reproducible and universal methods need to be developed. In the current study, we were able to design the best pair of universal PCR primes locating at the highly conserved regions among AAV1 through AAV8, AAV10 and AAV11. Among the reported AAV serotypes, AAV1 through 8 have been completely sequenced and characterized, but AAV9 was partially sequenced and only *cap* gene region of AAV9 sequences is available. Furthermore, AAV10 and 11 are also completely sequenced. Among all the reported AAV serotypes, the *rep* genes are highly conserved, whereas the *cap* genes are quite varied. We were able to locate the greatly conserved regions of the *rep* gene for all the characterized AAV serotypes. So we prepared the universal primers of those regions and then examined for their sensitivity, reliability and efficiency of PCR amplification. Although the universal primers were able to be aligned to AAV5 as the conserved region, several bases of AAV5 *rep* gene were not quite matched with the universal primers. However, the universal primers were able to amplify AAV5 as efficiently and sensitively as the other serotypes. Therefore, this simple titration protocol is greatly valuable for quantifying various AAV serotypes with one pair of the universal primers. Furthermore, this simple, sensitive and reliable PCR method using the universal primers can be greatly useful to detect various serotypes of AAV contaminants in therapeutic adenoviral vectors.

Acknowledgement

This study was supported by the grant from KFDA 09121-386, and partly by the Ministry of Knowledge Economy, A000200338.

References

1. Atchison, R. W., Casto, B. C. and Hammon, W. M. 1965. Adenovirus-Associated Defective Virus Particles. *Science* **149**, 754-756.
2. Aucoin, M. G., Perrier, M. and Kamen, A. A. 2008. Critical assessment of current adeno-associated viral vector production and quantification methods. *Biotechnol. Adv.* **26**, 73-88.
3. Bantel-Schaal, U. and zur Hausen, H. 1984. Characterization of the DNA of a defective human parvovirus isolated from a genital site. *Virology* **134**, 52-63.
4. Blouin, V., Brument, N., Toublanc, E., Raimbaud, I., Moullier, P. and Salvetti, A. 2004. Improving rAAV pro-

duction and purification: towards the definition of a scalable process. *J. Gene Med.* **6**, S223-228.
5. 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.
6. Chen, C. and Okayama, H. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell Biol.* **7**, 2745-2752.
7. Cho, Y. H., Park, H., Cho, E. S., Kim, W. J., Kang, B. S., Park, B. Y., Kim, Y. J., Lee, Y. I., Chang, S. I. and Park, K. 2007. A novel way of therapeutic angiogenesis using an adeno-associated virus-mediated angiogenin gene transfer. *Exp. Mol. Med.* **39**, 412-418.
8. Clark, K. R., Voulgaropoulou, F., Fraley, D. M. and Johnson, P. R. 1995. Cell lines for the production of recombinant adeno-associated virus. *Hum. Gene Ther.* **6**, 1329-1341.
9. Clark, K. R. 2002. Recent advances in recombinant adeno-associated virus vector production. *Kidney Int.* **61**, S9-15.
10. Collaco, R. F., Cao, X. and Trempe, J. P. 1999. A helper virus-free packing system for recombinant adeno-associated virus vectors. *Gene* **238**, 397-405.
11. Gao, G. P., Wilson, J. M. and Wivel, N. A. 2000. Production of recombinant adeno-associated virus. *Adv. Virus Res.* **55**, 529-543
12. Georg-Fries, B., Biederlack, S., Wolf, J. and zur Hausen, H. 1984. Analysis of proteins, helper dependence, and seroepidemiology of a new human parvovirus. *Virology* **134**, 64-71.
13. Grimm, D., Kern, A., Rittner, K. and Kleinschmidt, J. A. 1998. Novel tools for production and purification of recombinant adeno-associated virus vectors. *Hum. Gene Ther.* **10**, 2745-2760.
14. Grimm, D. and Kleinschmidt, J. A. 1999. Progress in adeno-associated virus type 2 vector production: promises and prospects for clinical use. *Hum. Gene Ther.* **10**, 2445-2450.
16. Hoggan, M. D., Blacklow, N. R. and Rowe, W. P. 1966. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. *Proc. Natl. Acad. Sci. USA* **55**, 1467-1474.
16. Kay, M. A., Manno, C. S., Ragni, M. V., Larson, P. J., Couto, L. B., McClelland, A., Glader, B., Chew, A. J., Tai, S. J., Herzog, R. W., Arruda, V., Johnson, F., Scallan, C., Skarsgard, E., Flake, A. W. and High, K. A. 2000. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat. Genet.* **24**, 257-261.
17. 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.
18. Kuck, D., Kern, A. and Kleinschmidt, J. A. 2007. Development of AAV serotype-specific ELISAs using novel monoclonal antibodies. *J. Virol. Methods* **140**, 17-24.
19. Le, C. T., Gray, G. C. and Poddar, S. K. 2001. A modified rapid method of nucleic acid isolation from suspension of matured virus: applied in restriction analysis of DNA from an adenovirus prototype strain and a patient isolate. *J. Med. Microbiol.* **50**, 571-574.
20. Matthews, L. C., Gray, J. T., Gallagher, M. R. and Snyder, R. O. 2002. Recombinant adeno-associated viral vector production using stable packaging and producer cell lines. *Methods Enzymol.* **346**, 393-413.
21. Mayginnes, J. P., Reed, S. E., Berg, H. G., Staley, E. M., Pintel, D. J. and Tullis, G. E. 2006. Quantitation of encapsidated recombinant adeno-associated virus DNA in crude cell lysates and tissue culture medium by quantitative, real-time PCR. *J. Virol. Methods* **137**, 193-204.
22. Merten, O. W., Geny-Fiamma, C. and Douar, A. M. 2005. Current issues in adeno-associated viral vector production. *Gene Ther.* **12**, S51-61.
23. Park, J. Y., Lim, B. P., Lee, K., Kim, Y. G. and Jo, E. C. 2006. Scalable production of adeno-associated virus type 2 vectors via suspension transfection. *Biotechnol. Bioeng.* **20**, 416-430.
24. Parks, W. P., Melnick, J. L., Rongey, R. and Mayor, H. D. 1967. Physical assay and growth cycle studies of a defective adeno-satellite virus. *J. Virol.* **1**, 171-180.
25. Qiao, C., Wang, B., Zhu, X., Li, J. and Xiao, X. 2002. A novel gene expression control system and its use in stable, high-titer 293 cell-based adeno-associated virus packing cell lines. *J. Virol.* **76**, 13015-13027.
26. Sommer, J. M., Smith, P. H., Parthasarathy, S., Isaacs, J., Vijay, S., Kieran, J., Powell, S. K., McClelland, A. and Wright, J. F. 2003. Quantification of adeno-associated virus particles and empty capsids by optical density measurement. *Mol. Ther.* **7**, 122-128.
27. Transfiguracion, J., Jorio, H., Meghrous, J., Jacob, D. and Kamen, A. 2007. High yield purification of functional baculovirus vectors by size exclusion chromatography. *J. Virol. Methods* **142**, 21-28.
28. Urabe, M., Ding, C. and Kotin, R. M. 2002. Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum. Gene Ther.* **13**, 1935-1943.
29. Urabe, M., Nakakura, T., Xin, K. Q., Obara, Y., Mizukami, H., Kume, A., Kotin, R. M. and Ozawa, K. 2006. Scalable generation of high-titer recombinant adeno-associated virus type 5 in insect cells. *J. Virol.* **80**, 1874-1885.
30. Veldwijk, M. R., Topaly, J., Laufs, S., Hengge, U. R., Wenz, F., Zeller, W. J. and Fruehauf, S. 2002. Development and optimization of a real-time quantitative PCR-based method for the titration of AAV-2 vector stocks. *Mol. Ther.* **6**, 272-278.
31. Yun, H. J., Cho, Y. H., Moon, Y., Park, Y. W., Yoon, H. K., Kim, Y. J., Cho, S. H., Lee, Y. I., Kang, B. S., Kim, W. J., Park, K. and Seo, W. 2008. Transcriptional targeting of gene expression in breast cancer by the promoters of protein regulator of cytokinesis 1 and ribonuclease reductase 2. *Exp. Mol. Med.* **40**, 345-353.

초록 : 다양한 adeno-associated virus (AAV) 혈청형의 효율성 높은 생산법과 새로운 공통적 정량법 개발

조영화[1] · 최예진[2] · 윤정희[1,4] · 김남희[2] · 최미라[2] · 최영국[1] · 김경희[1] · 이영일[3] · 이범준[4] · 박기랑[1]*
([1]주성대학교 주성유전자치료기술센터, [2]식품의약품안전평가원 생물의약품연구과, [3]수원대학교 공과대학, [4]충북대학교 수의과대학)

AAV는 매우 안전하고 효율적인 유전자전달방법으로 인정되어 왔다. 그러나, AAV가 가진 생물의약품으로서 단점은 효율적이고 재현성 높은 생산법이 취약하고, 또한 다양한 혈청형을 간단한 한 가지 공통적 방법으로 신뢰성 있게 정량하는 방법이 개발되어야 하는 것이다. 따라서, 본 연구에서는 AAV2와 아데노바이러스를 동시에 감염하는 종래의 생산법에 의한 효율성과 새로운 생산법, 즉 AAV2 감염 후 pHelper 플라스미드를 transfection 하는 방법을 통한 생산효율성을 비교하였고, HEK293과 293T를 생산세포주로 하여 시간에 따른 생산효율성도 분석하였다. 그 결과 AAV2와 pHelper DNA를 포함한 새로운 생산법은 기존의 방법에 비해 10배 이상 높은 생산효율성을 보였고, 293T에서 AAV2를 10 MOI로 감염한 후 5일째에 가장 높은 생산효율성을 보였는데, 생산세포 한 개 당 1.61×10^5 virus genomes (v.g.)을 생산하는 결과였다. 따라서 이 생산조건을 다른 혈청형 생산에 적용한 결과, 모든 혈청형에서 생산세포주 한 개 당 10^5 v.g. 이상을 생산하는 효율성을 보였다. 한편, 다양한 AAV 혈청형을 한 가지 공통적인 방법으로 정량하기 위해 the universal PCR 프라이머를 제작하였고, 그것을 이용하여 신뢰성 높고 10개 분자까지도 증폭이 가능한 결과를 모든 혈청형에서 얻었다. 그러므로 이 한 쌍의 정량용 the universal 프라이머는 임상시험용 아데노바이러스벡터에 존재하는 AAV오염을 검출하는 것에도 사용 가능하다.