Titre: Title:	Production du vecteur adénoviral dépendant d'un virus auxiliaire
Auteur: Author:	Edwige Dormond
Date:	2008
Type:	Mémoire ou thèse / Dissertation or Thesis
Référence: Citation:	Dormond, E. (2008). Production du vecteur adénoviral dépendant d'un virus auxiliaire [Ph.D. thesis, École Polytechnique de Montréal]. PolyPublie. https://publications.polymtl.ca/8148/

URL de PolyPublie: PolyPublie URL:	https://publications.polymtl.ca/8148/
Directeurs de recherche: Advisors:	Michel Perrier, & Amine A. Kamen
Programme: Program:	Unspecified

UNIVERSITÉ DE MONTRÉAL

PRODUCTION DU VECTEUR ADÉNOVIRAL DÉPENDANT D'UN VIRUS AUXILIAIRE

EDWIGE DORMOND
DÉPARTEMENT DE GÉNIE CHIMIQUE
ÉCOLE POLYTECHNIQUE DE MONTRÉAL

THÈSE PRÉSENTÉE EN VUE DE L'OBTENTION
DU DIPLÔME DE PHILOSOPHIAE DOCTOR (Ph.D.)
(GÉNIE CHIMIQUE)
AOÛT 2008

Your file Votre référence
ISBN: 978-0-494-46098-6
Our file Notre référence
ISBN: 978-0-494-46098-6

UNIVERSITÉ DE MONTRÉAL

ÉCOLE POLYTECHNIQUE DE MONTRÉAL

Cette thèse intitulée :

PRODUCTION DU VECTEUR ADÉNOVIRAL DÉPENDANT D'UN VIRUS AUXILIAIRE

présentée par : DORMOND Edwige

en vue de l'obtention du diplôme de : Philosophiae Doctor

a été dûment acceptée par le jury d'examen constitué de :

M. JOLICOEUR Mario, Ph.D., président

M. PERRIER Michel, Ph.D., membre et directeur de recherche

M. KAMEN Amine, Ph.D., membre et co-directeur de recherche

M. DE CRESCENZO Gregory, Ph.D., membre

M. BUTLER Michael, Ph.D., membre

DÉDICACE

À nos limites.

REMERCIEMENTS

Je souhaite tout d'abord remercier mes directeurs de recherche. Je tiens à exprimer ma grande gratitude au Dr. Amine Kamen pour son excellent encadrement dans un environnement de recherche très stimulant. J'ai énormément apprécié sa disponibilité, son intérêt et son dévouement pour partager les succès comme les moments difficiles du projet. J'ai grandement bénéficié de la confiance et de la liberté qu'il m'a accordée tout au long de mes recherches. Je remercie sincèrement mon directeur de recherche, le Prof. Michel Perrier, pour ses encouragements constants et son support.

J'aimerai remercier chaleureusement tous mes collègues du groupe des technologies de cellules animales, en particulier Normand Arcand, Eric Carpentier, Alice Bernier, Danielle Jacob, Gilles Saint-Laurent, Louis Bisson, Sylvie Perret, Roseanne Tom, le Dr. Yves Durocher, le Dr. Parminder Chahal et le Dr. Chung Fang Shen pour m'avoir aidé au quotidien et m'avoir si généreusement transmis leur expertise. Merci également pour les discussions scientifiques qui ont été très formatrices. J'ai particulièrement apprécié l'ambiance de travail conviviale.

Je tiens à remercier le Dr. Rénald Gilbert pour sa collaboration dans le projet.

Mes remerciements vont au Centre de Thérapie Expérimentale du Cancer de Montréal et au Fonds National Suisse pour les financements sous forme de bourses pour étudiant gradué.

Un grand merci aux anciens étudiants et étudiants du Secteur des Bioprocédés pour leur soutien à un niveau scientifique et personnel : Lucie Beaulieu, Maria de las Mercedes Segura, Marc Aucoin, Julie Shareck, Hans Drouin, Cyril Boucher et Sven Ansorge.

Je remercie ma famille qui est restée à mes côtés malgré la distance. Merci à mère pour m'avoir constamment encouragée à persévérer dans mes études. Merci à mes amis de part et d'autre de l'Atlantique pour leur présence si importante à mes yeux.

Je réserve mes plus belles pensées à Philippe. Merci simplement pour ton amour. Sans toi, 2008 ne serait pas une année exceptionnelle. Une vie commence au bout du chemin.

RÉSUMÉ

Le vecteur adénoviral est actuellement le véhicule viral le plus utilisé dans les protocoles cliniques de thérapie génique. Récemment, un nouveau vecteur adénoviral nommé vecteur adénoviral dépendant d'un virus auxiliaire (helper-dependent adenoviral vector, HDV) a été développé afin d'améliorer l'efficacité et la sûreté des traitements. Malgré le potentiel thérapeutique certain du HDV, la caractérisation de ce vecteur dans des protocoles cliniques est limitée par les difficultés liées à sa production. Cette thèse décrit le développement d'un processus intégré pour la production du HDV. Les étapes du processus sont définies par trois procédés distincts qui ont été établis pour produire à grande échelle des lots de vecteurs de grade clinique. Les procédés ont été étudiés en détail pour améliorer la compréhension de la formation des vecteurs.

La première étape du processus de production consiste en un procédé de secours du HDV utilisant une nouvelle méthode nommée adénofection. Cette méthode combine la transfection de la culture cellulaire par le HDV à l'infection par le virus auxiliaire (helper adenoviral vector, HV). Réalisée en suspension, l'adénofection réduit la durée du processus de production grâce à la génération du HDV à un titre plus élevé comparé aux méthodes conventionnelles. Le transfert de l'ADN du HDV est le facteur limitant de la production. Les complexes d'adénofection requièrent une quantité élevée d'ADN et de HV pour

produire le HDV à un titre maximum après seulement deux passages d'amplification (1×10^8 unités infectieuses/mL).

La deuxième étape du processus de production est un procédé d'amplification du HDV utilisant une méthode d'infection. Cette méthode repose sur le contrôle quantitatif de l'infection par le HDV et le HV pour garantir le titre de HDV (jusqu'à 2×10^8 unités infectieuses/mL) et limiter la contamination par le HV (jusqu'à 2%). Le titre de HDV maximum est obtenu lorsque le HV est ajouté à une multiplicité d'infection (quantité de HDV infectieux par cellule, multiplicity of infection, MOI) faible, ce qui limite aussi la contamination par le HV. Le titre de HDV augmente avec la MOI du HDV puis diminue au-delà d'une MOI de 5. La contamination par le HV est dépendante du ratio entre le HDV et le HV apportés au moment de l'infection. D'autre part, la compétition entre le HDV et le HV restreint l'amplification du HDV. Des stratégies de délai d'infection et de choc de température sont appliquées pour pallier cette limitation. Ces stratégies n'ont cependant pas eu d'influence significative sur le titre de HDV.

La troisième étape du processus de production repose sur la purification du HDV, procédé inclus dans le processus de production du HDV. Le procédé de purification combine l'avantage des méthodes chromatographiques pour la mise à l'échelle à l'avantage d'une méthode d'ultracentrifugation utilisant un gradient de densité d'iodixanol pour la séparation du HDV et du HV. Les taux de récupération du HDV (~75%), le ratio d'infectivité du HDV (~100%), la réduction

de la contamination par le HV (~facteur 7) et la pureté du matériel confirment l'efficacité du procédé. Par ailleurs, l'amplification du HDV ne dépend pas d'une éventuelle sélection par recombinase. La lignée cellulaire parentale est une alternative à la lignée cellulaire recombinase difficile à générer et à caractériser. Les titres de HDV et de HV étant similaires avec la lignée cellulaire parentale, le procédé de purification détaillé précédemment est requis pour réduire substantiellement la contamination par le HV.

Les procédés décrits dans cette thèse améliorent significativement la fiabilité et l'efficacité de la production du HDV. Ces avancées contribueront certainement à soutenir l'emploi thérapeutique du HDV à grande échelle.

ABSTRACT

Adenoviral vector is presently the most employed vehicle in gene therapy clinical trials. Recently, a new adenoviral vector called helper-dependent adenoviral vector (HDV) has been developed to improve the safety and efficacy of treatments. Even though the HDV has demonstrated a high therapeutic potential, further characterization of this vector has been hampered by production difficulties. This thesis describes the development of an integrated process for the production of HDV. The steps of the integrated process defined by three distinct processes have been established to allow clinical grade vector production at large scale. They were studied in details to improve the understanding of vector formation.

The first step of the integrated process consists of a HDV rescue process using a new method called adenofection. This method combines the transfection of cells by a helper-dependent adenoviral vector (HDV) with the infection by a helper adenoviral vector (HV). The adenofection of cells in suspension reduced process duration by generating the HDV at higher titer in comparison to conventional methods. The transfer of HDV DNA was the limiting factor of production. The adenofection complexes required high amounts of HDV DNA and HV to produce the HDV at a maximum titer following only two amplification passages (1×10^8 infectious units/mL).

The second step of the integrated process is a HDV amplification process using an infection method. This method relies on the quantitative control of infection by the HDV and HV to guarantee HDV titer (up to 2×10^8 infectious units/mL) and to limit contamination by the HV (to less than 2%). Maximum HDV titer was obtained when the HV was added at a low multiplicity of infection (number of infectious HDV per cell, MOI), which also limited the contamination by the HV. HDV titer increased with the MOI of HDV, then decreased above a MOI of 5. Contamination by the HV was dependent on the ratio between the HDV and HV provided at the infection time. Furthermore, the competition between the HDV and HV restricts HDV amplification. Strategies consisting of infection delay and cellular heat shock were evaluated to overcome this limitation. However, those strategies did not have a significant effect on HDV titer.

The third step of the integrated process relies on the purification of the HDV, a step included in the production process. The purification process combines the advantage offered by chromatographic methods for scale-up possibilities to the advantage of a rapid ultracentrifugation method using iodixanol density gradient for the separation of the HDV and HV. The recovery yield (~75%), HDV infectivity (~100%), reduction of contamination (~factor 7) and material purity confirmed the process efficiency. Furthermore, it was demonstrated that HDV amplification did not depend on an eventual recombinase selection. The parental cell line is an alternative to the recombinase cell line difficult to generate

and to characterize. The HDV and HV titers being similar with the parental cell line, the purification process previously detailed was required to substantially reduce the contamination by the HV.

The processes described in this thesis significantly improve the reliability and efficacy of HDV production. Those advances will certainly contribute to sustain the therapeutic use of the HDV at large scale.

TABLE DES MATIÈRES

LISTE DES TABLEAUX

LISTE DES FIGURES

LISTE DES SIGLES ET ABRÉVIATIONS

A549	Human lung epithelial cell line, lignée cellulaire épithéliale de poumon humain
A	Absorbance
AdV	Adenoviral vector, vecteur adenoviral
AEX	Anion-exchange, échange d'anion
ANOVA	Analysis of variance, analyse de la variance
ATP	Adenosine triphosphate, adénosine triphosphate
ARM	AdV reference material (humain serotype 5), materiel de reference AdV (serotype humain 5)
BCS	Bovine calf serum, serum de veau
BFU	Blue forming unit, unité de transduction bleue
BHK	Baby hamster kidney cell line, lignée cellulaire de rein de jeune hamster
CHO	Chinese hamster ovary cell line, lignée cellulaire d'vaire de hamster chinois
CMV	Cytomegalovirus, cytomégalovirus
CPE	Cytopathic effect, effet cytopathique
CRAdV	Conditionally replicating AdV, adenovirus conditionellement réplicatif
CsCl	Cesium chloride, chlorure de césium
CV	Column volume, volume de colonne
DEAE	Diethylaminoethyl, diéthylaminoéthyl
DMEM	Dulbecco's modified Eagle medium, milieu Eagle modifié par Dulbecco
DNA, ADN	Desoxyribonucleic acid, acide désoxyribonucléique
DO	Dissolved oxygen, oxygène dissous
DOE	Design of experiment, design d'expérience

DTT	1,4-dithiothreitol, 1,4-dithiothréitol
E	Early region of AdV genome, region précoce du genome AdV
EDTA	Ethylenediaminetetraacetic acid, acide éthylènediaminetétraacétique
FBS	Foetal bovine serum, serum de veau foetal
FDA	Food and drug administration
FGAdV	First generation AdV
FLP	Flipase
g	Gravitational constant, constante de gravité
GFP	Green fluorescent protein, protéine fluorescente verte
GTU	Green transfer unit, unité de transduction verte
HCl	Hydrochloric acid, acide chloridrique
HDV	Helper-dependent adenoviral vector, vecteur adenoviral dépendant d'un virus aide
HEK293	Human embryo kidney 293 cell line, lignée cellulaire de rein d'embryon humain
HeLa	Cervical cancer cell line, lignée cellulaire cervicale cancéreuse
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, acide 4-(2-hydroxyéthyl)-1- pipérazine éthane sulfonique
hpi	Hours post-infection, heures post-infection
HIV	Human immunodeficiency virus, virus de l'immunodéficience humaine
HPLC	High performance liquid chromatography, chromatographie liquide haute performance
HSFM	Hybridoma serum-free medium, milieu sans sérum pour hybridomes
hsp	Heat shock protein
HV	Helper virus, virus auxiliaire
ITR	Inverted terminal repeats, répétitions terminales inversées

IU, UI, IP, IVP	Infectious units, unités infectieuses, infectious particles, particules infectieuses, infectious viral particles, particules viral infectieuses
L	Late region of AdV genome, région tardive du genome AdV
MOI	Multiplicity of infection, multiplicité d'infection
NA	Non applicable
NaCl	Sodium chloride, chlorure de sodium
NSFM13	New serum free medium 13, milieu nouveau sans sérum 13
p	Probability of occurrence, probabilité d'occurrence
P	Passage
PAGE	Polyacrilamide gel electrophoresis, electrophorèse sur gel de polyacrilamide
PBS	Phosphate buffer saline, tampon phosphate salin
PCR	Polymerase chain reaction, réaction en chaîne de la polymérase
qPCR	Quantitative PCR, PCR quantitative
PEI	Polyethylenimine, polyéthylènimine
PER.C6	Human embryonic retinoblast cell line, lignée cellulaire de rétine d'embryon humain
PFU	Plaque forming unit, unité de formation de plaque
OUR	Oxygen uptake rate, vitesse de consommation d'oxygène
R^2	Determination coefficient, coefficient de détermination
RCA	Replication competent adenovirus, adenovirus à compétence de réplication
RP	Reverse phase, phase inverse
rpm	Round per minute, tours par minute
RSD	Relative standard deviation, déviation standard relative
SDS	Sodium dodecyl sulfate, dodécylsulfate de sodium
SEC	Size-eclusion chromatography, chromatographie d'exclusion stérique

SF	Serum free, sans sérum
SGAdV	Second generation AdV
SOP	Standard operating procedure, procédure d'opération standard
SPL	Sedimentation path length, distance de migration par sédimentation
TCA	Tricarboxylique acid, acide tricarboxylique
$TCID_{50}$	Tissue culture infected dose at 50%, dose infectieuse 50% sur culture de tissu
TGAdV	Third generation AdV
TOI	Time of infection, temps de l'infection
ΔTOI	Delay of infection, délai d'infection
tPA	Tissue plasminogen activator, activateur tissulaire du plasminogène
TU	Transducing unit, unité de transduction
TVP or VP	Total viral particles or viral particles, particules virales totales ou particules virales
UV	Ultraviolet
VG	Viral genomes, génomes viraux
Ψ	Encapsidation signal, signal d'encapsidation

AVANT-PROPOS

Ce travail de thèse est le résultat de quatre années de travaux de recherches à l'Institut de Recherche en Biotechnologie du Conseil National de Recherches Canada en collaboration avec le département de génie chimique de l'École Polytechnique de Montréal.

Cette thèse se divise en une introduction, quatre chapitres principaux et une section de discussion générale, conclusions et recommandations. Les quatre chapitres sont présentés sous forme d'articles qui ont été soumis à des journaux scientifiques avec comités de lecture.

Dans un premier chapitre, une revue de littérature fait état des connaissances concernant la production d'AdV de première, deuxième et troisième génération. *From the First to the Third Generation Adenoviral Vectors: what Parameters are Governing the Production Yield ?* a été soumis à la revue *Biotechnology Advances.*

Les chapitres 2 à 4 contribuent à définir, étudier et optimiser un processus intégré de production du HDV. Le chapitre 2 détaille le travail sur l'étape initiale de production du HDV dans laquelle le HDV est disponible sous la forme d'ADN. Il fait l'objet d'une première publication intitulée *An Efficient and Scalable Process for Helper-Dependent Adenoviral Vector Production using Polyethylenimine-Adenofection* actuellement en révision finale dans la revue

Biotechnology and Bioengineering. Dans cet article, une nouvelle méthode pour le procédé de secours est étudiée. La méthode se révèle efficace et adaptable en suspension. Des résultats complémentaires sont présentés et ouvrent la voie à l'utilisation du paramètre MOI comme outil de contrôle de la production pour l'étape subséquente d'amplification.

La génération d'un stock viral tel que présenté au chapitre 2 permet de réaliser les études portant sur l'étape d'amplification du HDV. Au chapitre 3, une deuxième publication intitulée *Identification of Critical Infection Parameters to Control Helper-Dependent Adenoviral Vector Production using QPCR* soumise dans la revue *Biotechnology and Bioengineering* est présentée. L'effet des paramètres d'infection sur la production du HDV et la contamination par le HV y est étudiée. Toujours dans le but de favoriser l'amplification du HDV au détriment du HV, une courte étude sur le choc thermique est décrite sous forme de résultats complémentaires.

Afin d'utiliser le HDV pour des applications cliniques, l'étape de purification pour éliminer la présence de sérum, la contamination par le HV et les impuretés dérivées des cellules hôtes est nécessaire. Un procédé de purification utilisant des méthodes chromatographiques couplées à une méthode d'ultracentrifugation par gradient de densité d'iodixanol est présenté sous forme d'article au chapitre 4. *An Efficient Process for the Purification of Helper-Dependent Adenoviral Vector and Removal of Helper virus by Iodixanol*

Ultracentrifugation a été soumis au journal *Analytical Biochemistry*. Les résultats complémentaires du chapitre 4 comparent la production du HDV avec les lignées cellulaires parentale et recombinase. Les productions sont utilisées dans le procédé de purification.

Ces travaux de recherche ont été présentés lors de conférences à caractère international et national.

Dormond E., Perrier M., Kamen A. 2008. Critical process parameter to control productivity in helper-dependent adenoviral vector production. *Vaccine Technology II.* Albufeira, Portugal, June 1-6. Poster

Dormond E., Bernier A., Jacob D., Chahal P.S., Perrier M., and Kamen A. 2008. Production and purification of helper-dependent adenoviral vector: comparison of parental and recombinase HEK293 cell line. *Cell Culture Engineering XI.* Coolum, Australia, April 11-16. Poster

Dormond E., Meneses-Acosta A., Jacob D., Durocher Y., Perrier M., and Kamen A. 2007. A scalable process for helper-dependent adenoviral vector production using PEI-derived transfection strategy in suspension culture. *The 20th Meeting of the European Society for Animal Cell Technology.* Dresden, Germany, June 17-20. Poster and proceeding

Meneses-Acosta A., Dormond E., Durocher Y., Gilbert R., and Kamen A. 2006. Optimization and scale-up of helper-dependent adenovirus production by FLP/frt system using the multiplicity of infection criteria. *The 14th Annual Congress of the European Society of Gene and Cell Therapy.* Athens, Greece, November 9-12. Poster

Dormond E., Durocher Y., Perrier M., and Kamen A. 2006. Helper-dependent adenoviral vector generation using an easy and scalable rescue process based on adenofection. *The 4th Annual Meeting of the Montreal Centre for Experimental Therapeutics in Cancer.* Montreal, Canada, October 26-27. Poster

Dormond E., Durocher Y., Perrier M., and Kamen A. 2006. Adenofection: a simple and efficient process for large-scale helper-dependent adenoviral vector production. *The 56th Canadian Chemical Engineering Conference.* Sherbrooke, Canada, October 15-18. Oral

Dormond E., Meneses-Acosta A., Durocher Y., Perrier M., and Kamen A. 2005. Helper-dependent adenoviral vector generation using an easy and scalable rescue process based on adenofection. *The 3rd Annual Meeting of the Montreal Centre for Experimental Therapeutics in Cancer.* Montreal, Canada, May 26-27. Poster

Dormond E., Meneses-Acosta A. and Kamen A. 2004. Strategy for scale-up of Gutless viral vector production. *The 2^{d} Bioprocess Perspectives Meeting*. Montréal, Canada, September 24. Oral

A titre de deuxième auteur, une publication portant sur la production de HDV intitulée Development of a suspension serum-free helper-dependent adenovirus production system and assessment of co-infection conditions est parue dans la revue Journal of Virological Methods.

INTRODUCTION

Les adénovirus (AdV) ont été découvert il y a plus d'un demi-siècle par Rowe et ses collègues (Rowe et al. 1953). L'AdV est un virion de 70 à 90 nm composé d'une capside et d'une enveloppe interne dans laquelle se trouve un complexe ADN-protéines. Le génome de l'AdV est un ADN linéaire double-brin d'environ 36 kb. Il se compose des éléments *cis*, éléments qui doivent être présents sur le génome pour assurer la croissance virale, tels le signal d'encapsidation (Ψ) et les répétitions terminales inversées (ITR, inverted terminal repeats), et des éléments *trans*, élements qui peuvent être supplémentés indépendamment de l'AdV, tels les gènes viraux. Les AdV sont spécifiques aux espèces et différents sérotypes ont été isolés à partir d'espèces mammifères tel l'homme et le primate. Entre autres, il existe 51 serotypes distincts d'AdV humains (Schenk 2001). L'AdV humain de type 5 dont il est question dans cette thèse est actuellement le sérotype ayant fait l'objet du plus grand nombre de recherche.

Initialement, les AdV de type humain ont servi de modèle pour la compréhension des mécanismes de base des cellules eucaryotes tels que la réplication, la traduction, la transcription et la cancérogénèse. Par ailleurs, la capacité des AdV à se modifier génétiquement durant leur croissance en culture (Lewis et al. 1966; Lewis and Rowe 1970; Pierce et al. 1968) a inspiré les

scientifiques pour utiliser des vecteurs modifiés génétiquement afin de transférer un gène de choix dans les cellules. La thérapie génique est ainsi née.

L'utilisation des AdV pour la thérapie génique humaine connait un engouement scientifique certain, justifié par les caractéristiques propres à ces vecteurs. Les AdV ont démontré un large tropisme cellulaire pour des cellules quiescentes et non-quiescentes. Ils n'ont pas démontré que leur génome s'intégrait au génome de la cellule hôte et ne sont donc pas oncogènes. Ils ont une grande capacité d'insertion pour le transgène thérapeutique. Ils peuvent être produit à haut titre. Ces caractéristiques font des AdV les vecteurs les plus utilisés dans les protocoles cliniques de thérapie génique, de thérapie cancéreuse et de vaccination, totalisant plus d'un quart des protocoles débutés à ce jour, soit 342 (www.wiley.co.uk/genetherapy/clinical/). Cependant, l'intérêt thérapeutique que les AdV suscitent se heurte souvent au manque de développement de leur production impliquant des coûts qui, à l'heure actuelle, dépasse la volonté d'investissement des industries. Près de vingt ans après l'approbation du premier protocole clinique impliquant un virus pour le traitement de patients souffrant du mélanome métastatique (www.wiley.co.uk/genetherapy/clinical/, essai clinique US-001), la commercialisation d'une thérapie virale tarde encore dans les pays occidentaux.

Suite au décès d'un patient traité avec l'AdV pour une déficience en ornithine transcarbamylase (Raper et al. 2003), l'efficacité et la sûreté des traitements

utilisant des AdV a fortement été remise en question. L'implication de la réponse immunitaire dans ce drame a conduit au développement d'AdV de nouvelles générations caractérisées par le retrait progressif des séquences *trans* sur le génome viral. Par rapport à l'AdV de première génération, les bénéfices apportés par l'AdV de seconde génération, dénué de quelques séquences *trans* sont peu claires. Ainsi, au début des années 1990, un AdV de troisième génération dépourvu de toutes les séquences *trans* est construit. Les avantages thérapeutiques de cet AdV de troisième génération sont notables. Comparés à l'AdV de première génération, la réponse immunitaire de l'hôte est réduite, l'expression du transgène est allongée dans le temps et l'espace disponible pour le transgène est considérablement augmenté. Le désavantage majeur de ce vecteur réside dans sa production, relativement complexe.

Dû à la particularité de sa construction, l'AdV de troisième génération, appelé aussi vecteur adénoviral dépendant d'un virus auxiliaire (helper-dependent adenoviral vector, HDV) est complémenté par un virus auxiliaire, un AdV de première génération (helper virus, HV). La production de HDV s'effectue généralement dans les cellules de rein d'embryon humain 293 (Human Embryo Kidney 293, HEK293). Ce système produit de nouvelles particules de HDV grâce à l'apport du génome HDV par le HDV lui-même, l'apport des séquences des protéines nécessaires à son encapsidation par le HV et l'apport des séquences initiant la réplication virale par la lignée cellulaire HEK293.

Actuellement, le potentiel thérapeutique du HDV est limité par deux aspects : la difficulté de production du HDV et la contamination de la production par le HV. Les productions sont réalisées avec des lignées cellulaires adhérentes, lesquelles sont cultivées en boîtes de Pétri. Les protocoles de production sont empiriques, c'est-à-dire qu'ils n'utilisent aucun principe de bio-ingénieurie permettant de garantir les rendements de production. Les conditions de production sont ainsi difficiles à réaliser à grande échelle et ne permettent de générer que de petites quantités de HDV à peine suffisantes pour effectuer des études pré-cliniques sur de petits modèles d'animaux. D'autre part, le matériel viral est contaminé par le HV. L'utilisation d'un système de sélection recombinase a grandement réduit la contamination par le HV. Cependant, la contamination par le HV n'est maîtrisée que par l'utilisation d'une lignée cellulaire HEK293 exprimant la recombinase de manière constitutive, lignée qui se doit d'être efficace à la fois pour produire le HDV et pour réduire la contamination par le HV. La caractérisation et l'isolement d'une telle lignée sont difficiles.

L'utilisation du HDV dans les protocoles cliniques d'envergure en vue d'étoffer sa caractérisation nécessite donc d'améliorer les méthodes de production du HDV. A cette fin, le développement de méthodes de production basées sur l'utilisation de paramètres faciles d'utilisation et prédictifs des rendements de production est souhaité. L'hypothèse principale de cette recherche est que la production du HDV est gouvernée par l'apport de HDV et de HV, lesquels

ajoutés à la culture cellulaire selon des quantités relatives et totales définies permettent de rencontrer des exigences de production identifiées.

L'objectif principal de cette recherche est de définir un processus intégré, efficace et adaptable à grande échelle pour la production du HDV de grade clinique. Le processus doit être défini par des procédés fiables et efficaces correspondant chacun à une étape de production. Pour parvenir à cet objectif, il est nécessaire d'identifier les paramètres clés régissant la production du HDV pour les étapes de secours et d'amplification. La maîtrise de ces paramètres permet ensuite d'optimiser la production en maximisant la production du HDV et en minimisant la contamination par le HV. La purification des vecteurs et leur séparation efficace est ensuite requise pour une utilisation clinique du HDV.

CHAPITRE 1: DE LA PREMIÈRE À LA TROISIÈME GÉNÉRATION DE VECTEURS ADÉNOVIRAUX: QUELS SONT LES PARAMÈTRES GOUVERNANT LE RENDEMENT DE PRODUCTION ?

1.1 Présentation de l'Article

Le but de cette thèse est de développer un processus pour la production du HDV à grande échelle. L'identification de paramètres qui définissent les étapes du processus et qui permettent un contrôle de la production est un sous-objectif important de cette thèse. Afin de mesurer ces paramètres, la production doit être caractérisée. Les connaissances acquises durant ces vingt dernières années, portant principalement sur la production d'AdV de première génération, sont autant de ressources qui peuvent aider au développement de procédés efficaces pour la production d'AdV de troisième génération.

Ce chapitre présenté sous forme d'article de revue fait état des connaissances concernant la production d'AdV. Il tente d'expliquer comment l'évolution des générations a affecté le développement de la production au niveau de la caractérisation, de l'amplification virale et des procédés. L'article *From First to Third Generation Adenoviral Vectors: what Parameters are Governing the Production Yield ?* a été soumis à la revue *Biotechnology Advances*.

1.2 From the First to the Third Generation Adenoviral Vector: what Parameters are Governing the Production Yield ?

Dormond E.[1,2], Perrier M.[2], and Kamen A.[1]

[1]Animal Cell Technology Group, Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montréal, Québec, Canada H4P 2R2

[2]Chemical Engineering Department, École Polytechnique de Montréal, Campus de l'Université de Montréal, 2500, chemin de Polytechnique, Montréal, Québec, Canada, H3T 1J4

Keywords : Adenovirus; Helper-dependent adenoviral vector; Production; Quantification.

1.2.1 Abstract

Human adenoviral vectors serotype 5 (AdVs) are presently the primary viral vectors used in gene therapy trials. Advancements in AdVs process development directly contribute to the clinical and commercialization of the AdV gene delivery technology. Notably, the development of AdV productions in suspension culture has driven the increase in AdV volumetric and specific productivity, therefore providing large quantities of AdV required for clinical studies. This review focuses on detailing the viral, cell and cell culture

deterministic aspects governing the productivity of the three generations of AdV vectors.

1.2.2 Introduction

Human adenoviral vector serotype 5 (AdV) is the most characterized virus among 51 others human serotypes of the same family and therefore its biology is well known (McConnell and Imperiale 2004; Tatsis and Ertl 2004). AdV has been considered a good candidate for therapeutic gene delivery in humans for a number of advantages including its wide cell tropism in quiescent and non-quiescent cells, its inability to integrate the host genome, its high capacity for the therapeutic gene insertion and its high production titer. Within the last two decades, the AdV genome has been progressively modified from the wild-type genome to improve its safety and efficacy in therapeutic applications. A decrease in the immunological response following vector administration has been achieved by a progressive removal of non-essential viral DNA regions. From the first generation of AdV (FGAdV) with the deletion of the replication region to the third generation of AdV (TGAdV) with the complete clearance of viral genes, an enhanced capacity for a therapeutic gene insertion from ~7 kb to ~30 kb has been achieved.

The number of AdV clinical trials increased from 1994 to 1999 and then dropped dramatically in 1999 (Figure 1.1) (www.wiley.co.uk/genetherapy/clinical/)

following the tragic death of a patient ongoing an AdV therapy for the treatment of an ornitnine transcarbamylase deficiency (Raper et al. 2003). Safety and efficacy concerns in AdV therapy were reevaluated by the National Institute of Health, USA and the group gave recommendations to strengthen the design, evaluation and conduct of AdV-based protocols (National Institute of Health 2002). Then, targets for gene therapy moved completely from monogenic diseases towards the more typical marketable diseases involving transient therapies. In this context, AdV is also an interesting candidate for vaccine technology, cancer, vascular dysfunctions and rheumatoid arthritis. In 2007, cancer therapy constituted the major field of AdV clinical applications (80%). Following Shenzhen SiBiono GenTech's commercial licensing in China recombinant AdV-p53 (Pearson et al. 2004), a similar product Introgen's Advexin, currently undergoing phase III clinical trials will likely be the first approved gene therapy in the USA. However, the recent decision by Merck & Co to stop a phase II AdV-based HIV vaccine clinical trial will certainly bring again a period of skepticism regarding AdV-based therapies.

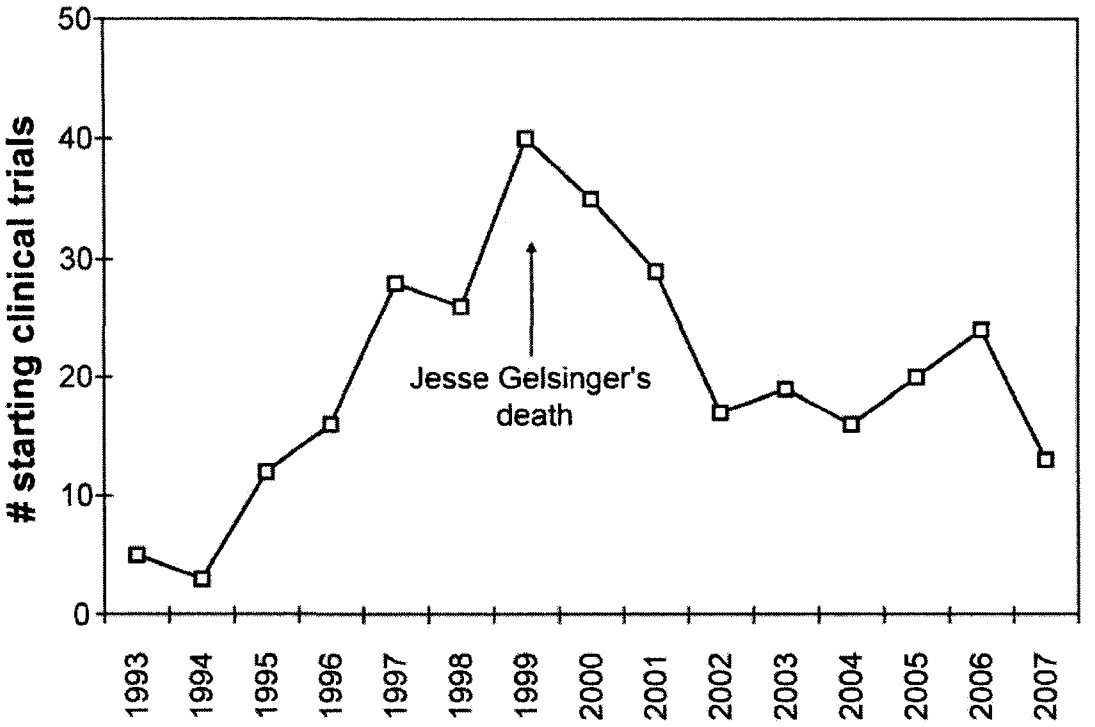

Figure 1.1 Evolution of number of starting AdV clinical trials from 1993 to 2007. From http://www.wiley.co.uk/genmed/clinical/. Updated march 2008

The actual need for large amounts of clinical-grade AdV (10^{12}-10^{13} viral particles/patient; 10^{10}-10^{11} plaque-forming units/patient) requires efficient and established processes for productions at large-scale capacity under good manufacturing practices. To develop robust production bioprocesses for AdV, up-to-date knowledge on overall production systems and on the critical parameters that affect the AdV yield are required.

This review provides a summary of the research activities relating to AdV production. Quantification assessment of AdV will be described first to

demonstrate the impact of the viral product, the virus-cell system, and the cell culture aspect on AdV productivity with specific concerns on the third generation of AdV. Finally, current research trends dedicated to the enhancement of production yields will also be discussed.

1.2.3 Adenoviral Vector Characterization

Detailed quantification assessments are required during the whole manufacturing process including viral amplification, purification and formulation to evaluate and to control process parameters. During production, quantification allows us to control viral amplification through the multiplicity of infection (MOI), ie. the number of infectious viral particles added per cell at infection. Quantification is also intended to monitor the production yield. In order to coordinate the wide variety of techniques, an AdV serotype 5 reference material (ARM) has been developed by the FDA, academic centers and industries and coordinated by the Williamsburg BioProcessing Foundation (Hutchins 2002; Hutchins et al. 2000). The initial purpose of such work was to generate and thoroughly characterize an AdV stock with representative assays to further help in validating new assays. This promoted an open exchange of standard operating procedures (SOP) among development and manufacturing groups contributing to a generalization of best practices in the field (Simek et al. 2002). However, the ARM, a FGAdV, has failed to serve as a universal standard for all AdV generations, despite some efforts to adapt it (Palmer and Ng 2004).

Quantification methods tended to be generation specific and since the establishment of the ARM, a wide variety of methods have been developed to fulfill the requirements for TGAdV characterization. The lack of uniformity in TGAdV quantification complicated a meaningful comparison of data in process development and pre-clinical studies.

A panel of methods to quantify AdV is routinely used in the development and manufacturing productions of this virus. They rely on physical, molecular or biological properties of the particles. The main features of the methods are described in Table 1.1. Three groups of characterization assays are commonly used: physical assays (viral particles, VP), infectivity assays (infectious viral particles, IVP), and replication competent AdV (RCA) assays. The VP assays are intended to measure the process yield, the IVP assays are useful to monitor the bioactivity of AdV and the RCA assays report the biological purity of the production.

Table 1.1 Characteristics of AdV assays

Assessment	Component or effect detected	Detection method	Development time	Assay time	Measurements characteristics		Adaptability of assay		References
					Limit of detection	Assay error	Different AdV generations	Different AdV transgene	
AdV-VP	DNA	Absorbance	---	---	+++	+[a]	No [b]	Yes	(Maizel et al. 1968) www.wilbio.com
		Fluorecence	++	---	+	-[a]	No [b]	Yes	(Murakami and McCaman 1999)
		AEX HPLC – Absorbance	+++	---	++	+	No [b]	Yes	(Klyushnichenko et al. 2001; Shabram et al. 1997; Transfiguracion et al. 2001))
		qPCR	+++	---	-	--[a]	Yes [c]	[d]	(Ma et al. 2001; Puntel et al. 2006)
	Protein	RP HPLC – hexon protein	+++	---	++	+	No [b]	Yes	(Lehmberg et al. 1999)
AdV-IVP or AdV-PFU		Colorimetric	+	+	+	++	Yes [e]	No	(Mittereder et al. 1996)
	Protein	Direct fluorescence	+	+	+	++	Yes [e]	No	(Côté et al. 1997; Gueret et al. 2002; Park and Lee 2000)
		Indirect fluorescence	+	+	+	++	No [b]	Yes	(Lusky et al. 1998; Weaver and Kadan 2000)

Table 1.1 Characteristics of AdV assays (continued)

Assessment	Component or effect detected	Detection method	Development time	Assay time	Measurements characteristics		Adaptability of assay		References
					Limit of detection	Assay error	Various AdV generations	Various AdV transgenes	
AdV-IVP or AdV-PFU	CPE	Plaque Assay/EPDA	+	+++	+	+++	No [f]	Yes	(Graham and van der Eb 1973; Mittereder et al. 1996; Nyberg-Hoffman et al. 1997) ww.wilbio.com
	Infecting DNA	Infection/qPCR	+++	++	++	+[a]	Yes [c]	Yes	(Wang et al. 2005)
RCA	DNA (Total RCA)	RCA-qPCR	+++	++	+	+[a]	NA	NA	(Lochmuller et al. 1994; Puntel et al. 2006; Zhang et al. 1995)
	CPE (Infective RCA)	RCA-plaque assay	+	+++	++	+++	NA	NA	(Fallaux et al. 1998; Hehir et al. 1996; Zhu et al. 1999)
	Infecting DNA (Infective RCA)	Infection/RCA-qPCR	+++	++	++	+[a]	NA	NA	(Ishii-Watabe et al. 2003; Schalk et al. 2007)

Notes: Positive signs for high values, negative signs for low values. [a] Viral DNA sample should be highly purified. [b] Assay will assess concentration/titer without distinguishing FGAdV from TGAdV. [c] Targeted sequences should be carefully chosen to distinguish AdV generations. [d] Targeted sequences should not be a part of transgene [e] Transgenes for FGAdV and TGAdV should code for different proteins. [f] For TGAdV sample, assay is only suitable for concentration assessment of FGAdV

1.2.3.1 Viral Particles Concentration

The VP concentration is commonly measured by assessing DNA absorbance at 260 nm of a purified lysed virion preparation using the extinction coefficient for a 36 kb wild-type AdV (Maizel et al. 1968). Upstream from ultra-violet (UV) detection, anion-exchange high performance liquid chromatograpy (AEX-HPLC) has been developed to determine AdV concentrations in non-purified samples (Klyushnichenko et al. 2001; Shabram et al. 1997; Transfiguracion et al. 2001). The relative standard deviation (RSD) is ~10% for crude samples and ~5% for purified samples. Size correction should be made if the viral DNA size varies from the usual 36 kb ARM standard used for calibration. For instance, this method is less precise for the determination of TGAdV concentrations. Non-purified samples from TGAdV productions usually contain an unknown mixture of FGAdV and TGAdV (30 and 36 kb AdV) harboring similar capsids. Nevertheless, the advantage of this HPLC method is its adaptation to the quantification of all AdV harboring anionic charged capsids at neutral pH. Reverse phase HPLC (RP-HPLC) protocol has also been developed to assess the VP concentration based on hexon quantification (Lehmberg et al. 1999). Fluorescent detection of AdV DNA has been shown to further improve the detection level of AdV by a factor of 20 (Murakami and McCaman 1999). Also considering that the limit of detection for TGAdV samples using AEX-HPLC is determined by the limit of the UV absorbance methods used, fluorescent dye

technology will certainly enhance the sensitivity of the methods. Although the initial cost for the set-up of an HPLC including development and validation of the instrumentation is high, FGAdV concentration has been routinely assessed by these established, fast and accurate methods in most of the development laboratories and manufacturing facilities. Real-time qPCR is deemed the most sensitive method for DNA detection although its sensitivity to contaminant requires the use of highly purified DNA samples. To improve specificity, TaqMan technology has been used for single or multiplex detection (Ma et al. 2001; Puntel et al. 2006). The development of qPCR is undoubtedly justified for the characterization of TGAdV samples. By using this technique, an accurate comparison of the concentration of FGAdV to the concentration of TGAdV has been rendered possible (Palmer and Ng 2005). The AdV concentrations are assessed on the same detection basis allowing a good base of comparison of the concentrations. For purified samples, reproducibility is high ~5% RSD. The costs of the equipment and consumables are high, and even higher when multi-fluorescent techniques are used. Total viral particles concentration can be reported as viral particles (VP)/mL, as total viral particles (TVP)/mL or as viral genomes (VG)/mL. For consistency, viral particle concentrations will be reported as VP/mL, in the remaining of the text.

1.2.3.2 Infectious Viral Particles Concentration

Infectious viral particles have been referred to as particles able to transduce cells in which they undergo one or more viral life steps such as DNA replication, transgene expression, viral growth, etc. Provided with this definition, the number of existing infectious assays for AdV is wide considering the diversity of methods used to detect their occurrence. During production development, most infectious assays use the HEK293 cell line as the target cell line taking advantage of its availability in laboratories. The most common infectious assay involves the detection of a cytolytic effect on cells following viral spread. The fastest infectious assay relies on the transfer of a specific gene coding for a protein and the subsequent detection of this protein. Other infectious assays have relied on molecular DNA detection techniques following viral DNA replication. The cytopathic effect assay has been the most widely used method to quantify FGAdV. It can be easily performed in most laboratories, requiring minimal equipment and was therefore chosen to characterize the infectious viral particle concentration of ARM. Target cells show a cytopathic effect as a consequence of mass production of cytotoxic viral proteins following the amplification of AdV. This assay has been usually performed in plaque-format or end-point dilution format (Graham and van der Eb 1973; Mittereder et al. 1996; Nyberg-Hoffman et al. 1997). The ARM protocol has been made available from the WilBio website (www.wilbio.com). This assay is highly insensitive as a consequence of the successive and dependent viral events that need to occur to observe a cytolytic

event. Hence concentrations are usually one log below other infectious titers (Mittereder et al. 1996; Nyberg-Hoffman et al. 1997). To limit assay variability, the SOP has to be strictly executed by a limited number of well-trained people resulting in a RSD lower than ~40% (Callahan 2002; Hutchins 2002). Moreover, the cytopathic effect assay is not appropriate in determining TGAdV concentrations as these vectors are devoid of viral coding sequences and are therefore replication-deficient in HEK293 cell lines. But, this assay is well suited to detect infectious viral particles concentration of FGAdV present in TGAdV lots. The cytopathic effect assay is applicable to FGAdV with no regards to its transgene. It has been therefore used at the pre-clinical and clinical stages, for vectors whose marker gene has been replaced by a therapeutic gene. In gene transfer assays (GTA), AdV transfers to cells a gene for which the corresponding protein is expressed and its activity is measured. With an expression cassette containing a reporter gene detectable in each cell such as the green fluorescent protein (GFP) under the cytomegalovirus (CMV) strong promoter control, the GTA is fast, sensitive and easy to perform. Following the infection of HEK293 cells with serial dilutions of the AdV sample in a plaque-like format or end-point dilution format, infected cells are detected either by direct monitoring such as fluorescent reading and colorimetric staining or by immunostaining (Côté et al. 1997; Lusky et al. 1998; Mittereder et al. 1996; Nyberg-Hoffman et al. 1997; Park and Lee 2000; Weaver and Kadan 2000). With direct detection methods, we generally obtained a RSD of ~30%

(unpublished results). The variability in concentration assessment is increased with differences in viral constructs, reporter gene and expression cassette characteristics, sensitivity of detection method, as well as target cell line and cell culture conditions (Kamen and Henry 2004; Mittereder et al. 1996; Nyberg-Hoffman et al. 1997). During process development, infectious viral particle concentration is not routinely monitored for FGAdV. However, specificity of the marker gene permits to distinguish the FGAdV from the TGAdV. When AdV is ready to be manufactured, the marker gene is replaced by the therapeutic cassette and immunoassays or cytopathic effect assays are then employed for viral titer determination. Combined infectious and qPCR assays have also been used to a lesser extent to monitor infectious titer (Wang et al. 2005). Following cell infection with samples at low MOI, AdV DNA is subjected to qPCR. This assay is limited by the qPCR instrument availability and the costs related to its use. Infectious viral particle concentration have been reported as infectious viral particles (IVP), plaque forming units (PFU), tissue culture infected dose at 50% ($TCID_{50}$), infectious units (IU), infectious particles (IP), transducing units (TU), green transfer units (GTU) in the case of the GFP reporter gene, blue-forming units (BFU) in the case of the β-galactosidase reporter gene, etc. Taking into account the various sensitivities of the different assays, the infectious viral particle concentrations will be reported as PFU for the cytopathic effect assays and as IVP for the gene transfer assays.

1.2.3.3 Ratio between Viral Particles and Infectious Viral Particles

The infectivity ratio, i.e the ratio between VP and IVP has been highly relevant to assess the quality of the vector stock (National Institute of Health 2002). Because the AdV capsid proteins are cytotoxic, the use of AdV stocks at low infectivity ratios limits the risk associated with the toxicity due to the total particles while it increases the therapeutic benefits provided by the infectious particles. In terms of the infectivity ratio of AdV lots, the FDA requirement has been strengthened from 1998 to 2002 (Simek et al. 2002) (Biological Response Modifiers Advisory Committee Meeting 30, 13 July 2001). The infectivity ratio of clinical lots should stand below 30:1 (VP:PFU). By quantifying VP by HPLC or by qPCR and IVP using GTA, we usually obtain an infectivity ratio of 3:1 to 10:1 (VP: IVP) for FGAdV and TGAdV (Kamen and Henry 2004; Meneses-Acosta et al. 2007).

1.2.3.4 Replication Competent Particles Determination

A replication competent adenovirus (RCA) is able of propagating in non-complementing cells as a consequence of the incorporation of the E1 region into its genome. The presence of these RCA particles in clinical lots is unwanted as they would be able to propagate uncontrollably in tissues leading to patient infection and enhancement of the immunological response (Imler et al. 1995; Lochmuller et al. 1994). The FDA has restricted the RCA presence in clinical lots

to 1 for every 3×10^{10} VP (Biological Response Modifiers Advisory Committee Meeting 30, 13 July 2001). Assessment of infectious RCAs consists of a serial cytopathic effect assay performed in E1-deleted cells. The presence of plaques is reported as the initial AdV dose applied on cells. The RCA assay is semi-quantitative and time-consuming usually performed over two to three weeks. Roller bottle cultivation mode is often required to increase the surface of adherent cells. Moreover, like all cytopathic-based assays, the cytopathic RCA assay suffers from high variability and underestimates the RCA concentration. In an attempt to facilitate the quantitative assessment of the RCAs, Ishii-Watabe et al. (2003) have developed a q-PCR assay which has shown more sensitivity and less variability as compared to the traditional cytopathic RCA assay. This method has been refined by Schalk et al. (2007) to diminish the constraints related to the handling of large cell culture volumes.

The interpretation of AdV quantification results is not straightforward as the methods for the same type of assessment have different sensitivities and therefore different meanings. Quantification results should be carefully used while keeping in mind the nature and limitations of these assays.

1.2.4 Adenoviral Vector Production

To get a better understanding of what governs the yield in AdV productions, we have classified the pertinent parameters described in the literature into two

categories. The first category concerns the parameters related to the AdV and the second one concerns the cell culture conditions.

1.2.4.1 Viral Parameters

Viral Production Systems

The AdV has been divided into four main types of constructs: the FGAdV, the conditionally replicating AdV (CRAdV), the second generation of AdV (SGAdV) and the TGAdV. The size of the type 5 AdV genome should stand between ~28 and ~38 kb to be efficiently packaged therefore enabling deletions and insertions on the original 36 kb genome (Bett et al. 1993; Parks and Graham 1997). Figure 1.2 and Table 1.2 provide a description of the AdV vector systems and an insight on productivity of such systems.

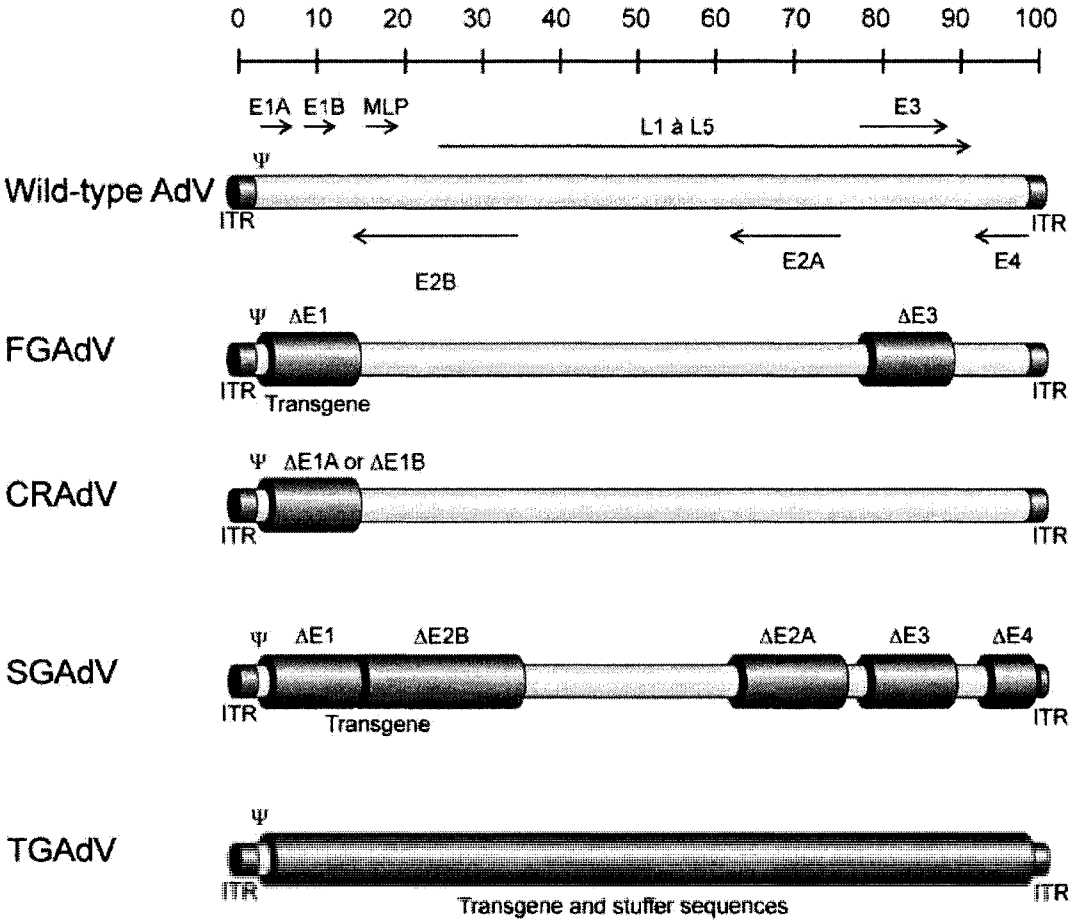

Figure 1.2 Schematic representation of wild-type AdV genome and different generations of AdV. Genomes are divided into 100 map units (28 to 38 kb). E1 to E4: early transcript units, L1 to L5: late transcript units, ITR: inverted terminal repeats, MLP: major late promoter, ψ: packaging signal

Table 1.2 Virus-cell system characteristics for production

Virus-Cell system	Vector generation	Construction specificity vs. wild-type AdV	Complementing cell system	Production yield	References
Helper-Independent	FGAdV	ΔE1 ± ΔE3	HEK293, PERC.6, HeLa Suspension	3×10^2–1×10^3 PFU/cell 5×10^2–6×10^3 IVP/cell 2×10^4–1×10^5 VP/cell	(Reviewed in Nadeau and Kamen 2003)
	C-RAdV	ΔE1A or ΔE1B, Tissue specific promoter	HEK293, HeLa, A549 Suspension	1-5 $\times 10^4$ VP/cell	(Longley et al. 2005; Yuk et al. 2004)
	SGAdV	ΔE1/E2 mutation or deletion ± ΔE3	HEK293	50% FGAdV (temperature mutation on E2A)	(Engelhardt et al. 1994)
			HEK 293-E2B	4×10^8 PFU/mL (temperature mutation on E2B)	(Amalfitano et al. 1996; Amalfitano and Chamberlain 1997)
			A549-E1-E2A	50% FGAdV 1-3 $\times 10^2$ PFU/cell	(Gorziglia et al. 1996)
			HEK293 E1-E2A	4×10^7 IVP/mL	(Lusky et al. 1998)
				1×10^9 IVP/mL	(Zhou and Beaudet 2000)
		ΔE1/ ΔE4 total or partial	HEK293-E4	2×10^8 PFU/mL	(Wang et al. 1995)
			HEK293- E4 partial	10-50% FGAdV 2-4 $\times 10^8$ IVP/mL	(Lusky et al. 1998)
		ΔE1/ΔE2A/ΔE3/total or partial ΔE4	HEK293-E2A + E4 transient transfection	7×10^2 VP/cell	(Gorziglia et al. 1999)
			HEK293-E2A-partial E4 HEK293-E2A-total E4	4×10^{11} VP/mL 4×10^{10} IVP/mL	(Andrews et al. 2001)
			HEK293-AdV amplicon E1-E4	50 % FGAdV 5×10^3 VP/cell 2×10^{12} VP/mL	(Catalucci et al. 2005)
Helper-Dependent system	TGAdV	All viral sequences deleted (except ITR and packaging signal Ψ _FGAdV loxP	HEK293-Cre Suspension	5×10^7 IVP/mL	(Parks et al. 1996)
				1-5 $\times 10^8$ IVP/mL 1.8 $\times 10^4$ VP/cell 1.2 $\times 10^3$ IVP/cell	(Ng et al. 2001; Palmer and Ng 2003)
			PERC6-Cre Suspension	1×10^{10} VP/mL 3×10^3 VP/cell	(Sakhuja et al. 2003)
		All viral sequences deleted (except ITR and packaging signal Ψ) + FGAdV/ΔE2A or /ΔE2B loxP	HEK293-E2A-Cre	1-5 10^{11} VP/mL 0.8-2 $\times 10^{10}$ IVP/mL	(Zhou et al. 2001)
			HEK293-E2B-Cre	1×10^8 IVP/mL	(Barjot et al. 2002; Hartigan-O'Connor et al. 2002)
		All viral sequences deleted (except ITR and packaging signal Ψ) + FGAdV frt	HEK293-FLP Suspension	1×10^8 IVP/mL	(Umana et al. 2001)
				2-5 $\times 10^8$ IVP/mL	(Ng et al. 2001)
				1-4 $\times 10^8$ IVP/mL	(Meneses-Acosta et al. 2007)

Table 1.2 Virus-cell system characteristics for production (continued)

Virus-Cell system	Vector generation	Construction specificity vs. wild-type AdV	Complementing cell system	Yield	References
Helper-Dependent system	TGAdV	All viral sequences deleted (except ITR and packaging signal Ψ) + ΔpIX FGAdV loxP	HEK293-Cre	6×10^7 IVP/mL	(Sargent et al. 2004)
		All viral sequences deleted (except ITR and packaging signal Ψ) + Baculovirus FGAdV loxP	HEK293-Cre	4×10^9 IVP/mL	(Cheshenko et al. 2001)

The AdV has been divided into four main types of constructs: the FGAdV, the conditionally replicating AdV (CRAdV), the second generation of AdV (SGAdV) and the TGAdV. The size of the type 5 AdV genome should stand between ~28 and ~38 kb to be efficiently packaged therefore enabling deletions and insertions on the original 36 kb genome (Bett et al. 1993; Parks and Graham 1997). Figure 1.2 and Table 1.2 provide a description of the AdV vector systems and an insight on productivity of such systems.

The FGAdV is devoid of the E1 region. This region codes for two important subunits E1A and E1B. E1A transactivates other early units, crucial for viral replication function which induces a propitious cell environment for viral replication and deregulates various cell cycle controls. E1B is mainly involved in the inactivation of apoptosis pathways. Most of the FGAdV constructs have also been deleted in the E3 region, which is not necessary for viral amplification and permits a transgene insert capacity of up to 8.2 kb. Because the E1 deletion

impairs AdV replication, FGAdV are produced in a cell line supplying the E1 sequence *in trans*. The HEK293 cell line developed by Graham et al. (Graham et al. 1977) has been the typical cell line used to produce AdV. The AdV production in HEK293 has been shown to generate RCA, directly attributable to the presence in HEK293 genome of a large AdV sequence containing the E1 region and which undergoes homologous recombination with the vector (Hehir et al. 1996; Lochmuller et al. 1994; Louis et al. 1997; Zhu et al. 1999). During production, the RCA presence is unwanted as it amplifies advantageously over non-RCA rendering the production process uncontrollable. A panel of novel human cell lines preventing or minimizing sequence overlaps between vector and cell genome have been used to reduce RCA occurrences (Fallaux et al. 1998; Fallaux et al. 1996; Gao et al. 2000; Imler et al. 1995; Schiedner et al. 2000; Xu et al. 2006). Also, the transfer of the pIX encoding gene to the cell genome has diminished sequence overlaps and contribute to RCA reduction (Hehir et al. 1996). Another alternative has consisted of artificially increasing FGAdV DNA size with stuffer DNA in the E3 region, rendering the E1-recombined viral DNA un-packageable (Bett et al. 1993).

CRAdV has been employed in cancer treatments (Everts and van der Poel 2005). The first type of CRAdV has been engineered in its E1 functions. Either the E1A or E1B region has been mutated which confers the capacity of the CRAdV to replicate in human tumor cells that harbor a defective retinoblastoma tumor suppressor functions or p53. The second type of CRAdV, less common,

contains a tissue specific promoter upstream from E1A for specific replication in target cells. Type 1 and 2 CRAdV have been efficiently produced in E1-containing cell lines. RCA-preventing cell lines such as HeLa or A549 cell lines have also been used for CRAdV production (Longley et al. 2005; Yuk et al. 2004).

Additional deletions to FGAdV have been proposed to reduce the *in vivo* immunogenicity associated with FGAdV. Together with E1 or E1/E3 deletions, full or partial deletion of E4 and full or partial deletion of E2 have given rise to SGAdV. A first modification of deleted-E1 and deleted-E1/E3 AdV concerns temperature sensitive mutations or partial or total deletions in the E2 region (Amalfitano et al. 1996; Amalfitano and Chamberlain 1997; Engelhardt et al. 1994; Gorziglia et al. 1996; Zhou et al. 1996). For AdV productions, this deletion is complemented by the cell line constitutively expressing the E2 region missing in the vector.

Additional lethal deletions in the E4 region were also done. To generate stable cell lines, the E4 region transactivated by E1 expression has been driven by an inducible promoter to keep the cell line viable during maintenance (Lusky et al. 1999; Wang et al. 1997; Yeh et al. 1996). A third type of SGAdV is deleted in all the early regions, i.e E1-E2-E3 and partially E4 (Andrews et al. 2001; Catalucci et al. 2005; Gorziglia et al. 1999). Due to these additional viral deletions, the SGAdV system has been shown to generate less RCAs (Hehir et al. 1996). The

major problem of the SGAdV concerns the isolation of a complementary cell line, a time-consuming process. Cell systems generally have suffered from low level of expression and also inappropriate expression timing that result in low vector productivity. Yields are usually lowered by a factor 2 to 10 as compared to FGAdV. Due to intrinsic difficulty in developing SGAdV systems, scarce data are available regarding production development of this vector.

Progressive deletions of viral coding regions for safety concerns have given rise to the TGAdV, completely devoid of non-essential viral genes. Compared to the FGAdV and SGAdV systems, the TGAdV or helper-dependent AdV relies on a helper-dependent system to be produced. AdV sequences required *in cis*, are the inverted terminal repeats (ITR) where DNA replication starts and the packaging signal (Grable and Hearing 1992). The 36 kb of space available for the transgene is filled with non-coding stuffer DNA. The helper functions are provided by a FGAdV also called helper AdV. Initial work on TGAdV pointed out a relative low yield in TGAdV and a high contamination in helper AdV (Kochanek 1999; Mitani et al. 1995). Because FGAdV and TGAdV carry the same envelope, only partial separation through density difference was achieved (Kumar-Singh and Chamberlain 1996; Mitani et al. 1995; Parks and Graham 1997). For this reason, the size of TGAdV never exceeds 32 kb allowing a better separation from the 36 kb FGAdV. By introducing a recombinase system, the TGAdV system was improved in terms of contamination (Hardy et al. 1997; Lieber et al. 1996; Parks et al. 1996). A recombinase recognition site is added

beside the packaging signal of the helper AdV, and renders this one un-packageable when infecting a HEK293 cell line constitutively expressing the recombinase. Three recombinase systems have been used: Cre/LoxP, FLP/frt and attB-attP/ΦC31. Cre/LoxP and FLP/frt have demonstrated similar efficiencies in the removal of FGAdV (Ng et al. 2001; Umana et al. 2001). Before purification, FGAdV contamination is ~1-10%. Some sequences present on the TGAdV genome have been shown to have an impact on TGAdV yield. The preferred stuffer DNA has been determined to be non-coding DNA of human origin with minimal repeated sequences (Parks et al. 1999; Sandig et al. 2000; Schiedner et al. 2002). The presence of the promoter for the E4 region has conferred an amplification advantage to TGAdV (Sandig et al. 2000). The construction of FGAdV has also been shown to influence TGAdV amplification (Zhou et al. 2002). An homology of sequences in the packaging signal should be avoided to limit the generation of recombinant FGAdV and TGAdV (Hardy et al. 1997; Sandig et al. 2000). TGAdV complementary cell lines have been derived from those producing FGAdV or SGAdV. Additionally, they constitutively express the recombinase Cre or/and FLP or ΦC31 (Alba et al. 2007; Ng et al. 2001; Parks et al. 1996; Umana et al. 2001). The generation of a cell line that efficiently supports the amplification of TGAdV and limits the FGAdV contamination is not straightforward. Investigators have shown that contamination by FGAdV is attributable to the AdV-mediated host cell shutoff (Ng et al. 2002). Others have demonstrated that high levels of recombinase

expression might impair the amplification of TGAdV (Hartigan-O'Connor et al. 2002). RCA emergence in TGAdV preparations is often associated with the presence at low concentration of the FGAdV as a helper vector, however, a production method requiring the presence of the two viral constructs during serial passages might increase the probability of an RCA occurrence. Solutions to prevent RCA are similar to those proposed for the FGAdV system, i.e., the use of cells with minimal homology such as the PERC.6 cell line (Sakhuja et al. 2003) or the addition of stuffer DNA in the E3 region of FGAdV DNA to limit encapsidation due to viral DNA size constraints (Parks et al. 1996).

Production data concerning the recently developed TGAdV are not available as extensively as for the FGAdV. Consequently, the next sections of this review are mostly dedicated to the production of FGAdV but should serve as a basis for TGAdV production approaches.

Although their impact is lesser, non-viral sequences have shown an effect on the AdV yield. The choice of transgene expression cassette containing the promoter, the transgene and polyA signal, dictated by the target tissues type and the target disease, have been shown to influence the AdV yield (Youil et al. 2003). Besides variation of titers provided by the difference in transgene type and sensitivity of transgene detection, promoter and transgene orientation has influenced AdV amplification.

Clinical requirements to produce less immunogenic AdV led to progressive deletions of all non necessary viral sequences. The ongoing elucidation of the AdV complex network functions has rendered possible the development of virus-cell system for AdV production where missing functions are complemented by helper functions provided by the cell line or a FGAdV. However, these systems gave rise to lower AdV yields compared to wild-type AdV system. A comprehensive research for the viral gene functionality and for the effect of viral modifications will continue to be helpful in order to improve AdV constructions. Also, development of improved stable cell lines will contribute to an enhancement of AdV productivity.

Viral Material for Production

Initial viral vector productions start from viral DNA. Within the late nineties, authors have dramatically improved the generation of recombinant AdV DNA (for a pertinent review, see Danthinne and Imperiale (2000)). Following vector linearization, complementary cells are transfected and AdV is recovered in its viral form. This step is often referred to as rescue. Transfection has been usually performed on adherent cell lines in static cultures which are limited by surface area for scale-up. Using HEK293 and PER.C6 cell lines, Blanche et al. (2000) have recovered around 3×10^8 VP/mL of FGAdV at 14 days post-transfection using Lipofectamine-mediated transfection. Recently, Subbramian et al. (2008) have described a method for the rapid preparation of a FGAdV. PERC.6 and

HEK293 cells, cultivated under static conditions, have been transfected by the calcium-phosphate co-precipitation method. Around 4×10^8 VP/mL (5×10^{10} VP per 1 tray Nunc cell factory) have been produced at day 6 post-transfection. TGAdV have usually been generated by transfection of cells with TGAdV DNA followed by infection with FGAdV (Parks et al. 1996) or by transfection with FGAdV DNA (Kochanek 1999). TGAdV yields at this step are low (10^2 to 10^6 BFU/mL) (Kumar-Singh and Chamberlain 1996; Parks et al. 1996). Adherent C7-Cre cells co-transfected with AdV DNA have been used to produce up to 10^7 IVP/mL at 8 to 12 days post-transfection (Hartigan-O'Connor et al. 2002). We have developed a highly efficient method compared to the original transfection/infection method, adaptable to suspension cell culture conditions (Dormond et al., to be published). The so-called adenofection process has enabled the generation of 10^6 IVP/mL at the rescue step.

Because infection is a more efficient method to transfer and express DNA in cells, productions of AdV have been more often reported from viral seed. Following AdV rescue as previously described, amplification by infection has enhanced AdV yield at least by a factor of 100 allowing the preparation of large viral stocks (Blanche et al. 2000; Schoofs et al. 1998). For TGAdV productions, amplification by infection has usually led to an increase in titer by a factor ~10-100. Up to six amplification rounds of co-infection have been required to get a maximum titer corresponding to ~1×10^8 IVP/mL or ~10^9 VP/mL. Using the

efficient adenofection rescue step, two amplification rounds were required to obtain 1.5×10^8 IVP/mL in a 3 L bioreactor (Dormond et al., to be published). The generation of a large stock of AdV allows careful study and the establishment of controlled bioprocesses where the AdV seed is not a variable *per se*.

Infection Parameters

The multiplicity of infection (MOI) has been referred to as the ratio of the number of IVP to the number of cells. This parameter has been defined by the Poisson distribution and has been originally used to calculate AdV titer. Its use as a parameter to control production processes is subjected to controversy. The Poisson distribution describes discrete events for which the MOI is less than 1. However, when describing experimental conditions, it is often used at values greater than 1. Detail commentaries are available in Shabram and Aguillar-Cordova (2000). Although controversial, the use of MOI is convenient when used in the acceptable range of both cell and virus concentrations. The MOI has been largely employed for AdV production as it determines viral stock production. In Schoofs et al. (1998), production of FGAdV is roughly identical at low (1) and high MOI (125) producing ~3000 PFU/cell. AdV titer has been increased for MOI from 0.01 to 1 and has remained constant for MOI from 1 to 200 (~1000 PFU/cell) (Park et al. 2004). Provided by two distinct AdV, components of TGAdV are assembled at an established stoichiometry for AdV.

In a previous work, we have shown that the establishment of a MOI-based strategy of infection allows to limit waste of viral material, improve TGAdV yield while limiting contamination by FGAdV (Dormond et al., to be published). Productivity of TGAdV has attained ~300 IVP/cell when MOI of TGAdV is 5 and MOI of FGAdV is 0.5.

Because the titer is maximal when cell viability is still high, AdV has been preferably recovered within the cell paste. AdV also has shown a better stability when stored in the cell fraction. Furthermore, downstream processing has been greatly facilitated by cell centrifugation or microfiltration, allowing therefore a reduction of processing volumes to up to 100 times thereby increasing the initial low virus concentration. Consequently, cell culture viability has often dictated the time of harvest which usually occurs between 36 to 72 hpi, the time at which viability is ~60 % (Kamen and Henry 2004). For TGAdV production, viability at harvest time has been usually higher as a result of a lower AdV yield (Dormond et al., unpublished results). The productivity peak has been reached at 48 hpi (Meneses-Acosta et al. 2007; Sakhuja et al. 2003).

1.2.4.2 Towards Improving the Viral Amplification Efficiency: Identification of the Limiting Steps

Viral amplification efficiency is dependent on the viral construction, viral binding properties and the cell line surface characteristics. A viral cycle begins with

infection involving virus diffusion, virus attachment to the cell surface through specific receptors present on the cell surface (Coxsackie adenovirus receptors and αv integrins) and AdV ligands (viral fiber and penton base proteins). Following AdV-cell binding, the virus is internalized through endocytosis, released efficiently from endosomes, translocated to the nucleus and unpackaged for DNA import into the nucleus. More details are available in specialized reviews (Meier and Greber 2003). AdV DNA delivery is recognized as a highly efficient method to transfer and express DNA. In order to evaluate the possible rate-limiting steps in a viral cycle process, quantitative experiments coupled to mathematical modeling have been shown to be useful tools. Viral diffusion has been identified as a limiting step of the infection process (Mittereder et al. 1996; Nyberg-Hoffman et al. 1997) and another limiting step has been shown to be the reversibility of adsorption (Gilbert et al. 2007). Increasing viral diffusion could possibly be achieved at low temperatures while endocytosis is blocked. More efficient interactions between AdV ligands and cell receptors might also improve the adsorption step. Such strategies involve modifications of the cell surface receptors through their number or their characteristic concomitant with the engineering of AdV proteins for cell targeting (reviewed inVarga et al. 2000). None of the steps from viral uptake to gene expression have been identified as rate-limiting (Varga et al. 2005). As an example, reporter protein expression has been detected as early as 6 hpi (Gilbert et al. 2000). Post-delivery events such as DNA replication, transcription,

translation and viral assembly are more probably rate-limiting compared to pre-delivery events. Deletions and transfers of specific viral regions from AdV DNA to the cellular genome have not compromised AdV amplification but are incontestably responsible for a decrease in the AdV yield. As an example, if deletion of the E3 region has not impaired viral amplification, its presence has conferred to AdV a significant growth advantage (Youil et al. 2003). The highly evolved efficiency of the AdV life cycle does not expect a high dependency on one single step which probably permitted the AdV persistence during evolution. A thorough knowledge on AdV biology should serve to design experiments and set-up models to systematically investigate the limitations of post-delivery events. This will further help in designing and engineering AdV constructs to generate enhanced production systems.

1.2.5 Cell Culture Parameters

It has been assumed by the scientific community involved in process development, that early work in this field should be done in readily scalable cell culture conditions in order to maximize development capabilities of the product. Since the early nineties, AdV productions in cell suspension cultures have been demonstrated to be feasible (reviewed in Nadeau and Kamen 2003).

1.2.5.1 Cell Culture Characteristics

Since the isolation of Graham's cell line, several daughter cell lines have been selected for their growth capacity, their ability to produce AdV or their adaptation to scalable culture mode. Adherent cell lines have shown a higher specific productivity compared to suspension-adapted cell lines (Iyer et al. 1999). However surface requirements for cell growth renders such processes difficult to scale-up to large volumes and high cell densities. Roller bottles and cell factory systems for cell cultivation could offer a large surface. Up to 10 cell factories have been used to produce FGAdV within the HEK293 B cell line (Okada et al. 2005). Specific productivity has been maintained with active gassing to up to 8×10^3 PFU/cell. Microcarrier supports have offered a better scalable solution to produce AdV with the adherent cell line. Scalability is however limited by initial cell inoculum size expanded on static vessels. With cytodex beads, up to 1.5×10^4 PFU/cell have been produced in small spinner flasks whereas 2×10^4 PFU/cell have been produced in small-scale static cultures (Wu et al. 2002). Lyer et al. (1999) have demonstrated that specific productivity was similar using Cytodex microcarrier beads in serum containing cultures and in suspension serum-free culture in a bioreactor. Recovery of AdV from cell pellet is however cumbersome requiring cell detachment from the beads by trypsinization. Nevertheless, normal scale-up capacity is attained under suspension cell culture conditions. The original HEK293 cell line has been adapted to suspension

through passage into nude mice (Graham et al. 1977). However, the majority of suspension cell lines have been obtained from gradual adaptation to suspension conditions (Côté et al. 1997; Garnier et al. 1994; Schoofs et al. 1998). Cell line stability over time is displayed by the stability of cell growth rate and AdV yield and is cell line-dependent. In long-term cultures, changes in cell growth rate have been reported (Côté et al. 1997; Garnier et al. 1994; Schoofs et al. 1998). Park et al. (2004) have demonstrated that growth rate and cell size instabilities for their HEK293M cell line affected the AdV yield. Similarly, PER.C6 cells at high passage numbers have displayed a larger cell diameter, a higher specific growth rate and metabolism. AdV volumetric productivity has been increased by a factor of 3 (Berdichevsky et al. 2008).

1.2.5.2 Cell Culture Conditions

The cell environment for AdV productions has been extensively studied because of its complexity for optimization purposes where cell growth has to be sustained with high AdV yield. The cell environment is characterized by medium formulation, physicochemical conditions, feeding strategies and various cellular stresses induced by the different culture modes. Media available for HEK293 cell line growth are usually under proprietary formulation. Their development is a labor-consuming task requiring high expertise. A medium contains hundreds of components and therefore procedures used to develop one are highly empirical and the final formulation is often suboptimal. A basal defined medium is

completed with amino-acids, trace elements and balanced at physiological pH with buffer. Certain components of animal origin might hinder the development of a large-scale process where production variability, final product contamination and safety issues regarding human injectable material are not desirable. Statistical analysis design has been shown to be useful in screening components and optimizing quantities (Liu and Wu 2007). Analysis of central cell metabolism has permitted to identify differences in consumption and production rates during growth and infection (Maranga et al. 2005; Nadeau et al. 2002). Rates of consumptions and productions are also altered by changes in medium composition. To summarize, an increase in consumption rates of amino-acids and oxygen demand have been noted when switching from growth to infection. Considering the cell requirement dependence to the process status, the development of a good medium is even more complex when both good cell growth and AdV production have to be satisfied to sustain large-scale productions. In answer to an increase in rates of extracellular fluxes following infection, various medium adjustments through control of physico-chemical conditions and feeding strategies have been implemented to batch cultures. Slightly acidic and basic pH control in a bioreactor have shown a drastic effect on nutrient/metabolite consumption/production rates and on AdV productivities (Jardon and Garnier 2003; Xie et al. 2002). The optimum pH value has been found to be about 7.2. Bioreactor control at low pCO2 (0.05 atm) has shown to positively affect AdV productivity and a three-fold enhancement in AdV

productivity has been obtained at 35°C (Jardon and Garnier 2003; Xie et al. 2002). Operation complexity and the cost of feeding processes have been concomitantly increased with AdV productivity from cocktail addition (fed-batch), to medium exchange at infection (fed-batch with discontinuous cell retention), to perfusion (fed-batch with continuous cell retention). Fed-batch strategies could be beneficial if factors limiting batch cultures are precisely identified. However, these strategies have been shown to be of limited success. Inhibitory effects of factors such as waste metabolite accumulation, pH acidification and an increase in osmolarity are also detrimental for maintaining specific productivity (Ferreira et al. 2005; Garnier et al. 1994; Nadeau et al. 1996; Nadeau et al. 2002). Although they are more difficult to implement at large-scale, medium replacement at infection time combined or not with pulse feedings have been shown to increase specific productivities (Ferreira et al. 2005; Garnier et al. 1994; Nadeau et al. 1996). Perfusion systems have allowed successful nutrient renewal and waste removal through continuous medium exchange and cell retention. Although particularly costly, perfusion addresses limitations occurring with previous feeding techniques. Plain capacity of perfusion systems has been attained at high cell densities for which productivity is not sustainable using previous fed-batch modes. This technique will be discussed below. Cellular shear stresses are common problems associated with the large-scale culture of cells. Sparging and agitation requirements for efficient mass transfer as well as cell retention systems in perfusion culture have increased cell sensitivity which

decreased their AdV yield. Requirements to cultivate cells in serum-free medium have also increased cell sensitivity, by depriving cells from protecting serum agents. Investigations on causes of AdV productivity decrease when using a sparging system have pointed to polysorbate-80, a surfactant present in the viral stock formulation buffer (Xie et al. 2003). Removal of polysorbate-80 in viral buffer formulation while increasing the surfactant Pluronic-F68® from 0.3 to 1 g/L in medium has permitted to maintain AdV productivities under sparging conditions applicable to the 10,000 L bioreactor-scale. Because infected cells are particularly fragile, gentle retention systems for perfusion devices are preferred. Although literature does not provide a comparative study in uses of retention devices for AdV production, the acoustic separator chamber (Henry et al. 2004) which is known to induce less cell shear stress (Voisard et al. 2003) compared to the hollow fiber system (Cortin et al. 2004; Yuk et al. 2004) would be a better choice for AdV production.

1.2.5.3 Cell Density at Infection

Cell density achievable at infection depends on cell intrinsic characteristics and also cell culture conditions, sections that were previously discussed. Studies on the cell environment have permitted to enhance specific AdV productivity through better cell culture environment control (medium formulation and feeding strategies). These strategies have also been developed to increase the cell density at infection to ultimately enhance volumetric productivity. The challenge

has been to keep the specific productivity obtained at low cell density constant without feeding. The cell density effect describes a drop in the specific virus production when cell density is above 0.5×10^6 cells/mL (Kamen and Henry 2004). Because of a potential huge gain in AdV yield, efforts to understand and overpass this limitation has gained interest by research groups. The major hypothesis behind the cell density effect is that nutrient depletion and metabolite accumulation hinders cell metabolism and renders them unable to support viral production at high cell densities. Consequently, in order to improve substrate renewal and metabolite removal, production modes have gained in complexity from batch to perfusion system. Fed-batch strategies have failed to maintain a high specific productivity above 2×10^6 cells/mL (Garnier et al. 1994). High cell density at high viability could only be attained using perfusion systems through a continuous renewal of medium. Compared to a batch cell culture infected at 0.5×10^6 cells/mL with medium replacement, specific productivity has been maintained at 1.8×10^4 VP/cell when infection was performed on a 3 L perfusion cell culture at ~3×10^6 cells/mL, with 2-3 volumes/day under serum-free conditions (Henry et al. 2004). Up to 4.1×10^{10} VP/mL has been produced under these conditions. Cortin et al. (2004) have infected 8×10^6 cells/mL at 35°C at 1 volume/day in 5% serum-containing medium to produce ~8×10^9 IVP/mL. CRAdV productions in HeLa cells have been successfully performed at infection densities of 5 to 14.6×10^6 cells/mL at 1.5-2 volumes/day, under serum-free

conditions. Productivities have reached 3-6 $\times$ 10^{11} VP/mL and 3-5 $\times$ 10^{4} VP/cell (Yuk et al. 2004). An other avenue to explain cell density effect has been studied by Zhang et al. (Zhang et al. 2006). They demonstrated that a decrease in the proportion of cells in S phase was also responsible for a decrease in specific productivity at high cell densities.

In order to decrease process development time-lines and to optimize productivity for large-scale productions, integrated process development is sought. Because clone characteristics, medium composition and culture mode are highly inter-dependent, it would be misleading to optimize each variable independently. Selection of best producing clone should be done under suspension conditions in a serum-free medium (Côté et al. 1997; Côté et al. 1998).

1.2.5.4 Towards Understanding Cell Physiological State at Infection

The cell physiological state is a global term that describes the metabolic characteristics of a cell during its growth and production phases. It depends on a variety of parameters such as cell line characteristics, cell culture conditions and cell density. Their effects on growth and production are usually studied independently. The status of infection has been monitored using a variety of non-invasive on-line measurements to provide useful information. The oxygen uptake rate (OUR) and the capacitance signal have been correlated to viable

cell growth prior to infection (Henry et al. 2004). At infection both signals are affected. An increase in respiration has been noted together with an increase in capacitance measurements (Garnier et al. 1994; Henry et al. 2004; Monica et al. 2000). This correlates well with an increase in cell metabolic activity and an increase in cell size upon infection. In an attempt to measure the kinetics of infection of a GFP-expressing AdV, a fluorescent probe has been designed by Gilbert et al. (2000). It was used to monitor the AdV production kinetics and as a tool to identify the appropriate harvest time. At-line monitoring of GFP cells by flow cytometry has given valuable information on AdV production kinetics (Sandhu and Al-Rubeai 2008). These monitoring tools have however been of limited success in providing indications on the relative efficiency of AdV productions.

Developing a good understanding of specific metabolic requirements for both cell growth and virus production remains the basis of further improvements in AdV productivity. Even so, knowledge of the cell metabolic requirements is not sufficient as fed-batch strategies partially alleviated production limitations. Understanding the effect of metabolites inhibition has led to perfusion system use. However, these limitations have only been partially alleviated. Analysis of extracellular metabolic fluxes relies on envisioning a cell as a black box. Intracellular metabolic fluxes are useful indicators in the investigation and understanding of mammalian cell physiology and its changes in response to environmental variations. Identification, quantification and analyzes of

intracellular metabolic fluxes have provided a rationale to develop better media and feed strategies but also to improve cell system and viral vectors with genetic tools. In the context of AdV productions, Kamen's group has dedicated continuing efforts to better understand the cellular physiology at infection time through metabolic flux analysis. Based on material balancing and isotope tracers for the measurement of carbon, Nadeau's model includes glycolysis fluxes, glutaminolysis, amino acids pathways and the pentose-phosphate cycle (Nadeau et al. 2000). A favorable metabolic state for AdV production has been defined by an increase in glycolytic fluxes, in TCA fluxes and in ATP production rates upon infection (Henry et al. 2005; Nadeau et al. 2000). This state might be prolonged at higher cell densities if the feed rate is adjusted (Henry et al. 2005). In better medium/feeding strategies, glucose enters more efficiently into the TCA cycle and glutaminolysis and amino-acid catabolism rates are reduced. Lower lactate and ammonia production rates have been consequently observed (Henry et al. 2005; Nadeau et al. 2000). Metabolic fluxes are therefore useful to determine culture conditions resulting in enhanced productivity. It provides a basis for a rational approach to improve medium and feeding strategies, to design better cell lines with improved TCA activity and ATP production rates and to implement on-line control of such fluxes.

1.2.6 Conclusion

Successful development of high yield AdV production processes definitely requires an integrated approach. Concomitant decision on cell line specifications, cell culture characteristics, cell culture density, viral parameters and viral construction with respect to final viral product identity should be taken into consideration to fully optimize AdV production. Better understanding of viral amplification and cell physiology will contribute to the elaboration of an improved viral cell system for high viral specific productivities at high cell density processes. This knowledge acquired during the production development of FGAdV will be highly relevant to improving TGAdV production, an AdV with improved *in vivo* capacity for gene therapy protocols.

1.2.7 Acknowledgements

E.D. was supported by a prospective researcher fellowship from the Swiss National Science Foundation (PBSK2--115853). The authors acknowledge Dr. Parminder S. Chahal and Alice Bernier for editorial comments.

1.2.8 Références

Alba R, Hearing P, Bosch A, Chillon M. 2007. Differential amplification of adenovirus vectors by flanking the packaging signal with attB/attP-PhiC31 sequences: implications for helper-dependent adenovirus production. *Virology* 367(1):51-8.

Amalfitano A, Begy CR, Chamberlain JS. 1996. Improved adenovirus packaging cell lines to support the growth of replication-defective gene-delivery vectors. *Proc Natl Acad Sci U S A* 93(8):3352-6.

Amalfitano A, Chamberlain JS. 1997. Isolation and characterization of packaging cell lines that coexpress the adenovirus E1, DNA polymerase, and preterminal proteins: implications for gene therapy. *Gene Ther* 4(3):258-63.

Andrews JL, Kadan MJ, Gorziglia MI, Kaleko M, Connelly S. 2001. Generation and characterization of E1/E2a/E3/E4-deficient adenoviral vectors encoding human factor VIII. *Mol Ther* 3(3):329-36.

Barjot C, Hartigan-O'Connor D, Salvatori G, Scott JM, Chamberlain JS. 2002. Gutted adenoviral vector growth using E1/E2b/E3-deleted helper viruses. *J Gene Med* 4(5):480-9.

Berdichevsky M, Gentile MP, Hughes B, Meis P, Peltier J, Blumentals I, Aunins J, Altaras NE. 2008. Establishment of higher passage PER.C6 cells for adenovirus manufacture. *Biotechnol Prog* 24(1):158-65.

Bett AJ, Prevec L, Graham FL. 1993. Packaging capacity and stability of human adenovirus type 5 vectors. *J Virol* 67(10):5911-21.

Blanche F, Cameron B, Barbot A, Ferrero L, Guillemin T, Guyot S, Somarriba S, Bisch D. 2000. An improved anion-exchange HPLC method for the detection and purification of adenoviral particles. *Gene Ther* 7(12):1055-62.

Callahan JD. 2002. A Statistical Analysis of Adenovirus Reference Material Assay Results. *Bioprocessing J* 1(3):43-7.

Catalucci D, Sporeno E, Cirillo A, Ciliberto G, Nicosia A, Colloca S. 2005. An adenovirus type 5 (Ad5) amplicon-based packaging cell line for production of high-capacity helper-independent deltaE1-E2-E3-E4 Ad5 vectors. *J Virol* 79(10):6400-9.

Cheshenko N, Krougliak N, Eisensmith RC, Krougliak VA. 2001. A novel system for the production of fully deleted adenovirus vectors that does not require helper adenovirus. *Gene Ther* 8(11):846-54.

Cortin V, Thibault J, Jacob D, Garnier A. 2004. High-titer adenovirus vector production in 293S cell perfusion culture. *Biotechnol Prog* 20(3):858-63.

Côté J, Bourget L, Garnier A, Kamen A. 1997. Study of adenovirus production in serum-free 293SF suspension culture by GFP-expression monitoring. *Biotechnol Prog* 13(6):709-14.

Côté J, Garnier A, Massie B, Kamen A. 1998. Serum-free production of recombinant proteins and adenoviral vectors by 293SF-3F6 cells. *Biotechnol Bioeng* 59:567-65.

Danthinne X, Imperiale MJ. 2000. Production of first generation adenovirus vectors: a review. *Gene Ther* 7(20):1707-14.

Engelhardt JF, Ye X, Doranz B, Wilson JM. 1994. Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. *Proc Natl Acad Sci U S A* 91(13):6196-200.

Everts B, van der Poel HG. 2005. Replication-selective oncolytic viruses in the treatment of cancer. *Cancer Gene Ther* 12(2):141-61.

Fallaux FJ, Bout A, van der Velde I, van den Wollenberg DJ, Hehir KM, Keegan J, Auger C, Cramer SJ, van Ormondt H, van der Eb AJ and others. 1998. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum Gene Ther* 9(13):1909-17.

Fallaux FJ, Kranenburg O, Cramer SJ, Houweling A, Van Ormondt H, Hoeben RC, Van Der Eb AJ. 1996. Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum Gene Ther* 7(2):215-22.

Ferreira TB, Ferreira AL, Carrondo MJ, Alves PM. 2005. Effect of re-feed strategies and non-ammoniagenic medium on adenovirus production at high cell densities. *J Biotechnol* 119(3):272-80.

Gao GP, Engdahl RK, Wilson JM. 2000. A cell line for high-yield production of E1-deleted adenovirus vectors without the emergence of replication-competent virus. *Hum Gene Ther* 11(1):213-9.

Garnier A, Cote J, Nadeau I, Kamen A, Massie B. 1994. Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells. *Cytotechnology* 15(1-3):145-55.

Gilbert PA, Garnier A, Jacob D, Kamen A. 2000. On-line measurement of GFP fluorescence for the monitoring of recombinant adenovirus production. *Biotechnol Lett* 22:561-7.

Gilbert PA, Kamen A, Bernier A, Garnier A. 2007. A simple macroscopic model for the diffusion and adsorption kinetics of r-adenovirus. *Biotechnol Bioeng* 98(1):239-51.

Gorziglia MI, Kadan MJ, Yei S, Lim J, Lee GM, Luthra R, Trapnell BC. 1996. Elimination of both E1 and E2 from adenovirus vectors further improves prospects for in vivo human gene therapy. *J Virol* 70(6):4173-8.

Gorziglia MI, Lapcevich C, Roy S, Kang Q, Kadan M, Wu V, Pechan P, Kaleko M. 1999. Generation of an adenovirus vector lacking E1, e2a, E3, and all of E4 except open reading frame 3. *J Virol* 73(7):6048-55.

Grable M, Hearing P. 1992. cis and trans requirements for the selective packaging of adenovirus type 5 DNA. *J Virol* 66(2):723-31.

Graham FL, Smiley J, Russell WC, Nairn R. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 36(1):59-74.

Graham FL, van der Eb AJ. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52(2):456-67.

Gueret V, Negrete-Virgen JA, Lyddiatt A, Al-Rubeai M. 2002. Rapid titration of adenoviral infectivity by flow cytometry in batch culture of infected HEK293 cells. *Cytotechnology* 38(1-3):87-97.

Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps ML. 1997. Construction of adenovirus vectors through Cre-lox recombination. *J Virol* 71(3):1842-9.

Hartigan-O'Connor D, Barjot C, Crawford R, Chamberlain JS. 2002. Efficient rescue of gutted adenovirus genomes allows rapid production of concentrated stocks without negative selection. *Hum Gene Ther* 13(4):519-31.

Hehir KM, Armentano D, Cardoza LM, Choquette TL, Berthelette PB, White GA, Couture LA, Everton MB, Keegan J, Martin JM and others. 1996. Molecular characterization of replication-competent variants of adenovirus vectors and genome modifications to prevent their occurrence. *J Virol* 70(12):8459-67.

Henry O, Dormond E, Perrier M, Kamen A. 2004. Insights into adenoviral vector production kinetics in acoustic filter-based perfusion cultures. *Biotechnol Bioeng* 86(7):765-74.

Henry O, Perrier M, Kamen A. 2005. Metabolic flux analysis of HEK-293 cells in perfusion cultures for the production of adenoviral vectors. *Metab Eng* 7(5-6):467-76.

Hutchins B. 2002. Development of a Reference Material for Characterizing Adenovirus Vectors. *Bioprocessing J* 1(1):25-8.

Hutchins B, Sajjadi N, Seaver S, Shepherd A, Bauer SR, Simek S, Carson K, Aguilar-Cordova E. 2000. Working toward an adenoviral vector testing standard. *Mol Ther* 2(6):532-4.

Imler JL, Bout A, Dreyer D, Dieterle A, Schultz H, Valerio D, Mehtali M, Pavirani A. 1995. Trans-complementation of E1-deleted adenovirus: a new vector to reduce the possibility of codissemination of wild-type and recombinant adenoviruses. *Hum Gene Ther* 6(6):711-21.

Ishii-Watabe A, Uchida E, Iwata A, Nagata R, Satoh K, Fan K, Murata M, Mizuguchi H, Kawasaki N, Kawanishi T and others. 2003. Detection of replication-competent adenoviruses spiked into recombinant adenovirus vector products by infectivity PCR. *Mol Ther* 8(6):1009-16.

Iyer P, Ostrove JM, Vacante D. 1999. Comparaison of manufacturing techniques for adenovirus production. *Cytotechnology* 30:169-72.

Jardon M, Garnier A. 2003. PH, pCO2, and temperature effect on R-adenovirus production. *Biotechnol Prog* 19(1):202-8.

Kamen A, Henry O. 2004. Development and optimization of an adenovirus production process. *J Gene Med* 6 Suppl 1:S184-92.

Klyushnichenko V, Bernier A, Kamen A, Harmsen E. 2001. Improved high-performance liquid chromatographic method in the analysis of adenovirus particles. *Journal of Chromatography B. Biomedical Sciences and Applications* 755(1-2):27-36.

Kochanek S. 1999. High-capacity adenoviral vectors for gene transfer and somatic gene therapy. *Hum Gene Ther* 10(15):2451-9.

Kumar-Singh R, Chamberlain JS. 1996. Encapsidated adenovirus minichromosomes allow delivery and expression of a 14 kb dystrophin cDNA to muscle cells. *Hum Mol Genet* 5(7):913-21.

Lehmberg E, Traina JA, Chakel JA, Chang RJ, Parkman M, McCaman MT, Murakami PK, Lahidji V, Nelson JW, Hancock WS and others. 1999. Reversed-phase high-performance liquid chromatographic assay for the adenovirus type 5 proteome. *J Chromatogr B Biomed Sci Appl* 732(2):411-23.

Lieber A, He CY, Kirillova I, Kay MA. 1996. Recombinant adenoviruses with large deletions generated by Cre-mediated excision exhibit different biological properties compared with first-generation vectors in vitro and in vivo. *J Virol* 70(12):8944-60.

Liu CH, Wu PS. 2007. Optimization of adenoviral production in human embryonic kidney cells using response surface methodology. *J Biosci Bioeng* 103(5):406-11.

Lochmuller H, Jani A, Huard J, Prescott S, Simoneau M, Massie B, Karpati G, Acsadi G. 1994. Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants (delta E1 + delta E3) during multiple passages in 293 cells. *Hum Gene Ther* 5(12):1485-91.

Longley R, Radzniak L, Santoro M, Tsao YS, Condon RGG, Lio P, Voloch M, Liu Z. 2005. Development of a Serum-free Suspension Process for the Production of a Conditionally Replicating Adenovirus using A549 Cells *Cytotechnology* 49(2-3):161-71.

Louis N, Evelegh C, Graham FL. 1997. Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line. *Virology* 233(2):423-9.

Lusky M, Christ M, Rittner K, Dieterle A, Dreyer D, Mourot B, Schultz H, Stoeckel F, Pavirani A, Mehtali M. 1998. In vitro and in vivo biology of recombinant adenovirus vectors with E1, E1/E2A, or E1/E4 deleted. *J Virol* 72(3):2022-32.

Lusky M, Grave L, Dieterle A, Dreyer D, Christ M, Ziller C, Furstenberger P, Kintz J, Hadji DA, Pavirani A and others. 1999. Regulation of adenovirus-mediated transgene expression by the viral E4 gene products: requirement for E4 ORF3. *J Virol* 73(10):8308-19.

Ma L, Bluyssen HA, De Raeymaeker M, Laurysens V, van der Beek N, Pavliska H, van Zonneveld AJ, Tomme P, van Es HH. 2001. Rapid determination of adenoviral vector titers by quantitative real-time PCR. *J Virol Methods* 93(1-2):181-8.

Maizel JVJ, White DO, Scharff MD. 1968. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 36(1):115-25.

Maranga L, Aunins JG, Zhou W. 2005. Characterization of changes in PER.C6 cellular metabolism during growth and propagation of a replication-deficient adenovirus vector. *Biotechnol Bioeng* 90(5):645-55.

McConnell MJ, Imperiale MJ. 2004. Biology of adenovirus and its use as a vector for gene therapy. *Hum Gene Ther* 15(11):1022-33.

Meier O, Greber UF. 2003. Adenovirus endocytosis. *J Gene Med* 5(6):451-62.

Meneses-Acosta A, Dormond E, Jacob D, Tom R, Bernier A, Perret S, St-Laurent G, Durocher Y, Gilbert R, Kamen A. 2007. Development of a suspension serum-free helper-dependent adenovirus production system and assessment of co-infection conditions. *J Virol Methods.*

Mitani K, Graham FL, Caskey CT, Kochanek S. 1995. Rescue, propagation, and partial purification of a helper virus-dependent adenovirus vector. *Proc Natl Acad Sci U S A* 92(9):3854-8.

Mittereder N, March KL, Trapnell BC. 1996. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J Virol* 70(11):7498-509.

Monica TJ, Montgomery T, Ayala JL, Schoofs GM, Whiteley EM, Roth G, Garbutt JJ, Harvey S, Castillo FJ. 2000. Monitoring adenovirus infections with on-line and off-line methods. *Biotechnol Prog* 16(5):866-71.

Murakami P, McCaman MT. 1999. Quantitation of adenovirus DNA and virus particles with the PicoGreen fluorescent Dye. *Anal Biochem* 274(2):283-8.

Nadeau I, Garnier A, Côté J, Massie B, Chavarie C, Kamen A. 1996. Improvement of recombinant protein production with the human adenovirus/293S expression system using fed-batch strategies. *Biotechnol Bioeng* 51:613-23.

Nadeau I, Gilbert PA, Jacob D, Perrier M, Kamen A. 2002. Low-protein medium affects the 293SF central metabolism during growth and infection with adenovirus. *Biotechnol Bioeng* 77(1):91-104.

Nadeau I, Jacob D, Perrier M, Kamen A. 2000. 293SF metabolic flux analysis during cell growth and infection with an adenoviral vector. *Biotechnol Prog* 16(5):872-84.

Nadeau I, Kamen A. 2003. Production of adenovirus vector for gene therapy. *Biotechnology Advances* 20(7-8):475-489.

National Institute of Health. 2002. Assessement of adenoviral vectors safety and toxicity: Report of the National Institutes of Health Recombinant DNA Advisory Committee. *Hum Gene Ther* 13(1):1-13.

Ng P, Beauchamp C, Evelegh C, Parks R, Graham FL. 2001. Development of a FLP/frt system for generating helper-dependent adenoviral vectors. *Mol Ther* 3(5 Pt 1):809-15.

Ng P, Evelegh C, Cummings D, Graham FL. 2002. Cre levels limit packaging signal excision efficiency in the Cre/loxP helper-dependent adenoviral vector system. *J Virol* 76(9):4181-9.

Nyberg-Hoffman C, Shabram P, Li W, Giroux D, Aguilar-Cordova E. 1997. Sensitivity and reproducibility in adenoviral infectious titer determination. *Nat Med* 3(7):808-11.

Okada T, Nomoto T, Yoshioka T, Nonaka-Sarukawa M, Ito T, Ogura T, Iwata-Okada M, Uchibori R, Shimazaki K, Mizukami H and others. 2005. Large-scale production of recombinant viruses by use of a large culture vessel with active gassing. *Hum Gene Ther* 16(10):1212-8.

Palmer D, Ng P. 2003. Improved system for helper-dependent adenoviral vector production. *Mol Ther* 8(5):846-52.

Palmer DJ, Ng P. 2004. Physical and infectious titers of helper-dependent adenoviral vectors: a method of direct comparison to the adenovirus reference material. *Mol Ther* 10(4):792-8.

Palmer DJ, Ng P. 2005. Helper-dependent adenoviral vectors for gene therapy. *Hum Gene Ther* 16(1):1-16.

Park MT, Lee GM. 2000. Rapid titer assay of adenovirus containing green fluorescent protein gene using flow cytometry analysis. *Bioprocess Engineering* 22:403-6.

Park MT, Lee MS, Kim SH, Jo EC, Lee GM. 2004. Influence of culture passages on growth kinetics and adenovirus vector production for gene therapy in monolayer and suspension cultures of HEK 293 cells. *Appl Microbiol Biotechnol* 65(5):553-8.

Parks RJ, Bramson JL, Wan Y, Addison CL, Graham FL. 1999. Effects of stuffer DNA on transgene expression from helper-dependent adenovirus vectors. *J Virol* 73(10):8027-34.

Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A* 93(24):13565-70.

Parks RJ, Graham FL. 1997. A helper-dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging. *J Virol* 71(4):3293-8.

Pearson S, Jia H, Kandachi K. 2004. China approves first gene therapy. *Nat Biotechnol* 22(1):3-4.

Puntel M, Curtin JF, Zirger JM, Muhammad AK, Xiong W, Liu C, Hu J, Kroeger KM, Czer P, Sciascia S and others. 2006. Quantification of high-capacity helper-dependent adenoviral vector genomes in vitro and in vivo, using quantitative TaqMan real-time polymerase chain reaction. *Hum Gene Ther* 17(5):531-44.

Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, Wilson JM, Batshaw ML. 2003. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* 80(1-2):148-58.

Sakhuja K, Reddy PS, Ganesh S, Cantaniag F, Pattison S, Limbach P, Kayda DB, Kadan MJ, Kaleko M, Connelly S. 2003. Optimization of the generation and propagation of gutless adenoviral vectors. *Hum Gene Ther* 14(3):243-54.

Sandhu KS, Al-Rubeai M. 2008. Monitoring of the adenovirus production process by flow cytometry. *Biotechnol Prog* 24(1):250-61.

Sandig V, Youil R, Bett AJ, Franlin LL, Oshima M, Maione D, Wang F, Metzker ML, Savino R, Caskey CT. 2000. Optimization of the helper-dependent adenovirus system for production and potency in vivo. *Proc Natl Acad Sci U S A* 97(3):1002-7.

Sargent KL, Ng P, Evelegh C, Graham FL, Parks RJ. 2004. Development of a size-restricted pIX-deleted helper virus for amplification of helper-dependent adenovirus vectors. *Gene Ther* 11(6):504-11.

Schalk JA, de Vries CG, Orzechowski TJ, Rots MG. 2007. A rapid and sensitive assay for detection of replication-competent adenoviruses by a combination of microcarrier cell culture and quantitative PCR. *J Virol Methods* 145(2):89-95.

Schiedner G, Hertel S, Johnston M, Biermann V, Dries V, Kochanek S. 2002. Variables affecting in vivo performance of high-capacity adenovirus vectors. *J Virol* 76(4):1600-9.

Schiedner G, Hertel S, Kochanek S. 2000. Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production. *Hum Gene Ther* 11(15):2105-16.

Schoofs G, Monica TJ, Ayala J, Howtitz J, Montgomery T, Roth G, Castillo FJ. 1998. A High-Yielding Serum-Free, Suspension culture Process to Manufacture Recombinant Adenoviral Vectors for Gene Therapy. *Cytotechnology* 28:81-89.

Shabram P, Aguilar-Cordova E. 2000. Multiplicity of infection/multiplicity of confusion. *Mol Ther* 2(5):420-1.

Shabram PW, Giroux DD, Goudreau AM, Gregory RJ, Horn MT, Huyghe BG, Liu X, Nunnally MH, Sugarman BJ, Sutjipto S. 1997. Analytical anion-exchange HPLC of recombinant type-5 adenoviral particles. *Hum Gene Ther* 8(4):453-65.

Simek S, Byrne BJ, Bauer SR. 2002. FDA perspectives on the use of the adenovirus reference material. *Bioprocessing J* 1(3):40-42.

Subramanian S, Kim JJ, Harding F, Altaras GM, Aunins JG, Zhou W. 2008. Scaleable production of adenoviral vectors by transfection of adherent PER.C6 cells. (8756-7938 (Print)).

Tatsis N, Ertl HC. 2004. Adenoviruses as vaccine vectors. *Mol Ther* 10(4):616-29.

Transfiguracion J, Bernier A, Arcand N, Chahal P, Kamen A. 2001. Validation of a high-performance liquid chromatographic assay for the quantification of adenovirus type 5 particles. *J Chromatogr B Biomed Sci Appl* 761(2):187-94.

Umana P, Gerdes CA, Stone D, Davis JR, Ward D, Castro MG, Lowenstein PR. 2001. Efficient FLPe recombinase enables scalable production of helper-dependent adenoviral vectors with negligible helper-virus contamination. *Nat Biotechnol* 19(6):582-5.

Varga CM, Tedford NC, Thomas M, Klibanov AM, Griffith LG, Lauffenburger DA. 2005. Quantitative comparison of polyethylenimine formulations and adenoviral vectors in terms of intracellular gene delivery processes. *Gene Ther* 12(13):1023-32.

Varga CM, Wickham TJ, Lauffenburger DA. 2000. Receptor-mediated targeting of gene delivery vectors: insights from molecular mechanisms for improved vehicle design. *Biotechnol Bioeng* 70(6):593-605.

Voisard D, Meuwly F, Ruffieux PA, Baer G, Kadouri A. 2003. Potential of cell retention techniques for large-scale high-density perfusion culture of suspended mammalian cells. *Biotechnol Bioeng* 82(7):751-65.

Wang F, Puddy AC, Mathis BC, Montalvo AG, Louis AA, McMackin JL, Xu J, Zhang Y, Tan CY, Schofield TL and others. 2005. Using QPCR to assign infectious potencies to adenovirus based vaccines and vectors for gene therapy: toward a universal method for the facile quantitation of virus and vector potency. *Vaccine* 23(36):4500-8.

Wang Q, Greenburg G, Bunch D, Farson D, Finer MH. 1997. Persistent transgene expression in mouse liver following in vivo gene transfer with a delta E1/delta E4 adenovirus vector. *Gene Ther* 4(5):393-400.

Wang Q, Jia XC, Finer MH. 1995. A packaging cell line for propagation of recombinant adenovirus vectors containing two lethal gene-region deletions. *Gene Ther* 2(10):775-83.

Weaver LS, Kadan MJ. 2000. Evaluation of adenoviral vectors by flow cytometry. *Methods* 21(3):297-312.

Wu SC, Huang GY, Liu JH. 2002. Production of retrovirus and adenovirus vectors for gene therapy: a comparative study using microcarrier and stationary cell culture. *Biotechnol Prog* 18(3):617-22.

Xie L, Metallo C, Warren J, Pilbrough W, Peltier J, Zhong T, Pikus L, Yancy A, Leung J, Aunins JG and others. 2003. Large-scale propagation of a replication-defective adenovirus vector in stirred-tank bioreactor PER.C6 cell culture under sparging conditions. *Biotechnol Bioeng* 83(1):45-52.

Xie L, Pilbrough W, Metallo C, Zhong T, Pikus L, Leung J, Aunins JG, Zhou W. 2002. Serum-free suspension cultivation of PER.C6(R) cells and recombinant adenovirus production under different pH conditions. *Biotechnol Bioeng* 80(5):569-79.

Xu Q, Arevalo MT, Pichichero ME, Zeng M. 2006. A new complementing cell line for replication-incompetent E1–deleted adenovirus propagation *Cytotechnology* 51(3):x-x.

Yeh P, Dedieu JF, Orsini C, Vigne E, Denefle P, Perricaudet M. 1996. Efficient dual transcomplementation of adenovirus E1 and E4 regions from a 293-derived cell line expressing a minimal E4 functional unit. *J Virol* 70(1):559-65.

Youil R, Toner TJ, Su Q, Casimiro D, Shiver JW, Chen L, Bett AJ, Rogers BM, Burden EC, Tang A and others. 2003. Comparative analysis of the effects of packaging signal, transgene orientation, promoters, polyadenylation signals, and E3 region on growth properties of first-generation adenoviruses. *Hum Gene Ther* 14(10):1017-34.

Yuk IH, Olsen MM, Geyer S, Forestell SP. 2004. Perfusion cultures of human tumor cells: a scalable production platform for oncolytic adenoviral vectors. *Biotechnol Bioeng* 86(6):637-42.

Zhang C, Ferreira TB, Cruz PE, Alves PM, Haury M, Carrondo MJ. 2006. The importance of 293 cell cycle phase on adenovirus vector production. *Enzyme and Microbial Technology* 39:1328-32.

Zhang WW, Koch PE, Roth JA. 1995. Detection of wild-type contamination in a recombinant adenoviral preparation by PCR. *Biotechniques* 18(3):444-7.

Zhou H, Beaudet AL. 2000. A new vector system with inducible E2a cell line for production of higher titer and safer adenoviral vectors. *Virology* 275(2):348-57.

Zhou H, O'Neal W, Morral N, Beaudet AL. 1996. Development of a complementing cell line and a system for construction of adenovirus vectors with E1 and E2a deleted. *J Virol* 70(10):7030-8.

Zhou H, Zhao T, Pastore L, Nageh M, Zheng W, Rao XM, Beaudet AL. 2001. A Cre-expressing cell line and an E1/E2a double-deleted virus for preparation of helper-dependent adenovirus vector. *Mol Ther* 3(4):613-22.

Zhou HS, Zhao T, Rao XM, Beaudet AL. 2002. Production of helper-dependent adenovirus vector relies on helper virus structure and complementing. *J Gene Med* 4(5):498-509.

Zhu J, Grace M, Casale J, Chang AT, Musco ML, Bordens R, Greenberg R, Schaefer E, Indelicato SR. 1999. Characterization of replication-competent adenovirus isolates from large-scale production of a recombinant adenoviral vector. *Hum Gene Ther* 10(1):113-21.

CHAPITRE 2: UN PROCÉDÉ EFFICACE ET ADAPTABLE À GRANDE ÉCHELLE UTILISANT L'ADÉNOFECTION PAR LA POLYÉTHYLENEIMINE POUR LA PRODUCTION DU VECTEUR ADÉNOVIRAL DÉPENDANT D'UN VIRUS AUXILIAIRE

2.1 Présentation de l'Article

La première étape de production d'un AdV consiste à former les premières particules virales. Dans la littérature, on nomme "secours" cette étape qui permet de récupérer le HDV sous la forme de virus à partir de l'ADN du HV. Généralement, le secours du HDV consiste en une transfection de la lignée cellulaire HEK293 par l'ADN du HDV, suivie d'une infection par le HV. Cette étape est particulièrement importante puisqu'elle génère les premières particules virales de HDV, lesquelles doivent être amplifiées par la suite. De son rendement dépend le rendement en HDV de l'étape d'amplification subséquente.

L'article présenté dans ce chapitre décrit l'étude d'une nouvelle méthode pour réaliser de manière efficace l'étape de secours du HDV. *An Efficient and Scalable Process for Helper-Dependent Adenoviral Vector Production using Polyethylenimine-Adenofection* a été soumis à la revue *Biotechnology and Bioengineering* et est actuellement en révision finale. Un des objectifs de ce chapitre est d'évaluer l'importance des paramètres régissant l'apport des

constituants du HDV. Le complexe d'adénofection formé de l'ADN du HDV, de polyéthylenimine (PEI) et de HV sous sa forme virale est utilisé. A l'aide de plans expérimentaux factoriels, les quantités relative et totale de ces entités sur la production du HDV est examinée. L'étude a révélé que la quantité d'ADN du HDV est un paramètre déterminant pour la production de HDV. L'apport simultané du HV avec l'ADN du HDV est important pour améliorer la transfection et vraisemblablement la réplication de l'ADN du HDV. La méthode d'adénofection adaptée en suspension démontre qu'elle facilite la génération de grandes quantités de HDV comparativement aux méthodes de transfection/infection utilisées jusqu'alors. Cette étude initie le travail portant sur la compréhension de la formation du HDV.

2.2 An Efficient and Scalable Process for Helper-Dependent Adenoviral Vector Production using Polyethylenimine-Adenofection

Dormond E.[1,2], Jacob D.[1], Durocher Y.[1], Gilbert R.[1], Perrier M.[2] and Kamen A.[1]

[1]Animal Cell Technology Group, Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montréal, Québec, Canada H4P 2R2

[2]Chemical Engineering Department, École Polytechnique de Montréal, Campus de l'Université de Montréal, 2500, chemin de Polytechnique, Montréal, Québec, Canada, H3T 1J4

2.2.1 Abstract

Safety requirements for adenoviral gene therapy protocols have led to the development of the third generation of vectors commonly called helper-dependent adenoviral vectors (HDVs). HDVs have demonstrated a high therapeutic potential; however, the poor efficiency and reliability of the actual production process hampers further large-scale clinical evaluation of this new generation of vector. The current HDV production methods involve a preliminary rescue step through transfection of adherent cell cultures by a HDV plasmid followed by a helper adenovirus (HV) infection. Amplification by serial co-infection of complementary cells allows an increase in the HDV titer. Using a HEK293 FLP/*frt* cell system in suspension culture, an alternative protocol to the current transfection/infection procedure was evaluated. In this work, the adenofection uses the HDV plasmid linked to the HV with the help of polyethylenimine and has shown to outperform standard protocols by producing higher HDV yield. The influence of complex composition on the HDV production was examined by a statistical design. The optimized adenofection and amplification conditions were successively performed to generate HDV at the 3 L bioreactor scale. Following only two serial co-infection passages, up to 1.44×10^8 HDV infectious units/mL of culture were generated, which corresponded to 26% of the total particles produced. This production strategy, realized in cell suspension culture, reduced process duration and therefore the probability of

vector recombination by introducing a cost-effective transfection protocol, ensuring production of high-quality vector stock.

Keywords: Helper-dependent adenoviral vector; Virus production; HEK293; PEI-transfection; Bioreactor

2.2.2 Introduction

During the past decade, gene therapy involving adenoviral vectors has gained considerable attention through its successes and failures (Branca 2005; National Institute of Health 2002; Peng 2005). The development of new adenoviral vectors has been a major task fed by the numerous advantages of this vector (Nadeau and Kamen 2003). The helper-dependent adenoviral vector (HDV) was constructed to overcome drawbacks of the first generation of adenoviral vectors. The HDV genome is devoid of all viral coding genes making a large space available for the insertion of a therapeutic transgene (up to 37 kb). Consequently, in treatment of various model diseases involving this vector, negligible *in vivo* toxicity and immunogenicity were observed (reviewed in Brunetti-Pierri and Ng 2006; Kochanek 1999; Morsy and Caskey 1999; Palmer and Ng 2005). The HDV genome harbors only cis-acting elements including the packaging signal (Ψ), inverted terminal repeats (ITRs), the transgene and additional sequences to improve its replication. Its genome construction and propagation in bacterial systems has been facilitated by work done by Chartier

et al. (1996). The HDV production in human embryonic kidney 293 cells (HEK293) requires trans-acting elements provided by the first generation of adenoviral vector called helper virus (HV) and by the host cell line (E1) (Graham et al. 1977). Initial attempts emphasized the necessity to develop cell systems capable of reducing the HV contamination. This has been achieved through the use of Cre/*loxP* (Hardy et al. 1997; Lieber et al. 1996; Parks et al. 1996) or FLP/*frt* recombinase systems (Ng et al. 2001; Umana et al. 2001). The HV harboring *loxP* or *frt* sites on both sides of Ψ loses the latter when replicating in HEK293 cells that express Cre or FLP respectively. Both the Cre/*loxP* and FLP/*frt* systems have shown similar efficiencies in reducing the HV contamination and in amplifying the HDV (Ng et al. 2001; Umana et al. 2001).

The standard HDV production consists of a multi-step process (Figure 2.1). The initial step, commonly known as the rescue step, aims to recover HDV from HDV DNA. Transfection of producer cells with the linearized HDV genome, i.e excised from the bacterial sequence, is followed by the HV infection 8 to 18 h post-transfection. Typical transfection protocols involve calcium phosphate-DNA co-precipitation (Hartigan-O'Connor et al. 2002b; Ng et al. 2002b; Sakhuja et al. 2003; Sandig et al. 2000) or DNA complexation with commercial liposomes (Oka and Chan 2005). However, protocols requiring adherent cell cultures are problematic when transfering to large-scale operations. The viral lysate containing the HDV is recovered when a cytopathic effect is visible, usually 48 to 72 h post-infection. At this step, because the HDV titer is low (10^2 to 10^5

Infectious Units (IU) of HDV/mL) (Kumar-Singh and Chamberlain 1996; Ng et al. 2001; Parks et al. 1996), further amplification of the HDV is required. To achieve this goal, an increasing number of adherent cells are co-infected with the HV and a defined volume of the viral lysate obtained from the previous step (Hartigan-O'Connor et al. 2002a; Ng et al. 2002b). Using this volume-based protocol for amplification, at least four to six passages are needed to attain a maximal HDV titer (about 10^8 IU of HDV/mL). Additional passages cause cyclic fluctuation in the HDV titer (Hartigan-O'Connor et al. 2002a; Ng et al. 2001; Ng et al. 2002b). Furthermore, favored by homologies between viral genomes but also by the number of passages, various HDV and HV recombinants are produced (Hartigan-O'Connor et al. 2002a; Sakhuja et al. 2003; Sandig et al. 2000).

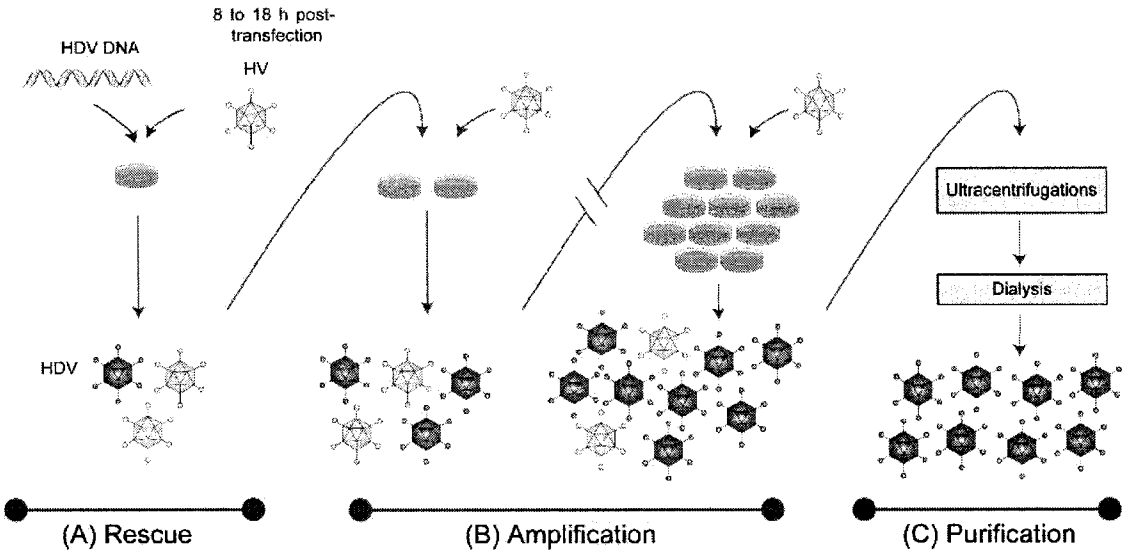

Figure 2.1 Production process of HDV which consists of (A) the rescue step, (B) the amplification step and (C) the purification step

Despite major advances in cell system development and *in vivo* testing, limitations concerning the HDV production restrict its possible use in gene therapy protocols, where high-quality clinical-grade vectors should be produced in large amounts under good manufacturing process conditions (Lusky 2005). Because the HDV production is carried out via empirical protocols, the reliability as well as the efficiency of the production are questionable. Moreover, because volumetric productivity is limited by surface area, standard protocols requiring adherent cell cultures are not suitable for large-scale production of the HDV.

In this study, we have shown how to rescue and amplify the HDV using an optimized production process in suspension that is easily transferable to large-scale volumes. An efficient method to transfer the large HDV DNA to cells,

which takes advantage of the HV infection, was evaluated and optimized in suspension cell cultures. Following a reduced number of amplification steps, an HDV stock was generated in a 3 L bioreactor using a cost-effective, efficient and scalable process.

2.2.3 Materials and Methods

2.2.3.1 FLPe Cell Line

A stable FLPe cell line was obtained following transfection of suspension-growing HEK293 SF-3F6 cells (Côté et al. 1997; Meneses-Acosta et al. 2007). Stable clones were selected for their ability to excise the Ψ of HV genomes, to limit the HV contamination and to amplify the HDV. FLPe cells were typically cultivated in low-calcium HSFM medium (Gibco, Ontario, Canada) supplemented with 10 mM HEPES buffer, 1% BCS and 0.75 µg/mL puromycin (Durocher et al. 2002). Cells were maintained as a suspension culture in shake flasks at 37°C in a humidified incubator with 5% CO_2 and agitation (120 rpm). Every 2 or 3 days, cells were subcultured to maintain exponential growth. Haemacytometer counts using the erythrosine dye exclusion technique were used to assess cellular densities.

2.2.3.2 Plasmid and Viruses

The HDV plasmid, pHCAgfp (32 kb) was a generous gift from Dr. V. Sandig. It carries a *gfp* expression cassette driven by the cytomegalovirus promoter, two ITRs adjacent to the bacterial sequence, the Ψ and the E4 promoter region of the adenovirus type 5. pHCA*gfp* was amplified in *E.coli* DH5-α and purified using Giga-Prep columns (Qiagen, Ontario, Canada). When necessary, the HDV DNA was PmeI-linearized to liberate the viral sequence. The purified DNA was quantified by UV absorbance in 50 mM Tris-HCl pH 8.0 ensuring that A_{260}/A_{280} was always between 1.80 and 1.95.

The HV, provided generously by Dr. P. Lowenstein, is an E1/E3 deleted adenoviral vector available in viral form (Umana et al. 2001). It bears two parallel *frt* sites flanking the Ψ. A high-titer stock of the HV was produced by infecting HEK293 SF-3F6 cells in a 3 L bioreactor. In the transfection/infection and the adenofection protocols, the HV was purified by double CsCl banding (one step gradient at 100,000 × g, 4°C for 1.5 h and one linear gradient at 100,000 × g, 4°C for 24 h) followed by dialysis against 10 mM Tris-HCl pH 7.9, 1 mM $MgCl_2$. In co-infection protocols, a viral lysate of the HV was employed.

2.2.3.3 Rescue Step via Transfection / Infection or PEI-Adenofection

At a 6-well plate scale, FLPe cells were seeded in fresh medium at 0.5×10^6 cells per well 24 h prior to transfection and adenofection or at 1×10^6 cells per well 1 h prior to transfection and adenofection.

At a 250 mL shake flask scale, FLPe cells were seeded in fresh medium at 0.5×10^6 cells/mL in a 50 mL working volume 1 h prior to adenofection.

Transfection complexes were formed in low-calcium HSFM containing 10 mM HEPES. Unless stated otherwise, 1 μg of HDV plasmid/mL of cell culture was mixed with 3 μg of linear 25 kDa PEI (Polysciences, Warrington, PA) per mL of cell culture or 6 μL of Lipofectamine 2000 (Invitrogen, Ontario, Canada) in one-tenth of the cell culture volume (200 μL or 5 mL). Complexes were incubated at room temperature for 10 min and added to FLPe cells. Medium for adherent FLPe cells in 6-well plates was changed prior to transfection and adenofection with HSFM containing 10 mM HEPES and replaced 5 h post-transfection and adenofection with complete medium. FLPe cells were infected with the purified HV 12 h post-transfection at a multiplicity of infection (MOI) of 5 HV IU/cell.

PEI-adenofection complexes were formed following the preparation of PEI-transfection complexes. After DNA-PEI complexation, the purified HV was added at a MOI of 5 HV IU/cell (unless stated otherwise). The resulting

complexes were left to stand for 10 min before being added to the cells. The viral lysates were collected 48 h post-infection in all cases and subjected to one freeze/thaw prior to being used in amplification step. This step will be further referred to as passage 0 (P0).

2.2.3.4 Amplification Step via Co-Infection

For the HDV serial amplification, the viral lysate from a previous passage and the HV were used to co-infect FLPe cells in fresh medium. Cells were seeded as stated above in 6-well plates (using adherent or suspension culture mode) or in shake flasks. The volume of viral lysate added was equal to one-tenth of the final culture volume (200 µL or 5 mL). HV was added at a MOI of 1 HV IU/cell. The viral harvest was done at 48 h post-infection. Three passages of amplification, further referred to as passage 1 (P1), passage 2 (P2) and passage 3 (P3) were successively performed following the protocol described above.

For the HDV production in a 3 L bioreactor, the optimized adenofection complexes were used to rescue and further amplify the HDV for two passages using the optimized infection conditions. P1 was done in a 2 L shake flask with a working volume of 400 mL. Briefly, 40 mL of P0, frozen and thawed once, was used to co-infect FLPe cells, resuspensed in fresh medium at 0.5×10^6 cells/mL with the HV at a MOI of 0.5 HV IU/cell. Harvest was done 48 h post-infection. P2 was performed in a 3 L Chemap CF-3000 bioreactor (Mannedorf, Switzerland).

The temperature was maintained at 37°C with a water jacket. The bioreactor was equipped with three surface baffles and two marine impellers. Agitation was maintained at 100 rpm. Oxygen, nitrogen and carbon dioxide were supplied at 300 mL/min by surface aeration to maintain the dissolved oxygen (DO) at 40% of air saturation. Carbon dioxide was replaced 24 h post-infection by 1 N NaOH to maintain the pH at 7.2. On-line control was performed via a thermocouple, a pH probe, a DO probe and individual mass flow controllers all connected to a control unit. FLPe cells were inoculated at 0.3×10^6 cells/mL in the bioreactor 24 h prior to infection. At infection time, the medium was completely renewed and cells were resuspended to 0.5×10^6 cells/mL for a final volume of 2800 mL. Co-infection was done with 280 mL of the viral lysate from P1 and the HV lysate at a MOI of 0.5 HV IU/cell. Cells were harvested at 48 h post-infection.

2.2.3.5 Experimental Design

A design of experiment (DOE) was performed to maximize the HDV yield using the adenofection protocol. To do so, the stoichiometry of adenofection complexes was examined: the concentration of HDV DNA in culture ([HDV DNA]), the mass ratio of PEI to DNA ([PEI]/[DNA]) and the MOI of HV were chosen as variables. Experiments were conducted in 6-well plate suspension cultures. Adenofection efficiency and HDV titer at P3 were chosen as responses. A full 2-level factorial design was first performed. It was augmented by axial points to generate a face-centered central composite design

(Montgomery 2005), that allowed for the determination of the optimum point location. Statistical analysis of the results and optimum finding were completed using Statistica 6.0 software (Statsoft, Tulsa, OK). Adenofection experiments were performed within one week to minimize the variability associated with cell transfection.

2.2.3.6 Assessment of Transfection and Adenofection Efficiency

The efficiency of HDV DNA transfection and HDV DNA adenofection in FLPe cells was assessed by the number of cells expressing GFP at 48 h post-transfection or adenofection. For this purpose, FLPe cells were harvested, centrifuged at 300 × g for 5 min and resuspended in 2% *p*-formaldehyde (Polysciences, Warrington, PA) in PBS. Following a 1 h incubation at 4°C, cells were filtered through a 60 µm mesh prior to flow cytometry analysis (EPICS^{TM} XL-MCL flow cytometer, Beckman Coulter, FL). EXPO 32 software was used to gate at least 10,000 events and determine the number of GFP-positive cells.

2.2.3.7 Quantification of Viral Vectors

The HDV was quantified by infecting target cells in suspension culture (HEK293 E cells in low-calcium HSFM, 10 mM HEPES, 1% BCS, 50 µg/mL G-418 (Durocher et al. 2002)). At least two different dilutions of the viral lysate were used to infect HEK293 E cells inoculated at 0.5×10^6 cells/mL in 12-well plates

with fresh media. At 20 h post-infection, cells were counted and fixed with 2% *p*-formaldehyde in PBS. Ratios of GFP-positive to total cells were assessed using flow cytometry analysis as described above. Only ratios between 3 and 30% were retained to estimate the HDV titer (Côté et al. 1997). The limit for an accurate detection was 10^5 IU of HDV/mL.

The HV titers were assessed by the end-point dilution method. Briefly, HEK293 A cells routinely maintained in adherent culture in DMEM+ (Wisent, Québec, Canada) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate and 5% FBS, were seeded at 0.03×10^6 cells/mL in 96-well plates and infected 24 h later with serial dilutions of viral lysate. The cytopathic effect was determined by visual observation 14 days post-infection. Positive wells were scored and the HV titer was estimated according to the calculation by Reed and Muench (O'Reilly et al. 1994).

The total viral particle (TVP) concentrations were determined using anion-exchange high performance liquid chromatography coupled to UV detection spectroscopy (Klyushnichenko et al. 2001). Lysates stored at –80°C were thawed and centrifuged at 4,500 × g for 2 min. Supernatants were filtered through a 0.45 µm membrane syringe filter before injection. The TVP concentration was corrected for the difference in vector size between the wild type adenovirus standard used to make the standard curve and the HDV (Ng et al. 2002b).

The viral genomes (VG) titers of the HDV and the HV were determined by a SYBR-Green I quantitative PCR assay (qPCR). Specific set of primers for the HDV and the HV were designed with the help of Clone Manager Professionnal Suite v.7 (Sci-Ed Software, Cary, NC). Primer sequences were as follows: HDV forward 5'-AGCTCACAGGCTGTAGTTTG-3' (bp 2347 to 2366 from HCAgfp stuffer region) and HDV reverse 5'-GGATCACTTGCACGGTTTAG-3' (bp 2551 to 2570); HV forward 5'-CCGCAGTTGACAGCATTACC-3' (bp 21402 to 21422 from the wild type adenovirus 5 genome) and HV reverse 5'-CGGACCACGTCAAAGACTTC-3' (bp 21601 to 21620). A standard curve was generated for each qPCR run. To do so, a plasmid containing the HDV and the HV targeted sequences was constructed. The reaction was performed in the Light Cycler instrument (Roche Diagnostics, Québec, Canada). Reactions were done in a total volume of 20 µL containing 18 µL of PCR Mix (12 µL H_20, 1.6 µL $MgCl_2$ 25 mM, 1.2 µL forward primer 5 µM, 1.2 µL reverse primer 5 µM, 2 µL 10× Master Mix Light-Cycler Fast-Start SYBR Green I purchased from Roche Diagnostics) and 2 µL of template (serial dilution of standard from 10^9 to 10^2 molecules or unknowns or water for negative control). Conditions for the reaction were a pre-incubation period at 95°C for 10 min; 40 cycles of amplification at 95°C for 10 s, 61°C for 7 s and 72°C for 10 s; a melting curve at 95°C, 70°C for 15 s and 95°C with a ramp of 0.10°C/s; a final cooling step at 40°C. Analysis of data was done using the Light Cycler 480 software. Specificity

of the reaction was confirmed by melting curves analysis and runs of qPCR products on agarose gels.

2.2.4 Results

2.2.4.1 Evaluation of Adenofection in 6-Well Plate Adherent Cell Cultures

The adenofection protocol was compared to standard transfection/infection protocols for its ability to deliver HDV DNA and to produce HDV in adherent cell culture. The transfection or the adenofection efficiencies of FLPe cells using the circular or the linearized HDV DNA was first examined (Table 2.1). As expected, greater transfection or adenofection efficiencies were achieved using the circular HDV DNA; however, since the corresponding HDV titers were undetectable at P2, the linearized HDV DNA was used in all subsequent studies. Furthermore, PEI-adenofection complexes generated higher transfection levels compared to PEI-transfection complexes.

Table 2.1 Rescue protocols in adherent cell culture

Rescue Protocol	HDV DNA form	Transfection efficiency (%)	Amplification of HDV at P2
Lipofectamine 2000 - Transfection / Infection at 12 h post-transfection	Circular	57 (± 18)	No
	Linearized	42 (± 15)	Yes
PEI - Transfection / Infection at 12 h post-transfection	Circular	36 (± 8)	No
	Linearized	12 (± 6)	Yes
PEI - Adenofection	Circular	41 (± 5)	No[a]
	Linearized	23 (± 3)	Yes

Notes : The various rescue protocols were performed using the circular or the linearized form of HDV DNA. The transfection efficiency was assessed 48 h post-transfection in all cases. The amplification was performed by three serial passages of co-infection. The means are given for triplicate wells of two independent experiments (n=6) with the standard deviation in parenthesis
[a] Variable from flask to flask; values close to detection limit (10^5 IU of HDV/mL)

Since the HDV titer could not be accurately determined at P0, serial amplification passages were done to allow accurate titer determination and to assess the capacity of rescue protocols to produce the HDV. HDV quantification revealed that compared to the Lipofectamine- and the PEI-transfection/infections, the adenofection allowed for a maximum titer of $1.02 \times 10^8 \pm 3.14 \times 10^7$ IU of HDV/mL in only two amplification passages whereas a third amplification passage was required to attain a similar level for the former two protocols (Figure 2.2). Surprisingly, the Lipofectamine-mediated transfection/infection, which gave a higher transfection level (Table 2.1) resulted

in a similar viral production as the PEI-mediated transfection/infection (Figure 2.2).

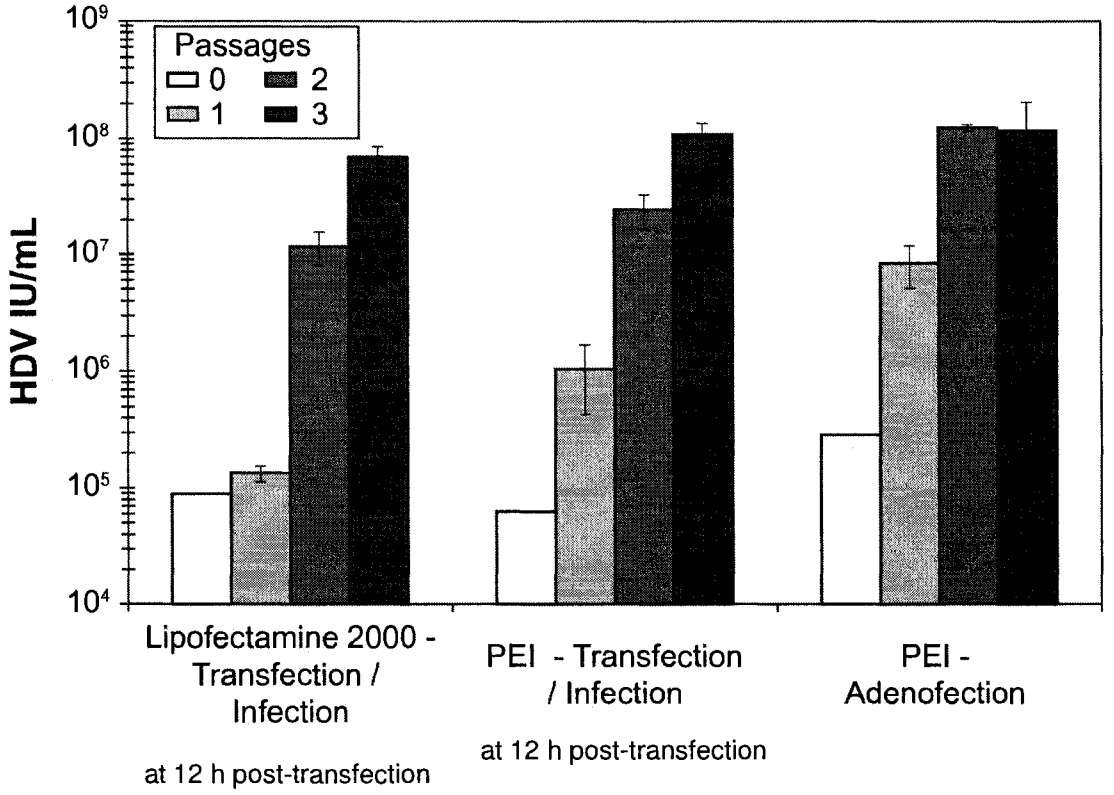

Figure 2.2 Rescue and amplification of the HDV via various rescue protocols in adherent cell culture. The HDV titers in IU of HDV/mL are represented for three serial passages of amplification. The means are represented for triplicate wells of two independent experiments with errors bars being the standard deviation (n = 6). Error bar is not represented for the HDV titer at P0 (limit of detection)

2.2.4.2 Evaluation of Adenofection in 6-Well Plate Suspension Cell Cultures

Adenofection experiments were also conducted in 6-well plate suspension cultures. It was observed that even if the adenofection efficiency at P0 was lower for adherent (23 ± 3%) than for suspension cultures (33 ± 2%), the HDV titers were always lower in the latter case. In adherent cell cultures, the adenofection-based protocol allowed a maximum titer at P2 (Figure 2.2), whereas in suspension culture the titer constantly increased from P0 to P3 to finally reach $4.65 \times 10^7 \pm 1.23 \times 10^7$ IU of HDV/mL at P3 (the mean is the value from triplicate and duplicate wells of two independent experiments with the standard deviation for n=5).

2.2.4.3 Evaluation of Adenofection in Shake Flask Suspension Cell Cultures

The adenofection protocol was further evaluated in larger volumes of suspension cell cultures. Figure 2.3 indicates similar kinetics of HDV amplification to that obtained in the adherent 6-well plate experiments. The HDV titer was maximum at P2 ($9.89 \times 10^7 \pm 9.43 \times 10^6$ IU of HDV/mL) and declined at P3. The evolution of viral titers is displayed in Figure 2.3. The total viral particle concentrations remained constant from P0 to P2 (about 3×10^8 TVP/mL). The HV titers were also stable from P0 to P2 and remained below 10^5 IU of HV/mL.

Consequently, the HDV infectivity ratio (IU of HDV/TVP) increased constantly to 27%, while the HV contamination ratio in terms of IU (IU HV/IU HDV) decreased to 0.1% at P2. At P3, a decrease in the TVP concentration and the HDV titer was observed and the HDV infectivity ratio showed high variability.

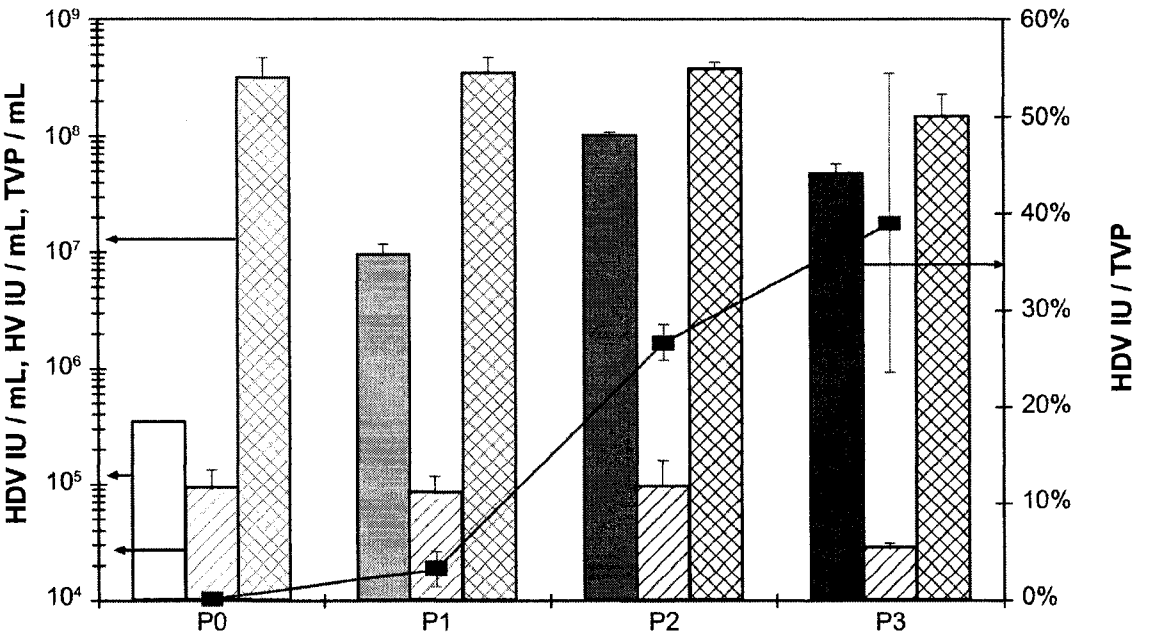

Figure 2.3 Rescue and amplification of the HDV via adenofection using HDV DNA in suspension cell culture. The viral titers (HDV IU/mL in full bars, HV IU /mL in hatched bars, TVP/mL in squared bars) and the HDV infectivity ratio (black squares) are shown for P0 to P3. The means are represented for duplicate shake flasks of two independent experiments with error bars being the standard deviation (n = 4). Error bar is not represented for the HDV titer at P0 (limit of detection)

2.2.4.4 Optimization of Adenofection Complexes Composition

The rescue step is the major bottleneck in the HDV production (Hartigan-O'Connor et al. 2002a); therefore, to improve the overall process, the poor productivity of the initial rescue step should be overcome by optimizing the adenofection protocol. The composition of the adenofection complexes needs to be optimized in order to adenofect a maximum number of cells and to provide the HDV DNA and the HV in an appropriate ratio for the HDV production. Hence, the effect of the concentrations of HDV DNA, PEI and HV was studied. The range of those variables were chosen with respect to common values found in literature for the PEI-transfections in HEK293 cells (Durocher et al. 2002) and for the HDV generation (Hartigan-O'Connor et al. 2002b; Ng et al. 2002b; Oka and Chan 2005) (Table 2.2). Considering the limitation for the HDV amplification observed in preliminary experiments performed in 6-well plate suspension cell cultures, three amplification passages were performed to get a detectable titer, constantly increasing from P0 to P3 (data not shown), for all the adenofection conditions.

Table 2.2 Description of variables and applied range in the DOE

Variable description	Variable names	Applied levels		
		Low (-1)	Center (0)	High (+1)
Concentration of HDV DNA in culture (μg/mL)	[HDV DNA]	0.5	1.25	2
Mass ratio of PEI to HDV DNA	[PEI]/[HDV DNA]	2	3	4
Number of infectious units of HV/cell (HV IU/cell)	MOI of HV	1	5.5	10

In order to extract the maximum of information in a minimal set of experiments, sequential DOEs were applied. A full factorial design with center points showed a significant curvature ($p<0.05$). Therefore, additional points (axial points with center points) were included to produce contour plots of the response as functions of the variables. Complete design and responses are provided in Table 2.3. At P3, adenofection efficiency showed response ranges with a fiftyfold increase (1.3 to 49.6%) and a HDV titer having a difference of 2 orders of magnitude (1.01×10^5 to 5.88×10^7 IU of HDV/mL). This indicates that the number of adenofected cells and the HDV titer at P3 are highly sensitive to the concentrations of HDV DNA, PEI and HV. A good correlation between the adenofection efficiency and the HDV titer at P3 was obtained, i.e, the highest adenofection efficiencies corresponded to the highest HDV titers (Figure 2.4). For low adenofection efficiencies (below 10%) variable HDV titers were obtained at P3. Adenofection efficiency was further employed as the response to be maximized.

Table 2.3 Description of the central composite experimental design face-centered

	Coded levels of variables			Responses	
	[HDV DNA]	[PEI]/[HDV DNA]	MOI of HV	Adenofection efficiency (%)	HDV titer at P3 (HDV IU/mL)
Full Factorial Design	-1	-1	-1	3	2.01×10^5
				1	1.01×10^5
	1	-1	-1	30	2.58×10^7
				32	2.95×10^7
	-1	1	-1	9	2.85×10^7
				10	5.03×10^6
	1	1	-1	33	9.84×10^6
				26	1.50×10^7
	-1	-1	1	6	4.66×10^6
				3	1.31×10^5
	1	-1	1	47	3.86×10^7
				43	3.22×10^7
	-1	1	1	33	3.42×10^7
				20	1.28×10^7
	1	1	1	50	5.88×10^7
				48	5.66×10^7
Center Points	0	0	0	39	3.30×10^7
				41	2.55×10^7
				41	1.58×10^7
Axial Points	-1	0	0	22	1.22×10^7
				23	8.57×10^6
	1	0	0	39	2.51×10^7
				42	1.71×10^7
	0	-1	0	21	9.8×10^6
				21	4.13×10^6
	0	1	0	26	8.40×10^6
				38	1.66×10^7
	0	0	-1	13	7.65×10^6
				21	8.70×10^6
	0	0	1	32	1.40×10^7
				37	1.87×10^7
Center Points	0	0	0	32	2.62×10^7
				36	3.36×10^7
				31	1.85×10^7

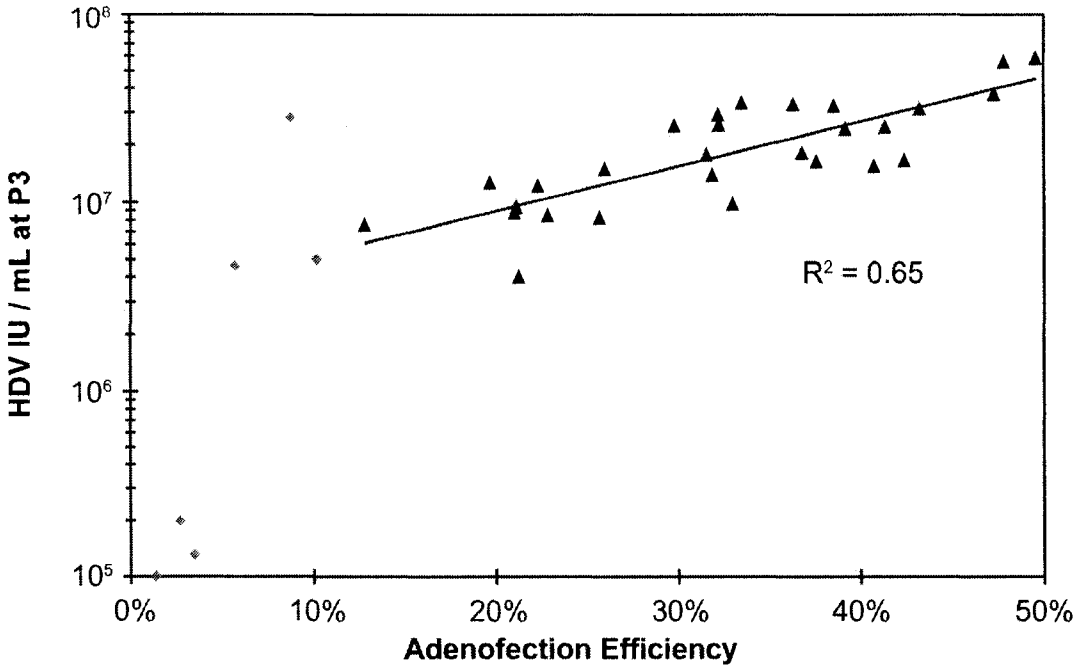

Figure 2.4 Relationship between the adenofection efficiency at P0 and the HDV titer at P3. A good degree of correlation is observed above an adenofection efficiency of 10%

With respect to the ANOVA hypothesis of normality of data, normality of error distribution and constant variance, a predictive quadratic equation combining all significant terms (variables and their combinations) ($p<0.05$) was constructed. The goodness-of-fit, $R^2 = 0.91$, indicated that experimental data were in good agreement with equation predictions. The relative contributions of the significant terms are presented in a Pareto chart (Figure 2.5). Far from the others, the most important variable was [HDV DNA]. In decreasing contribution order, the MOI of HV and [PEI]/[HDV DNA] follow as first-order terms. A dose-dependent increase in the number of adenofected cells was seen with [HDV DNA], [PEI]/[HDV DNA]

and the MOI of HV (Figure 2.6 A, B, C). However, the effects of the MOI of HV and [PEI]/[HDV DNA] were partially modified by the negative effect of second-order terms (MOI of HV)^2, ([PEI]/[HDV DNA])^2 and by the effect of interaction terms (Figure 2.5 and 2.6 A, B, C) . The presence of a quadratic term is reflected by the fact that, at high values of the variables, the effect on the response is more important than at low values of the variables. In Figure 2.6 A, B and C, the combined effect of first and second-order terms for both MOI of HV and [PEI]/[HDV DNA] was shown by the presence of a maximum of adenofection efficiency in the upper range of variables.

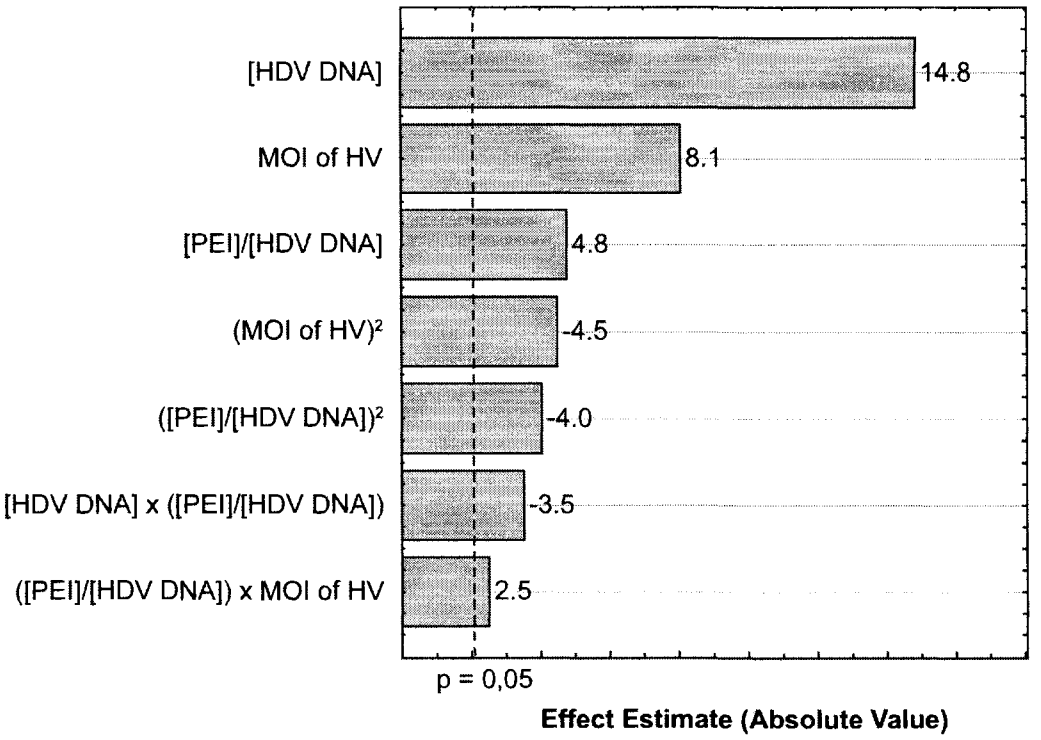

Figure 2.5 Pareto chart for variable contribution in the adenofection efficiency response. The values indicated at right of bars represent levels and signs of the variable contribution. (positive value: increase of the response when the variable is augmented, negative value: decrease of the response when the value of variable is increased)

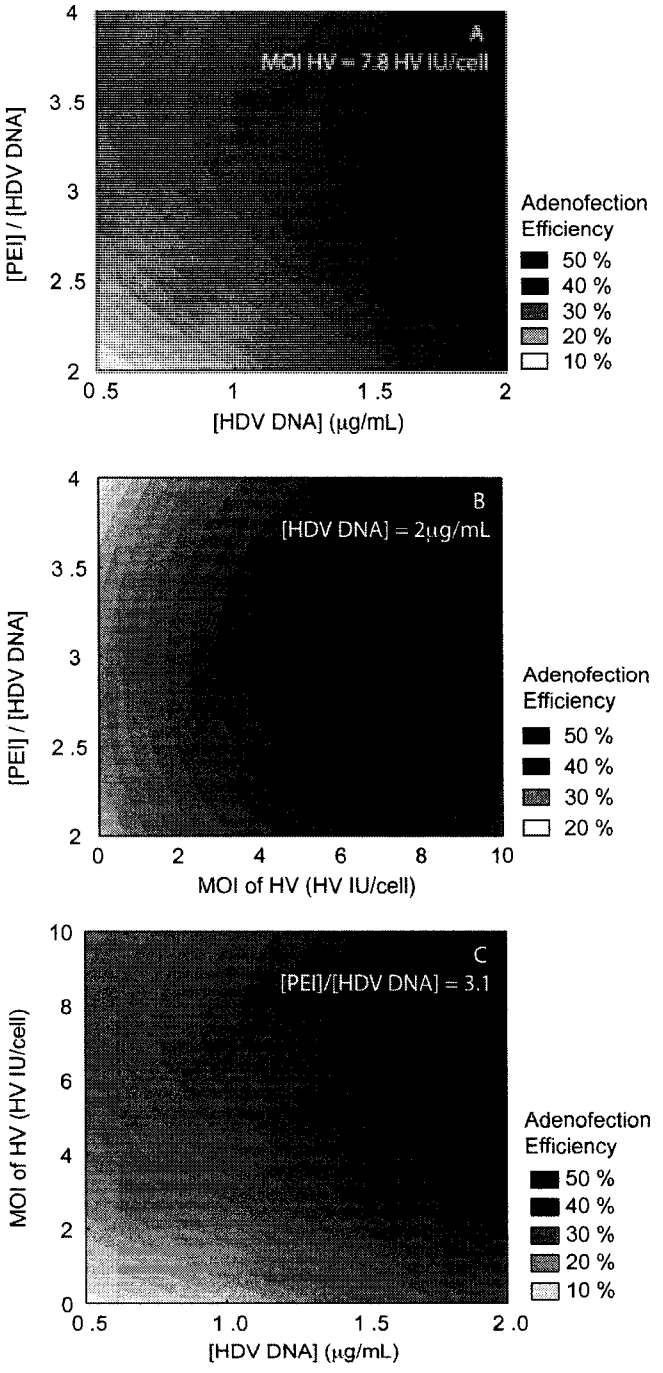

Figure 2.6 Response surfaces of the adenofection efficiency. A, B, C are countour plots as functions of two variables with the third one held constant

The significant interaction between [HDV DNA] and [PEI]/[HDV DNA] calculated in Figure 2.5 is seen partially in Figure 2.6 A. There is a less drastic change in adenofection efficiency when [PEI]/[HDV DNA] was varied at high levels of [HDV DNA] rather than at low levels of [HDV DNA]. In Figure 2.6 B however, the interaction between [PEI]/[HDV DNA] and MOI of HV is completely hidden. In fact, those former interaction terms ([HDV DNA] × [PEI]/[HDV DNA] and [PEI]/[HDV DNA] × MOI of HV), are confounded by the higher impact of [HDV DNA], [PEI]/[HDV DNA], $([PEI]/[HDV\ DNA])^2$, MOI of HV and $(MOI\ of\ HV)^2$ which represent more than 80% of the response variability.

Overall, the number of adenofected cells was the highest at the highest level of [HDV DNA], and with [PEI]/[HDV DNA] and the MOI of HV at levels within the upper range tested. The predicted maximum adenofection efficiency (51%) was found to be formed with complexes having the following composition: [HDV DNA] = 2 μg/mL, [PEI]/[HDV DNA] = 3.1, MOI of HV = 7.8 HV IU/cell.

2.2.4.5 Scale-Up of the HDV Production

In order to evaluate the scalability of the overall process, the optimized adenofection protocol previously established was tested in combination with the viral amplification procedure to produce HDV in a 3 L bioreactor. Using the optimized complexes for adenofection of FLPe cells in shake flasks, the efficiency of adenofection obtained was 37.5 ± 0.6%. For P1 of amplification,

FLPe cells were co-infected with the maximum volume of lysate acceptable, defined as one-tenth of total volume of culture (HDV MOI of <1 HDV IU/cell). For P2 amplification, the bioreactor cell culture should have been co-infected with an optimum MOI of HDV of 5 HDV IU/cell (to be published); however, the corresponding volume of lysate exceeded the defined maximum volume. Consequently, FLPe cells were infected with the maximum volume of viral lysate (280 mL) from P1 corresponding to a HDV MOI of 2 HDV IU/cell. For P1 and P2, the HV was added at the optimal MOI of 0.5 HV IU/cell (to be published). Viral amplification dynamics are displayed in Figure 2.7. Similar trends in the TVP concentration, the HDV titer and the HDV infectivity ratio were observed for both the 3 L bioreactor and shake flask experiments. Characterization of the HDV bioreactor stock is shown in Table 2.4. The final HDV titer in the bioreactor was 1.44×10^{8} HDV IU/mL. A total of 4.02×10^{11} HDV IU of was produced corresponding to 1.60×10^{12} TVP. The HDV infectivity ratio was 26% and the HV contamination ratio was 7.68% in terms of viral genomes or 0.27 % in terms of IU.

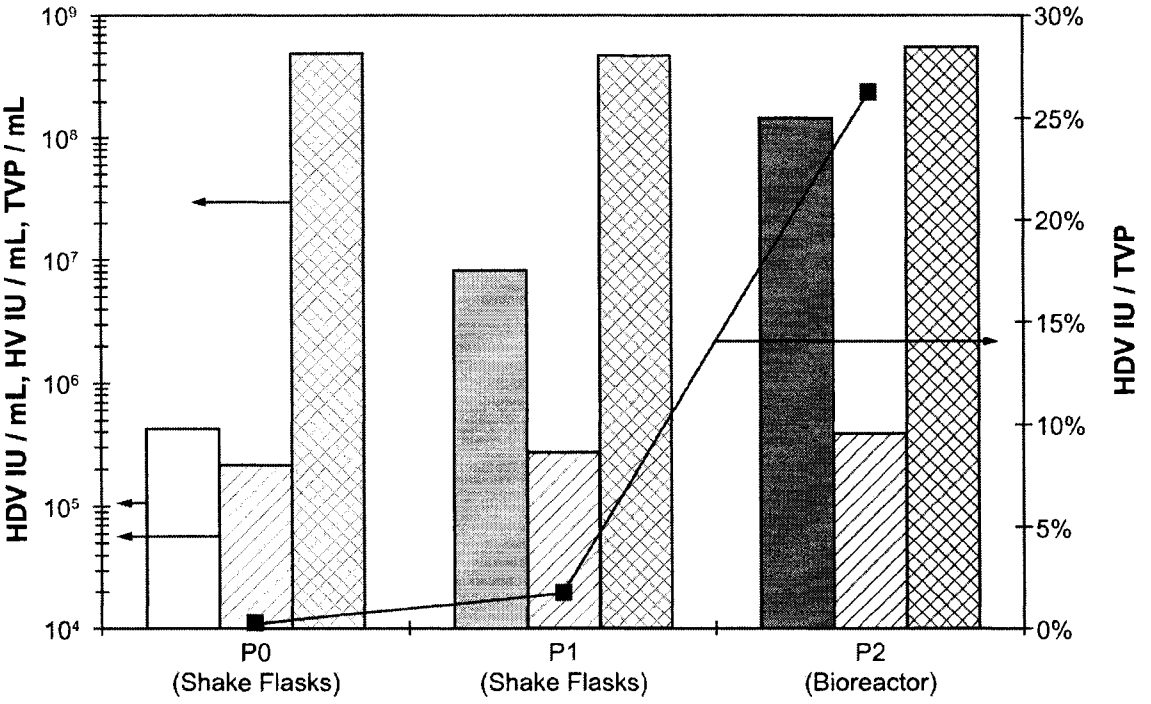

Figure 2.7 Large-scale amplification of the HDV. The adenofection complexes were formed as described in Materials and Methods however using 2 µg of HDV DNA/mL of culture, 6.2 µg of PEI/mL of cell culture and the HV at MOI of 7.8 HV IU/cell. P0 was performed in 50 mL of working volume in shake flasks. P1 was done in 400 mL of working volume in shake flasks. P2 was conducted in 2800 mL of working volume using a 3 L Chemap bioreactor. The viral titers (HDV IU/mL in full bars, HV IU/mL in hatched bars, TVP/mL in squared bars) and the HDV infectivity ratio (black squares) are shown for P0 to P2. The means are represented for duplicate shake flask experiments (n = 2) (P0 and P1) and for a single bioreactor experiment (n = 1) (P2)

Table 2.4 Characterization of the 3 L bioreactor stock (raw lysate)

	HDV	HV	HDV infectivity ratio (%)	HV contamination ratio (%)
IU/mL	1.44×10^8	3.82×10^5	NA	0.27
TVP/mL	5.48×10^8		26	NA
VG/mL	5.47×10^8	4.20×10^7	27	7.68

2.2.5 Discussion

Large-scale production of HDV is necessary to conduct pre-clinical tests in large animal models; however, such tests are restricted by a HDV production process involving an initial rescue step performed in non-scalable culture mode and based largely on empirical amplification protocols. In an attempt to overcome those drawbacks, we developed a PEI-derived transfection method for cells in suspension, which efficiently allows transfection of large DNA constructs and generates HDV.

In preliminary experiments, from a process perspective, the circular HDV DNA was used which required less manipulation after the bacterial plasmid purification. In previous studies, the circular DNA yielded higher *in vivo* and *in vitro* transfection efficiencies (Cherng et al. 1999; von Groll et al. 2006). However, in our case, the production of the HDV was less efficient with the circular DNA. In the best of cases, extensive amplification was required to get a significant HDV titer (data not shown). Eventually this confirmed the fact that the adenoviral particles are more easily produced when the adenoviral DNA harbors

ITRs at DNA extremities, probably providing an easier access for the replication factors (Tamanoi and Stillman 1982; van Bergen et al. 1983). The use of the linearized HDV DNA was therefore a prerequisite for an efficient HDV production. In the transfection/infection protocols, similar effectiveness in the HDV production using either the Lipofectamine 2000- or the PEI-transfection/infection protocols was somewhat surprising considering that the Lipofectamine 2000- outperformed the PEI-transfection efficiencies under standard transfection conditions. Even if the medium was renewed post-transfection, the high cytotoxicity of Lipofectamine 2000 might be detrimental to the formation of progeny viruses. Nevertheless, the adenofection, a proven method to efficiently transfer large DNA constructs into cells such as the 30 kb-HDV DNA (Baker and Cotten 1997; Campeau et al. 2001), outperformed the standard rescue procedures. Consistent with these results and previous works, it is believed that compared to the PEI-transfection, the PEI-adenofection not only enhanced the number of transfected cells (Baker et al. 1997; Campeau et al. 2001; Diebold et al. 1999; Meunier-Durmort et al. 1997) but also improved the HDV DNA nuclear translocation due to a higher endosmolytic activity of complexes (Cotten et al. 1992; Curiel et al. 1991; Yoshimura et al. 1993). This would lead to a better availability of the HDV DNA for further maturation into the HDV particles. Moreover, to produce a new HDV particle, both the HDV DNA and the HV are required in a cell. The adenofection complex is thought to bring simultaneously the HDV DNA and the HV to a same producer cell.

Consequently, the probability of producing more HDV particles is increased and instead of three, only two amplification passages were required to attain 10^8 IU of HDV/mL in adherent cell cultures. In 6-well plates, lower HDV yields were observed in suspension than in adherent cell cultures. Although the exact reason of this limitation remains unknown, cellular stresses induced by the use of 6-well plates under agitation might be responsible for a lower production. This aforementioned limitation was overcome in shake flasks culture as HDV yields were similar to those obtained in 6-well plate adherent cultures. Moreover, the study of the viral amplification suggested that the level of total viral particle production is similar at each passage. Considering the low infectious titers in the initial passages, the majority of the total viral particles produced at P0 and P1 are thought to be defective viral particles. At the maximum HDV yield, the HV contamination ratio in the raw lysate was inferior to 0.1% in terms of IU, which is similar to what has been previously reported using similar assays, indicating the efficiency of the FLPe selection system (Meneses-Acosta et al. 2007; Umana et al. 2001). The decrease of both the total viral particle concentration and the HDV titer obtained at P3 might be a consequence of a suboptimal infection scheme resulting from a volume-based amplification protocol (see discussion below).

While the production benefit of the adenofection was demonstrated in suspension culture, further understanding was sought to get better control of the adenofection protocol. The study of the stoichiometry of the complexes using a

DOE was useful in understanding the relative importance of the complexes components and to optimize the HDV yield.

Variability associated with the HDV amplification through three serial passages did not allow us to accurately correlate the HDV titer at P3 to the stoichiometry of adenofection complexes. Reasoning that only HDV DNA-adenofected cells are capable of producing HDV, the adenofection efficiency would therefore be a good but also an easy-to-get indicator of the HDV production at P0. This hypothesis was confirmed by the correlation between the HDV titer at P3 and the adenofection efficiency above 10%. Below 10% however, the HDV is amplified with great variability as a result of its rescue at low titer.

Previous studies highlighted that adenofection complexes are efficiently delivered into the cells thanks to a combined and also reciprocal enhancement effect of PEI and adenovirus at multi-levels (cell binding, entry, transport, DNA release, transcription) (Baker et al. 1997; Dunphy et al. 1999). Some researchers suggested that the carrier adenovirus binds to the PEI-DNA complexes via the negative charge of hexons and the global positive charge of the condensed DNA (Baker et al. 1997). Other studies demonstrated that the number of adenofected cells and transgene expression were dependent on the composition of complexes (Baker et al. 1997; Campeau et al. 2001; Meunier-Durmort et al. 1997). The titration of HDV DNA, PEI and the HV was done within the appropriate range of values enabling the generation of useful information.

For instance, the statistical analysis underlined the crucial function of the HV in the adenofection process. It further confirmed the role of the adenovirus in the enhancement of the transfection efficiency. A rapid decline in the adenofection efficiency was observed below those values in HDV DNA concentration, PEI/DNA ratio and MOI of HV. Compared to Durocher et al. (2002) who used a similar cell-transfection system, our optimal PEI/DNA ratio is higher. Those remarks are in accordance with Baker et al. (1997) and Campeau et al. (2001). They suggested to adenofect cells with PEI-DNA complexes of higher positive charge (as compared to PEI-DNA transfection complexes) to bind efficiently to the adenovirus. Complexes of higher charge or higher viral load have less effect (as compared to lesser charge or lesser viral load) on the variation of adenofection efficiency or transgene expression but are more cytotoxic. An optimal composition for the complexes was found in a minimal set of experiments and was further used to produce HDV in a 3 L bioreactor.

The experiment conducted in a bioreactor demonstrated the effectiveness and reliability of the developed HDV production process at 3 L scale. Consistent with the use of the optimized adenofection and the co-infection conditions, the HDV was amplified within two passages. The lower adenofection level compared to DOE prediction was still acceptable considering the experiment-to-experiment variability of the PEI-based transfection.

Currently, the HDV amplification uses a volume-based protocol, which is highly empirical and thus, impossible to rely on for scale-up from a bioengineering perspective. Reliable bioprocesses aim to define a set of parameters able to predict the overall performance of production. The MOI is the most characterized parameter describing co-infection protocols. It has been shown to be highly associated to the quality and the yield of bioproducts (Aucoin et al. 2006; Palomares et al. 2002). However, if the MOI has been only partially examined (Hartigan-O'Connor et al. 2002a), it has never been utilized as a predictive parameter during the HDV production starting from the HDV DNA (Palmer and Ng 2003; Sakhuja et al. 2003). In a separate study (to be published), we determined the optimal MOI for HV (0.5 HV IU/cell) and HDV (5 HDV IU/cell). During the HDV amplification, whenever possible, the optimal co-infection conditions were used, i.e, optimal MOI of HV was applied whereas optimal MOI of HDV was never attained. An infection using a volume-based protocol is appropriate for initial amplification passages at the quantity of HDV is limiting. However, as identified by Ng et al (2001), at higher amplification passages, a volume based-protocol would lead to an over-optimal MOI of HDV infection. It results in a waste of the HDV infecting material as well as in a lowered HDV titer.

Compared with other studies, the HDV final titer in the bioreactor is roughly similar; however, an HDV infectivity ratio is 3 to 4 times higher than reported elsewhere (Palmer and Ng 2003) has been achieved. Since total viral particles

mediate acute dose-dependent toxicity (reviewed in Palmer and Ng 2005), the HDV infectivity ratio is highly relevant to assess the quality of vector stock where high ratios are desirable (National Institute of Health 2002). We also observed a good concordance between VG and TVP concentrations, which comforts us in using the two quantifications methods. It appears that the HV contamination ratio in raw lysate is high compared to what has been previously reported (Palmer and Ng 2003; Sakhuja et al. 2003). However the purity of material tested and/or the HV quantification methods employed by others could explain those differences. Semi-purified or purified materials are partially cleared of the HV, therefore underestimating the HV contamination in raw lysates. In addition, major differences in principle and sensitivity of the HDV and the HV assays do not always allow a direct comparison between the HV and the HDV titers and tends to minimize the HV contamination (Mittereder et al. 1996). DNA-based quantification methods are therefore more appropriate to compare the HV to the HDV titer in order to assess the HV contamination (Puntel et al. 2006). Previously, the HDV production has been scaled-up in a 2 L bioreactor; however, this was achieved with extensive amplification of HDV in adherent cell culture (Sakhuja et al. 2003). Palmer and Ng (2003) produced HDV in a 3 L spinner-flask within four amplification passages; however, the process was mostly done in adherent cell cultures as well. Our suspension process permitted the production of a high quality HDV vector stock in a 3 L controlled bioreactor within only two amplification passages, which is both time-saving and restricts

possible viral recombinations (Hartigan-O'Connor et al. 2002a; Sakhuja et al. 2003).

From a manufacturing perspective, this process was completely realized in cell suspension mode from rescue to amplification in low serum-containing medium. Recent developments in large-scale transfection strategies and media optimization allowed operations in commercial serum-free medium. For the first time, the PEI-adenofection was employed in suspension cell culture for production purpose. The PEI-adenofection has been validated with great success for the HDV production, in a simple, time-saving and cost-effective way considering the simultaneous use of PEI and virus compared to a commercial transfection reagent. Moreover, because it was easily performed and optimized in suspension cultures, it might be adaptable to larger volumes considering the PEI-transfection scalability for the production of adeno-associated virus up to 3 L (Durocher et al. 2007). We are presently looking forward to producing the HDV at an even larger bioreactor scale within our facilities where production of the first generation of adenoviral vector has been scaled-up to 100 L (Kamen and Henry 2004). Taken together, these advances will be highly relevant to produce high-quality grade HDV to sustain the therapeutic development of this viral vehicle.

2.2.6 Acknowledgements

E.D. was supported by a graduate student fellowship from the Montreal Center for Experimental Therapeutics in Cancer (MCETC/FRSQ/CIHR Strategic training program in Cancer Experimental Therapeutics) and a prospective researcher fellowship from the Swiss National Science Foundation (PBSK2--115853). The authors gratefully acknowledge Dr. Volker Sandig for the HDV construct; Dr. Pedro Lowenstein for the HV gift; Sylvie Perret and Gilles Saint-Laurent for FLPe cell line development; Alice Bernier, Roseanne Tom, Normand Arcand and Lucie Bourget for expert technical support; Eric Carpentier for pertinent advices and Marc Aucoin for critical review of this manuscript.

2.3 Résultats Complémentaires

2.3.1 Introduction

L'amplification du HDV est restreinte par les faibles quantités de HDV produites aux premiers passages. L'utilisation d'un lysat cellulaire plus concentré en HDV permettrait d'augmenter la quantité de HDV infectant une culture cellulaire et donc d'améliorer l'amplification du HDV. Le nombre de passages d'amplification pour atteindre le titre maximum de HDV devrait ainsi être réduit.

2.3.2 Matériels et Méthodes

L'expérience est réalisée en flacons agités selon les conditions décrites au *Chapitre 2, 2.2.3 Materials and Methods*. Pour l'adénofection, l'ADN du HDV est utilisé sous sa forme circulaire ou linéaire. Le lysat cellulaire est placé tel quel (1×) à –80°C ou concentré préalablement 10 fois (10×) par centrifugation à 300 × g pendant 5 min. Après un cycle de gel/dégel, le lysat cellulaire 1× est ajouté directement à la culture cellulaire tandis que le lysat cellulaire 10× est trituré par pipetage, centrifugé (4,500 × g, 2 min) et seul le surnageant est ajouté. Une évaluation de la perte de HDV entraînée par l'utilisation du lysat cellulaire 10× est menée. La viabilité des cultures cellulaire n'est pas affectée par l'utilisation du lysat 10×. Elle est de 80% (± 5%) en utilisant le lysat 1× (n=10) et 87% (± 5%) en utilisant le lysat 10× (n=10). Le HDV est concentré conformément au facteur de concentration du lysat. Un maximum de perte de 25% est cependant observé lors du relargage des particules virales suite aux étapes de trituration et de culottage des débris cellulaires. Le facteur d'amplification est le ratio entre les UI de HDV produites et les UI de HDV introduites au moment de l'infection

2.3.3 Résultats et Discussion

La Figure 2.8 présente les titres obtenus du P0 au P4 en utilisant l'ADN circulaire (A) ou l'ADN linéaire (B). A la Figure 2.8 A et B, les titres obtenus à partir de l'adénofection avec l'ADN circulaire (A) sont deux ordres de grandeur

en dessous des titres obtenus à partir de l'adénofection avec l'ADN linéaire. Ces résultats sont conformes à ceux présentés à la Table 2.1. Aux premiers passages d'amplification (P0 à P3 Figure 2.8 A, P0 à P1 Figure 2.8 B), les titres pour le lysat 10× sont peu différents de ceux pour le lysat 1×. Pour les passages suivants (P4 Figure 2.8 A, P2 à P3 Figure 2.8 B), l'utilisation d'un lysat concentré contribue même à réduire le titre du HDV. Le titre relativement bas obtenu à partir de l'adénofection avec l'ADN circulaire stagne à ~3 × 10^6 UI de HDV/mL entre le P3 et le P4 avec le lysat 10× (Figure 2.8 A). En utilisant l'adénofection avec l'ADN linéaire, le titre diminue puis augmente à nouveau entre le P2 et le P4 avec les deux lysats (Figure 2.8 B). Le titre maximum est ~1-2 × 10^8 UI de HDV/mL.

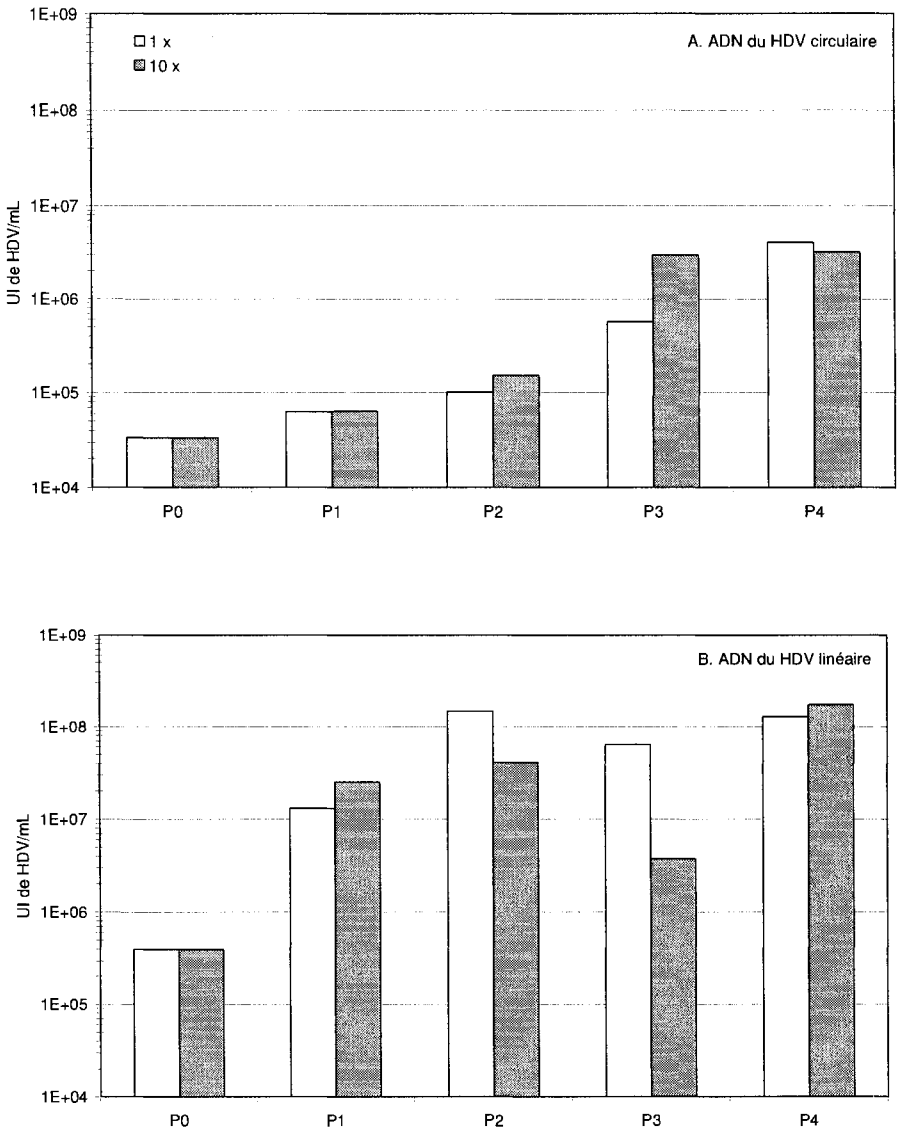

Figure 2.8 Secours et amplification du HDV en culture cellulaire en suspension. L'adenofection a été réalisée avec l'ADN circulaire (A) ou linéaire (B). L'amplification a été réalisée avec le lysat cellulaire 1× (barres blanches) ou 10× (barres grises). Les titres viraux (UI de HDV/mL) sont représentés pour P0 à P4 pour les lysats cellulaire 1×. Les valeurs moyennes sont données pour des duplicata de cultures (n = 2)

Une analyse des conditions d'amplification est montrée à la Figure 2.9. La figure reporte le titre de HDV en fonction de la MOI de HDV calculée à partir du titre du lysat utilisé pour l'infection. Pour l'adénofection avec l'ADN linéaire, les résultats indiquent l'obtention d'un titre maximum pour une MOI de HDV entre 2 et 10. De manière surprenante, les résultats de l'adénofection avec l'ADN circulaire sont différents des résultats de l'adénofection avec l'ADN linéaire. Il est difficile de justifier ces observations. Éventuellement, l'utilisation de l'ADN du HDV sous la forme circulaire génère des recombinants HDV ayant une capacité d'amplification limitée. Cette hypothèse reste cependant à vérifier.

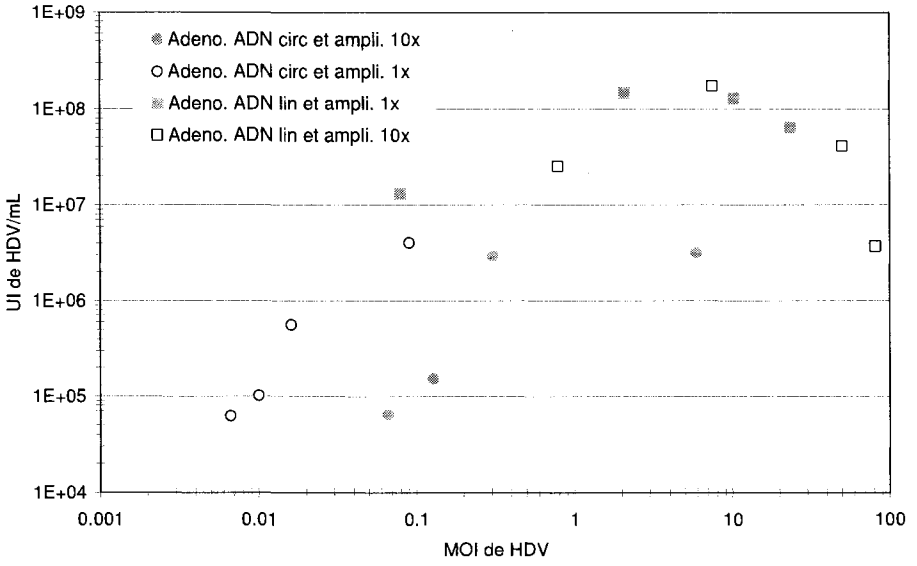

Figure 2.9 Relation entre la MOI de HDV et le titre de HDV

Le Tableau 2.5 reporte le facteur d'amplification obtenu à chaque passage. Ce facteur est une mesure du gain viral considérant la quantité initiale de HDV

apportée à la cellule au moment de l'infection et la quantité finale de HDV produite. L'amplification est maximum au P1 (324.8) pour les conditions d'adénofection avec l'ADN linéaire et d'amplification avec le lysat 1×. Au P2, le facteur d'amplification diminue (117.1) suggérant que le système atteint une limite de production. Le P2 correspond effectivement au titre maximum de HDV. Les valeurs des facteurs d'amplification pour le lysat 10× sont beaucoup plus faibles.

Tableau 2.5 Facteurs d'amplification pour le secours et l'amplification du HDV en culture cellulaire en suspension

		Adénofection ADN circulaire	Adénofection ADN linéaire
Amplification 1x	P0	NA	NA
	P1	18.9	324.8
	P2	20.8	117.1
	P3	70.5	4.3
	P4	91.8	25.5
Amplification 10x	P0	NA	NA
	P1	1.9	63.5
	P2	3.1	1.0
	P3	23.9	2.4
	P4	1.3	67.3

2.3.4 Conclusions

Ces résultats complémentaires confirment les résultats obtenus au Chapitre 2, 2.1 An Efficient and Scalable Process for Helper-Dependent Adenoviral Vector Production using Polyethylenimine-Adenofection, à savoir que l'utilisation de l'ADN circulaire n'est pas efficace pour produire le HDV. Pour des raisons non-élucidées, même les passages d'amplification répétés ne permettent pas d'atteindre des titres de HDV de l'ordre de 1×10^8 UI/mL. D'autre part, les résultats pour l'adénofection avec l'ADN linéaire indiquent que l'utilisation d'un

lysat concentré selon une méthode d'amplification par volume est trop aléatoire pour atteindre plus rapidement le titre maximum de HDV. La fenêtre de MOI conduisant à des titres maxima est relativement étroite. L'utilisation d'un lysat concentré peut mener à infecter la culture cellulaire avec des MOI trop élevées, ce qui diminue le titre de HDV. L'utilisation d'un lysat concentré ne permet pas d'atteindre la capacité d'amplification obtenue avec le lysat 1×. Si, aux premiers passages, la MOI de HDV utilisée se rapproche de la fenêtre de MOI optimale, la MOI du HV peut éventuellement être trop élevée et mener à amplifier faiblement le HDV.

En conclusion, la production de HDV peut être en partie maîtrisée par un ajustement de la MOI du HDV. Une identification des paramètres contrôlant la production de HDV fait l'objet d'une étude plus approfondie dans le chapitre suivant.

2.4 Références

Aucoin MG, Perrier M, Kamen AA. 2006. Production of adeno-associated viral vectors in insect cells using triple infection: optimization of baculovirus concentration ratios. *Biotechnol Bioeng* 95(6):1081-92.

Baker A, Cotten M. 1997. Delivery of bacterial artificial chromosomes into mammalian cells with psoralen-inactivated adenovirus carrier. *Nucleic Acids Res* 25(10):1950-6.

Baker A, Saltik M, Lehrmann H, Killisch I, Mautner V, Lamm G, Christofori G, Cotten M. 1997. Polyethylenimine (PEI) is a simple, inexpensive and effective reagent for condensing and linking plasmid DNA to adenovirus for gene delivery. *Gene Ther* 4(8):773-82.

Branca MA. 2005. Gene therapy: cursed or inching towards credibility? *Nat Biotechnol* 23(5):519-21.

Brunetti-Pierri N, Ng P. 2006. Progress towards the clinical application of helper-dependent adenoviral vectors for liver and lung gene therapy. *Curr Opin Mol Ther* 8(5):446-54.

Campeau P, Chapdelaine P, Seigneurin-Venin S, Massie B, Tremblay JP. 2001. Transfection of large plasmids in primary human myoblasts. *Gene Ther* 8(18):1387-94.

Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M. 1996. Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. *J Virol* 70(7):4805-10.

Cherng JY, Schuurmans-Nieuwenbroek NM, Jiskoot W, Talsma H, Zuidam NJ, Hennink WE, Crommelin DJ. 1999. Effect of DNA topology on the transfection efficiency of poly((2-dimethylamino)ethyl methacrylate)-plasmid complexes. *J Control Release* 60(2-3):343-53.

Côté J, Bourget L, Garnier A, Kamen A. 1997. Study of adenovirus production in serum-free 293SF suspension culture by GFP-expression monitoring. *Biotechnol Prog* 13(6):709-14.

Cotten M, Wagner E, Zatloukal K, Phillips S, Curiel DT, Birnstiel ML. 1992. High-efficiency receptor-mediated delivery of small and large (48 kilobase gene constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles. *Proc Natl Acad Sci U S A* 89(13):6094-8.

Curiel DT, Agarwal S, Wagner E, Cotten M. 1991. Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. *Proc Natl Acad Sci U S A* 88(19):8850-4.

Diebold SS, Lehrmann H, Kursa M, Wagner E, Cotten M, Zenke M. 1999. Efficient gene delivery into human dendritic cells by adenovirus polyethylenimine and mannose polyethylenimine transfection. *Hum Gene Ther* 10(5):775-86.

Dunphy EJ, Redman RA, Herweijer H, Cripe TP. 1999. Reciprocal enhancement of gene transfer by combinatorial adenovirus transduction and plasmid DNA transfection in vitro and in vivo. *Hum Gene Ther* 10(14):2407-17.

Durocher Y, Perret S, Kamen A. 2002. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res* 30(2):E9.

Durocher Y, Pham PL, St-Laurent G, Jacob D, Cass B, Chahal P, Lau CJ, Nalbantoglu J, Kamen A. 2007. Scalable serum-free production of recombinant adeno-associated virus type 2 by transfection of 293 suspension cells. *J Virol Methods*.

Graham FL, Smiley J, Russell WC, Nairn R. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 36(1):59-74.

Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps ML. 1997. Construction of adenovirus vectors through Cre-lox recombination. *J Virol* 71(3):1842-9.

Hartigan-O'Connor D, Barjot C, Crawford R, Chamberlain JS. 2002a. Efficient rescue of gutted adenovirus genomes allows rapid production of concentrated stocks without negative selection. *Hum Gene Ther* 13(4):519-31.

Hartigan-O'Connor D, Barjot C, Salvatori G, Chamberlain JS. 2002b. Generation and growth of gutted adenoviral vectors. *Methods Enzymol* 346:224-46.

Kamen A, Henry O. 2004. Development and optimization of an adenovirus production process. *J Gene Med* 6 Suppl 1:S184-92.

Klyushnichenko V, Bernier A, Kamen A, Harmsen E. 2001. Improved high-performance liquid chromatographic method in the analysis of adenovirus particles. *Journal of Chromatography B. Biomedical Sciences and Applications* 755(1-2):27-36.

Kochanek S. 1999. High-capacity adenoviral vectors for gene transfer and somatic gene therapy. *Hum Gene Ther* 10(15):2451-9.

Kumar-Singh R, Chamberlain JS. 1996. Encapsidated adenovirus minichromosomes allow delivery and expression of a 14 kb dystrophin cDNA to muscle cells. *Hum Mol Genet* 5(7):913-21.

Lieber A, He CY, Kirillova I, Kay MA. 1996. Recombinant adenoviruses with large deletions generated by Cre-mediated excision exhibit different biological properties compared with first-generation vectors in vitro and in vivo. *J Virol* 70(12):8944-60.

Lusky M. 2005. Good manufacturing practice production of adenoviral vectors for clinical trials. *Hum Gene Ther* 16(3):281-91.

Meneses-Acosta A, Dormond E, Jacob D, Tom R, Bernier A, Perret S, St-Laurent G, Durocher Y, Gilbert R, Kamen A. 2007. Development of a suspension serum-free helper-dependent adenovirus production system and assessment of co-infection conditions. *J Virol Methods*.

Meunier-Durmort C, Grimal H, Sachs LM, Demeneix BA, Forest C. 1997. Adenovirus enhancement of polyethylenimine-mediated transfer of regulated genes in differentiated cells. *Gene Ther* 4(8):808-14.

Mittereder N, March KL, Trapnell BC. 1996. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J Virol* 70(11):7498-509.

Montgomery DC. 2005. *Design and analysis of experiments*. In: Wiley, editor. New York. p 431-2.

Morsy MA, Caskey CT. 1999. Expanded-capacity adenoviral vectors--the helper-dependent vectors. *Mol Med Today* 5(1):18-24.

Nadeau I, Kamen A. 2003. Production of adenovirus vector for gene therapy. *Biotechnology Advances* 20(7-8):475-489.

National Institute of Health. 2002. Assessement of adenoviral vectors safety and toxicity: Report of the National Institutes of Health Recombinant DNA Advisory Committee. *Hum Gene Ther* 13(1):1-13.

Ng P, Beauchamp C, Evelegh C, Parks R, Graham FL. 2001. Development of a FLP/frt system for generating helper-dependent adenoviral vectors. *Mol Ther* 3(5 Pt 1):809-15.

Ng P, Parks RJ, Graham FL. 2002. Preparation of helper-dependent adenoviral vectors. *Methods Mol Med* 69:371-88.

O'Reilly DR, Miller LK, Luckow VA. 1994. *Baculovirus Expression Vectors: A Laboratory Manual.* New York: Oxford University Press.

Oka K, Chan L. 2005. Construction and characterization of helper-dependent adenoviral vectors for sustained in vivo gene therapy. *Methods Mol Med* 108:329-50.

Palmer D, Ng P. 2003. Improved system for helper-dependent adenoviral vector production. *Mol Ther* 8(5):846-52.

Palmer DJ, Ng P. 2005. Helper-dependent adenoviral vectors for gene therapy. *Hum Gene Ther* 16(1):1-16.

Palomares LA, Lopez S, Ramirez OT. 2002. Strategies for manipulating the relative concentration of recombinant rotavirus structural proteins during simultaneous production by insect cells. *Biotechnology and Bioengineering* 78(6):635-44.

Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A* 93(24):13565-70.

Peng Z. 2005. Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. *Hum Gene Ther* 16(9):1016-27.

Puntel M, Curtin JF, Zirger JM, Muhammad AK, Xiong W, Liu C, Hu J, Kroeger KM, Czer P, Sciascia S and others. 2006. Quantification of high-capacity helper-dependent adenoviral vector genomes in vitro and in vivo, using quantitative TaqMan real-time polymerase chain reaction. *Hum Gene Ther* 17(5):531-44.

Sakhuja K, Reddy PS, Ganesh S, Cantaniag F, Pattison S, Limbach P, Kayda DB, Kadan MJ, Kaleko M, Connelly S. 2003. Optimization of the generation and propagation of gutless adenoviral vectors. *Hum Gene Ther* 14(3):243-54.

Sandig V, Youil R, Bett AJ, Franlin LL, Oshima M, Maione D, Wang F, Metzker ML, Savino R, Caskey CT. 2000. Optimization of the helper-dependent adenovirus system for production and potency in vivo. *Proc Natl Acad Sci U S A* 97(3):1002-7.

Tamanoi F, Stillman BW. 1982. Function of adenovirus terminal protein in the initiation of DNA replication. *Proc Natl Acad Sci U S A* 79(7):2221-5.

Umana P, Gerdes CA, Stone D, Davis JR, Ward D, Castro MG, Lowenstein PR. 2001. Efficient FLPe recombinase enables scalable production of helper-dependent adenoviral vectors with negligible helper-virus contamination. *Nat Biotechnol* 19(6):582-5.

van Bergen BG, van der Ley PA, van Driel W, van Mansfeld AD, van der Vliet PC. 1983. Replication of origin containing adenovirus DNA fragments that do not carry the terminal protein. *Nucleic Acids Res* 11(7):1975-89.

von Groll A, Levin Y, Barbosa MC, Ravazzolo AP. 2006. Linear DNA low efficiency transfection by liposome can be improved by the use of cationic lipid as charge neutralizer. *Biotechnol Prog* 22(4):1220-4.

Yoshimura K, Rosenfeld MA, Seth P, Crystal RG. 1993. Adenovirus-mediated augmentation of cell transfection with unmodified plasmid vectors. *J Biol Chem* 268(4):2300-3.

CHAPITRE 3: IDENTIFICATION DES PARAMÈTRES CRITIQUES D'INFECTION POUR CONTRÔLER LA PRODUCTION DU VECTEUR ADÉNOVIRAL DÉPENDANT D'UN VIRUS AUXILIAIRE PAR QPCR

3.1 Présentation de l'Article

Dans le processus intégré de production du HDV, l'étape d'amplification fait suite à l'étape de secours. Elle consiste à infecter les cultures avec le HV et un volume défini de lysat cellulaire contenant le HDV obtenu au passage précédent. La co-infection est itérée jusqu'à l'obtention d'un titre maximum de HDV. Ce procédé d'amplification par volume de culture est empirique et conduit à produire le HDV avec un rendement aléatoire.

Ce chapitre se compose de l'article intitulé Identification of Critical Infection Parameters to Control Helper-Dependent Adenoviral Vector Production using QPCR soumis à la revue Biotechnology and Bioengineering. L'article rapporte le développement d'un procédé fiable pour l'étape amplification du HDV. Comme au chapitre précédent, les quantités relatives et totales de HDV et de HV ajoutés au moment de l'infection sont étudiés. Cependant, à l'étape d'amplification par infection, ces quantités sont manipulées par des paramètres d'infection comme la MOI et le ratio entre les vecteurs. Grâce à un outil quantitatif servant à la caractérisation de la production, on observe que la MOI et le ratio entre les vecteurs contrôlent le titre de HDV et le niveau de contamination par le HV.

D'autres paramètres d'infection comme le délai d'infection sont manipulés en vue de favoriser spécifiquement l'amplification du HDV. Les conditions optimales d'infection sont identifiées. De cette étude découle une meilleure compréhension de la formation des deux vecteurs, ce qui permet d'entrevoir une nouvelle approche de production utilisant la lignée cellulaire parentale.

3.2 Identification of Critical Infection Parameters to Control Helper-Dependent Adenoviral Vector Production using QPCR

Dormond E.[1,2], Perrier M.[2], and Kamen A.[1]

[1]Animal Cell Technology Group, Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montréal, Québec, Canada H4P 2R2

[2]Chemical Engineering Department, École Polytechnique de Montréal, Campus de l'Université de Montréal, 2500, chemin de Polytechnique, Montréal, Québec, Canada, H3T 1J4

Keywords : Adenovirus, Helper-dependent adenoviral vector, Helper virus, Multiplicity of Infection, Time of Infection, Contamination

3.2.1 Abstract

Helper-dependent adenoviral vectors (HDVs) are the most promising adenoviral vectors for gene therapy treatments as well as vaccination strategies. However, the lack of a robust and efficient strategy to produce the HDV at high titers constitutes a major obstacle hindering the use of this promising technology at the clinical level. The HDV production requires a double infection of a recombinase-expressing HEK293 cell line with the HDV and a helper virus (HV). To limit lots contamination by HV, encapsidation of HV is prevented by the recombinase action. A real-time PCR assay was developed to accurately characterize the production of this system. Infection strategies to enhance the HDV yield and reduce the contamination by HV were investigated. The multiplicity of infection (MOI) was identified as a critical parameter to simultaneously improve the HDV yield and reduce the contamination by HV. HDV to HV MOI ratio dictated the HDV yield whereas the HV accumulation was controlled by the MOI of HV. Delaying infection with the HV did not improve the HDV yield.

3.2.2 Introduction

In viral vectored gene therapy applications, a major drawback remains the availability of viral vector lots in sufficient quantity and quality to support pre-clinical and clinical trials. Compared to other vectors, the high yield production of

adenoviral vectors (AdV) makes these vectors particularly attractive for use as gene delivery vectors. The third generation AdV referred to as helper-dependent adenoviral vector (HDV) has demonstrated substantial *in vivo* benefits over earliest constructions. Still, the full exploitation of HDV potential suffers from a lack of focused efforts on the development of production processes.

The HDV is a 30 kb vector devoid of the major viral coding sequences. It accommodates up to 37 kb of transgene. Contrary to previous AdV constructions, it displays safer *in vivo* toxicity and immunogenicity profiles (reviewed in Brunetti-Pierri and Ng 2008; Palmer and Ng 2005). For its production in a recombinase-expressing HEK293 cell line, a first generation AdV called helper virus (HV) is required. In this system, the viral genome is supplied by the HDV while the structural proteins and replication elements are supplied by the HV and the HEK293 cell line. The HEK293 cell line expresses constitutively a recombinase (e.g. FLP) which cleaves DNA at the recognition sites (*frt*) flanking the packaging signal of the HV (Alba et al. 2007; Meneses-Acosta et al. 2007; Ng et al. 2001; Umana et al. 2001). Without its packaging signal, the HV genome is theoretically not integrated into a capsid. However, any remaining HV has to be efficiently removed from the final HDV preparation since it is considered as a contaminant.

The HDV production consists of a transfection of the producer cells with the HDV DNA followed by an infection with the HV. Then, serial amplification

passages allow increase of the HDV titer through co-infection of cells with the HDV-containing lysate and the HV. Previous studies have mostly focused on the improvement of the HDV yield and the reduction of the contamination by HV through the design of better viral constructs (Parks et al. 1996; Sandig et al. 2000) and the development of better producer cell lines (Barjot et al. 2002; Hartigan-O'Connor et al. 1999; Zhou et al. 2001). We recently developed an improved protocol referred to as adenofection to significantly reduce the number of amplification steps required to attain a high HDV yield (Dormond et al., to be published). Due to the lack of accurate and routine characterization of HDV production, a volume-based protocol is usually employed to amplify the HDV. Using this approach, the multiplicity of infection (MOI) of HDV, i.e the quantity of infecting units per cell, is expected to rise unpredictably assuming an increase of the HDV titer. At the early amplification steps for which the MOI of HDV is generally below 1 IU/cell, HDV titer increased logarithmically with the passage number. Some authors have proposed to infect cells with a MOI of HDV above 1 IU/cell, thereby ensuring a synchronous infection and a better HDV yield (Hartigan-O'Connor et al. 2002a). At late passages, a drop in the HDV titer suggested that the high MOI of HDV saturates the cellular machinery (Ng et al. 2002a). In a one-degree-of-freedom experiment, we have shown that the HDV production was maximum at a MOI of HDV equal to 5 (Meneses-Acosta et al. 2007).

Infection parameters such as the MOI are commonly used to maximize and reproduce AdV yields (Park et al. 2004; Schoofs et al. 1998). Moreover, in vector producing systems using multiple infections, the ratio of input viruses at the infection time point has been correlated to the yield and the quality of the final product (Aucoin et al. 2006; Meghrous et al. 2005; Palomares et al. 2002). In the HDV system, an incorrect stoichiometry between the two input viruses may also lead to a waste of viral material, a reduced HDV yield and an increased contamination by HV.

However, the establishment of reliable correlations between infection conditions and HDV production characteristics is hampered by the lack of appropriated quantification methods. Indeed, the well-established methods for the characterization of AdV suffer from a number of shortcomings. HPLC methods have been developed for the absolute quantification of the first generation AdV (Klyushnichenko et al. 2001; Shabram et al. 1997; Transfiguracion et al. 2001). However, assessment of concentration by HPLC consists of an overall quantification of vectors without providing indication on contamination level by HV, an important characteristic of the production of HDV. Moreover, the quantification is biased by the calibration curve usually done with a 36 kb wild-type adenovirus whereas the sample contains an unknown mixture of 30 kb HDV and 37 kb HV. Also, the low detection limit of HPLC (~10^8 VP/mL) hinders the accurate determination of the TVP concentration of HDV in samples, which is usually in the range of 10^8-10^9 VP/mL. Due to large differences in sensitivity,

the infectivity assays used to determine the HV and the HDV titers, do not allow an accurate assessment of the contamination by HV. Consequently, the reported contamination by HV underestimates the true contamination (Palmer and Ng 2005). DNA-based assays such as dot blot, Southern blot and qPCR assays have emerged as sensitive and reliable assays to measure the contamination by HV using a same unit of measurement (Kreppel et al. 2002; Palmer and Ng 2004; Puntel et al. 2006). Moreover, the qPCR assay surpasses the hybridization assays by providing an absolute quantification of the HDV and the HV. In Puntel et al. (2006), a qPCR assay based on the TaqMan technology was developed. This procedure is however difficult to exploit routinely, mainly due to its high cost.

Here, a routine duplex real time quantitative PCR assay was developed to accurately evaluate HDV production characteristics. Infection parameters served to fine tune the stoichiometry between components forming the HDV to ultimately increase the HDV yield and reduce the contamination by HV. First, the combined effect of the individual MOIs was examined. Second, a differential time of co-infection was tested. The advantages of a rational co-infection strategy to control the HDV production are presented.

3.2.3 Materials and Methods

3.2.3.1 Cells

The HEK293SF-FLPe cell line is a stable cell line expressing FLPe and has been described previously (Meneses-Acosta et al. 2007). The HEK293SF-FLPe cell line was maintained in HSFM medium supplemented with 10 mM HEPES, 1% BCS and 0.75 µg/mL puromycin at 37°C in a 5% CO_2 humidified incubator. The cells were maintained in suspension at 120 rpm in shake flasks and passaged three times a week at 0.2 x 10^6 cells/mL. The cell density and viability were assessed by haemacytometer counts using the erythrosine dye exclusion method.

3.2.3.2 Viruses

The HDV construct (HCAgfp), a generous gift of Dr. V. Sandig from Probiogen AG Germany, is a 30 kb adenoviral vector similar to the construction reported elsewhere (Sandig et al. 2000). A GFP expression cassette under the control of a CMV promoter is used as a marker. The HV, an E1/E3-deleted adenoviral vector, was kindly provided by Dr. P. Lowenstein (Umana et al. 2001). It carries *frt* sites flanking the packaging signal.

A high-titer stock of the HV was produced by infecting HEK293SF (Côté et al. 1998) cells in a 3 L bioreactor. Briefly, the cells were seeded at 0.25 × 10^6

cells/mL in fresh NSFM13 medium (Nadeau et al. 2002) and infected one day later with HV at a MOI of 5 IU/cell. At 48 hours post-infection (hpi), the cell lysate was collected and centrifuged at 300 × g for 10 min. The cell pellet was diluted in one-twentieth of the original cell culture volume. The HV was released in the supernatant by three freeze/thaw cycles and cell debris pelleting at 4,500 × g for 2 min. Unless stated otherwise, supernatant of HV was used. The HDV stocks were obtained following the so-called adenofection protocol (Dormond et al., to be published). Briefly, the HDV rescue (P0) was performed by adenofecting (2 µg/mL of HDV DNA, 6.2 µg/mL of linear 25 kDa PEI and MOI of HV of 7.8 IU/cell) the HEK293SF-FLPe cells seeded at 0.5×10^6 cells/mL in 25 mL of fresh medium. For the adenofection, CsCl-purified HV was used (one step gradient at 100,000 × g, 4°C for 1.5 h and one linear gradient at 100,000 × g, 4°C for 24 h followed by dialysis against 10 mM Tris-HCl pH 7.9, 1 mM $MgCl_2$). At 48 hpi, 2.5 mL of freezed-thawed P0 lysate was used to co-infect cells with a MOI of HV of 0.5 IU/cell in the same conditions (P1). The harvest was done at 48 hpi. P2 was performed similarly to P1 and was used as the HDV stock. The HDV stocks were generated in duplicated flasks from P0 to P2 (HDV stock 1 and HDV stock 2).

3.2.3.3 Infection Experiments

The HEK293SF-FLPe cells were amplified in a 2 L shake flask at 500 mL working volume. The cells were resuspended in 25 mL of fresh medium and distributed in 125 mL shake flasks at 0.5×10^6 cells/mL.

For conciseness, MOI of HDV and MOI of HV will be further referred to without units. In the MOI experiments, co-infection was carried out at MOI of HDV and MOI of HV of 0.1, 0.5, 1, 2, 5, 10. A total of 36 infected cell cultures were processed at the same time. The harvest was done at 48 hpi. Samples were aliquoted and frozen at –80°C for subsequent analysis. The experiment was repeated a second time with the HDV stock 2, obtained under similar conditions (n=2).

In the ΔTOI experiments, duplicated cell cultures were infected with HDV at a MOI of 5. The HV infection was carried out at 0, 1.5, 3, 4.5 and 6 hpi at a MOI of 0.5. The control consists of HV uninfected cell cultures. Samples were taken daily until 96 hpi with respect to the HV infection, processed immediately or frozen at –80°C for further analysis. The co-infection experiment (ΔTOI = 0 hpi) was repeated twice with the HDV stock 1 and 2 (n=4). The delayed infection experiment was done once (n=2).

3.2.3.4 Quantification Methods

GFP Positive Cells and Total GFP

Cells were resuspended in 2% *p*-formaldehyde in PBS. After 1 h of fixation at 4°C, at least 10,000 events were analyzed by flow cytometry using the Coulter EPICS^{TM} XL-MCL cytometer and EXPO32 software to determine the percentage of GFP-positive cells and the mean fluorescence. Total GFP was assessed by multiplying the number of GPF-positive cells with the mean fluorescence.

Infective HDV by GTA

The HDV IU were quantified by GFP gene transfer assay (GTA) on target cells in suspension culture. The HEK293E cells were seeded at 0.5×10^6 cells/mL in 12 well plates with HSFM medium, 10 mM HEPES, 1% BCS, 50 µg/mL G-418 (Durocher et al. 2002). Dilution of unknown (100 µL) was applied on the cells. At 24 hpi, cells were counted and resuspended in 2% *p*-formaldehyde in PBS and analyzed by flow cytometry as described in the previous section. A minimum of two dilutions showing 3 to 30% GFP-positive cells were taken into account for the titer calculation as previously described (Côté et al. 1997).

Infective HV by CPE-EPDA

The HV IU were quantified by a cytopathic effect (CPE) detection following end-point dilution assay (EPDA) on infected target cells. The HEK293A cells were seeded at 0.03×10^6 cells/mL in 96-well plates with DMEM+, 2 mM L-glutamine, 1 mM sodium pyruvate and 10% FBS. One day later, each column received a logarithmic dilution of unknown. A non-infected column was used as a negative control. The positive wells were scored 14 days post-infection and the HV titer was estimated according to the calculation by Reed and Muench (O'Reilly et al. 1994).

Total Viral Particles by HPLC

The total viral particle (TVP) concentrations of the HDV and the HV stocks were determined by an anion-exchange high performance liquid chromatography method coupled to UV detection spectroscopy (Klyushnichenko et al. 2001). The lysates stored at –80 °C were thawed and centrifuged at 4,500 × g for 2 min. The supernatants were filtered through a 0.45 µm GHP-membrane syringe filter before injection. When necessary, the TVP concentrations were corrected for the difference in vector size between the HDV (30 kb) and the ATCC wild-type adenovirus reference material (36 kb) used to make the HPLC standard curve (Ng et al. 2002b).

Viral Genomes by qPCR

The cell lysates were thawed at 37°C and centrifuged for 2 min at 4500 × g to remove the cell debris. The supernatants (200 µL) were treated with 1 µL of Dnase I 57 U/µL(Invitrogen, Grand Island, NY) in Dnase I buffer for 30 min at 37°C. The samples were mixed with 5 µL of 0.5 M EDTA and Dnase I was inactivated at 65°C for 30 min. The viral genomes were extracted using the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Laval, QC, Canada) following the manufacturer's instructions.

To assess the HDV and the HV viral genome concentrations, a standard plasmid for quantification was constructed. The HV genome was obtained by the Hirt extraction protocol. Briefly, the CsCl-purified HV was incubated in a 0.5% SDS solution containing 0.5 µg/µL of proteinase K (Roche Diagnostics, Laval, QC, Canada) for 1 h at 37°C. The extraction was performed using the phenol/chloroform and chloroform procedures. The HDV and HV elongated qPCR products were amplified by standard PCR using the pHCAgfp and the extracted HV genome as templates. Restriction site sequences were added to the 3'-end of each primer sequence (HDV forward: AAAGTTTAAACGCCCAGGTAGTAAATGTCTC containing PmeI sequence, HDV reverse: containing EcoR I sequence, HV forward: AAAAAGCTTGGCCTACCCTGCTAACTTCC containing Hind III sequence, HV reverse AAAGCGGCCGCAGGTACACGGTTTCGATGAC containing Not I

sequence). The PCR cycling conditions were 94°C for 5 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 40 s; 72°C for 7 min and final temperature 40°C. Each product was gel purified, digested, desalted and ligated sequentially into pTT vector (Durocher et al. 2002). The resulting standard plasmid pTT3-HDV-HV (6,718 bps) was amplified in *E.coli* and purified by Qiagen Maxiprep (Qiagen, Mississauga, ON, Canada). The standard stock was quantified by UV spectroscopy Plasmid preparations with absorbance ratios 260 nm/280 nm greater than 1.8 were considered pure and accepted for further studies.

A description of the duplex real-time qPCR in terms of reaction conditions, quantification and reaction specificity is shown in Table 3.1.

For design of primers, the stuffer region of the HDV and the hexon region were chosen to target the HDV and the HV, respectively. Specific primers for both the HDV and the HV were designed with the help of Clone Manager Software v.7 (Sci-Ed Software, Cary, NC) to ensure a high specificity for the HDV and the HV, respectively in a same qPCR run. The primer sequences and the qPCR product position and length are reported in Table 3.1. The qPCR was done using the Light Cycler instrument (Roche diagnostics, Manheim, Germany). The amplification conditions described in Table 3.1 were performed in a total volume of 20 µL containing 18 µL of PCR Mix (12 µL H_20 PCR grade, 1.6 µl $MgCl_2$ 25 mM, 1.2 µL forward primer 5 µM, 1.2 µL reverse primer 5 µM, 2 µL 10 x Master

Mix Light-Cycler Fast-Start SYBR Green I (Roche diagnostics, Laval, QC, Canada) and 2 µL of template (standard or samples)).

The HDV and the HV were quantified based on the standard curves generated at each qPCR run with duplicated logarithmic dilutions of pTT3-HDV-HV as described in Table 3.1. The quantitative analysis of data, the efficiency of the qPCR run (slope of the standard curve) and the goodness-of-fit of the standard curve (R^2 value) were assessed using the Light Cycler software (Table 3.1). The specificity of reaction was ensured by analyzing the melting curve and the agarose gel of qPCR product (Table 3.1). Results are expressed in HDV viral genomes, in HV viral genomes (VG/mL) or in total viral genomes (TVG/mL) (addition of HDV and HV viral genome concentrations).

Table 3.1 Description of the duplex real-time qPCR in terms of reaction conditions, quantification and specificity of reaction

			HDV	HV
qPCR conditions	Primer sequence (5'-3') Forward		AGCTCACAGGCTGTAGTTTG	CCGCAGTTGACAGCATTACC
	Primer sequence (5'-3') Reverse		GGATCACTTGCACGGTTTAG	CGGACCACGTCAAAGACTTC
	Product position and size		2551 to 2570 – 224 bps	21402 to 21422 – 220 bps
	Amplification (40 cycles)	Denaturation	95°C for 10 s	
		Annealing	61°C for 7 s	
		Elongation, quantification	72°C for 10 s, single fluorescence acquisition at end	
Quantification	Standard curve	Range	10^8 to 10^3 molecules/2 µL (1:10 dilutions)	10^7 to 10^2 molecules/2 µL (1:10 dilutions)
		Slope	-3.6 < Slope < 3.1	
		R^2	$R^2 > 0.99$	
Reaction specificity	Melting curve	Condition	70°C for 10 s, 70 °C to 95°C at 0.10°C/s with continuous fluorescence acquisition	
		Primer dimers [a]	no	no
		Non-specific products [b]	no	no
		Melting of products	87.2°C < T < 87.5°C	
	Agarose gel	Primer dimers [c]	no	no
		Non-specific products [d]	no	no
		Bands	Single band at ~ 200 bps	Single band at ~ 200 bps

Notes: [a] Primer dimers are usually produced when a fluorescence shift is observed at T<T of product on the melting curve [b] Non-specific products are usually produced when a fluorescence shift is observed at T>T product on the melting curve. [c] Bands for primer dimers are usually observed above the product band (>200 bps) on the agarose gel. [d] Bands for non-specific products are usually observed below the product band (<200 bps) on the agarose gel

In the text, "titer" refers to the infectious concentration in IU/mL (for both the HDV and the HV) while the term "concentration" refers to the sum of infectious and non-infectious concentration in VG/mL or in TVP/mL. The concentration in TVP/mL and the HV titer in IU/mL are only used to characterize the viral stocks.

Unless otherwise stated, the infectivity and the contamination by HV are ratios of HDV IU to TVG and HV VG to HDV VG, respectively.

3.2.4 Results

3.2.4.1 Viral Stocks Characterization

For the HDV stock 1 and 2, the TVG concentrations determined by qPCR are in accordance with the TVP concentrations determined by HPLC (Table 3.2). Also, the HDV stock 1 had similar HDV and HV VG concentrations compared to HDV stock 2. Noticeably, qPCR provided information on both the HDV and HV concentrations whereas HPLC gave an overall concentration value taking into account the two vectors. Based on the VG concentrations, the contamination by HV for the HDV stocks were ~3-5% (Table 3.2). In comparison, the contamination by HV in terms of IU showed much lower values ranging from 0.03% to 0.08% and was subject to a greater variability. The latter observation was due to the two-fold difference noted in the HV titers when comparing the HDV stocks, whereas the HV concentrations in VG were almost similar for both stocks. Importantly, the contamination by HV in term of IU was determined by two distinct infectivity assays, whereas the contamination by HV in terms of VG was assessed using the same assay (see Material and Methods section).

Table 3.2 Characteristics of viral stocks used in infection experiments

	Units	HDV stock 1 [a]	HDV stock 2 [a]	HV stock
Titers	IU of HDV/mL	1.08×10^8	1.09×10^8	NA
	IU of HV/mL	8.65×10^4	3.71×10^4	2.31×10^9
Concentration by q-PCR	VG of HDV/mL	4.18×10^8	3.10×10^8	NA
	VG of HV/mL	1.28×10^7	1.46×10^7	1.31×10^{11}
	TVG/mL	4.31×10^8	3.25×10^8	NA
Concentration by HPLC	TVP/mL	3.51×10^8	3.45×10^8	1.36×10^{11}
Infectivity	UI / VG	(HDV) 25%	(HDV) 34%	1.76%
	UI / TVP	(HDV) 31%	(HDV) 32%	1.70%
HV contamination	IU of HV/IU of HDV	0.08%	0.03%	NA
	VG of HV/VG of HDV	3.06%	4.71%	NA

Notes : [a] HDV stock 1 and 2 are stocks generated in duplicated flasks as described in Materials and Methods. NA : non applicable

The HDV yield of the two HDV stocks showed a similar high HDV titer ~108 IU/mL (Table 3. 2). A good infectivity ratio of ~30% also characterized the stocks. Because of the low contamination by HV, the infection of the HEK293SF-FLPe cell line with the HDV stocks was not contributing to the overall MOI of HV. Therefore, in the infection experiments, the MOI of HV was estimated based on the addition of the HV stock only.

3.2.4.2 MOI Experiment

HDV

Figure 3.1 A and B presents the HDV titers relatively to the MOI of HDV and the MOI ratios, respectively. The HDV titers are distributed over a ~3 log range, from 3×10^5 (MOI of HDV 0.1 & MOI of HV 10) to 2×10^8 UI/mL (MOI of HDV 5 & MOI of HV 0.5) as shown in Figure 3.1 A. Therefore, a substantial improvement could be obtained by simply adjusting the MOI of the two viruses. Also, for a specific MOI of HDV, the HDV titer was dependent on the MOI of HV. In the range of the MOIs evaluated, the maximum HDV titer was systematically obtained for a MOI of HV equal to 0.5, independently of the MOI HDV. When the MOI of HV was increased, the MOI of HDV also had to be increased to maintain or increase the HDV titer. This interdependence is more obvious in Figure 3.1 B where HDV titer is plotted against the MOI ratios. The HV titers for a MOI of HV equal to 0.1 were systematically lower when compared to the titers for HV at a MOI of 0.5. We hypothesized that under this condition, a limitation of cell infection with the HV does not sustain the production of HDV. For a MOI ratio of HDV over HV between 0.01 and 10, the larger the ratio was, the higher the HDV titer was. At high MOI of HDV and low MOI of HV (ratios ~10-100), a decrease in HDV titers was noted. Above the limiting infection conditions for the HV (MOI>0.1) and below a MOI of HDV equal to 10, we observed a good correlation between the HDV titers and MOIs ratios.

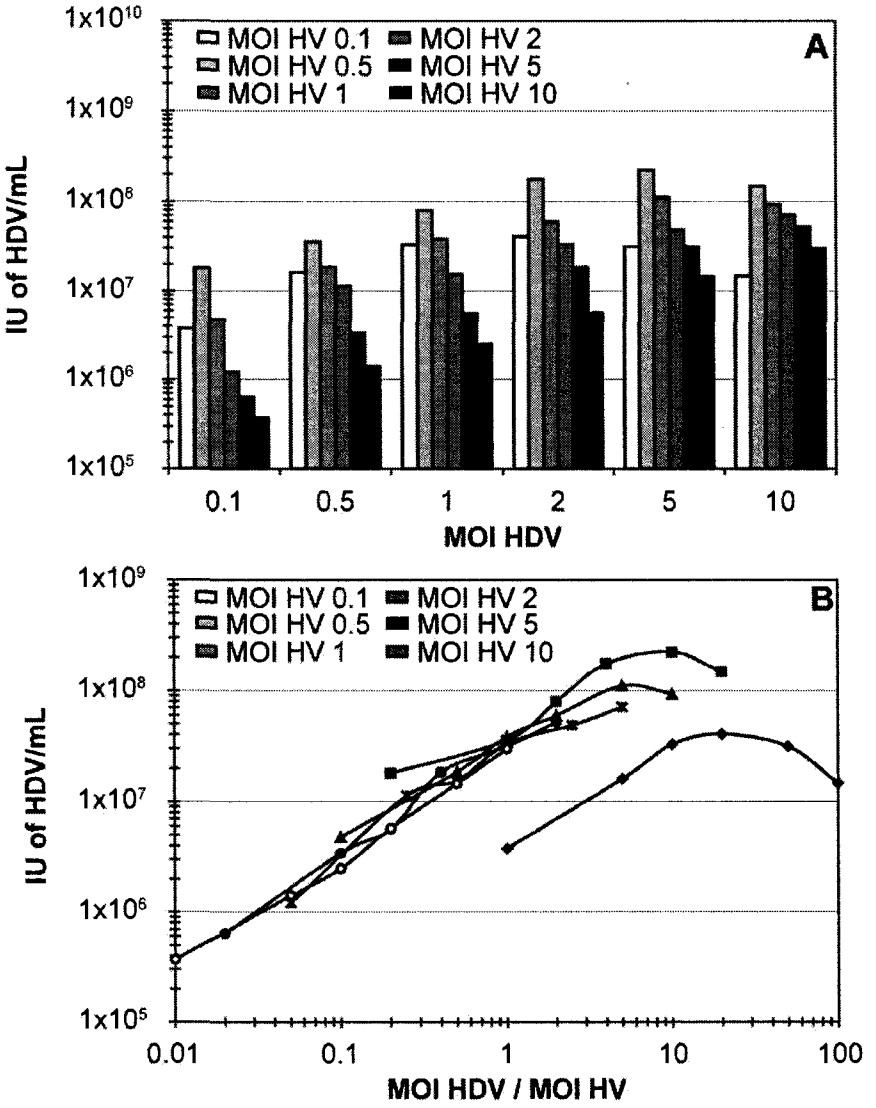

Figure 3.1 HDV titer in IU of HDV/mL at 48 hpi. (A) HDV titer at various MOI of HDV and MOI of HV. (B) Correlation between HDV titer and MOI ratios

The concentration of HDV versus the MOI of HDV and the MOI ratios are presented in Figure 3.2 A and B, respectively. The HDV concentrations were found to be proportional to the HDV titers, i.e no obvious dependence on the MOIs value was noted (data not shown). Therefore, as expected, the results in

term of HDV concentrations (Figure 3.2 A and B) led to the same observations as those previously made for the HDV titers (Figure 3.1 A and 3.1 B).

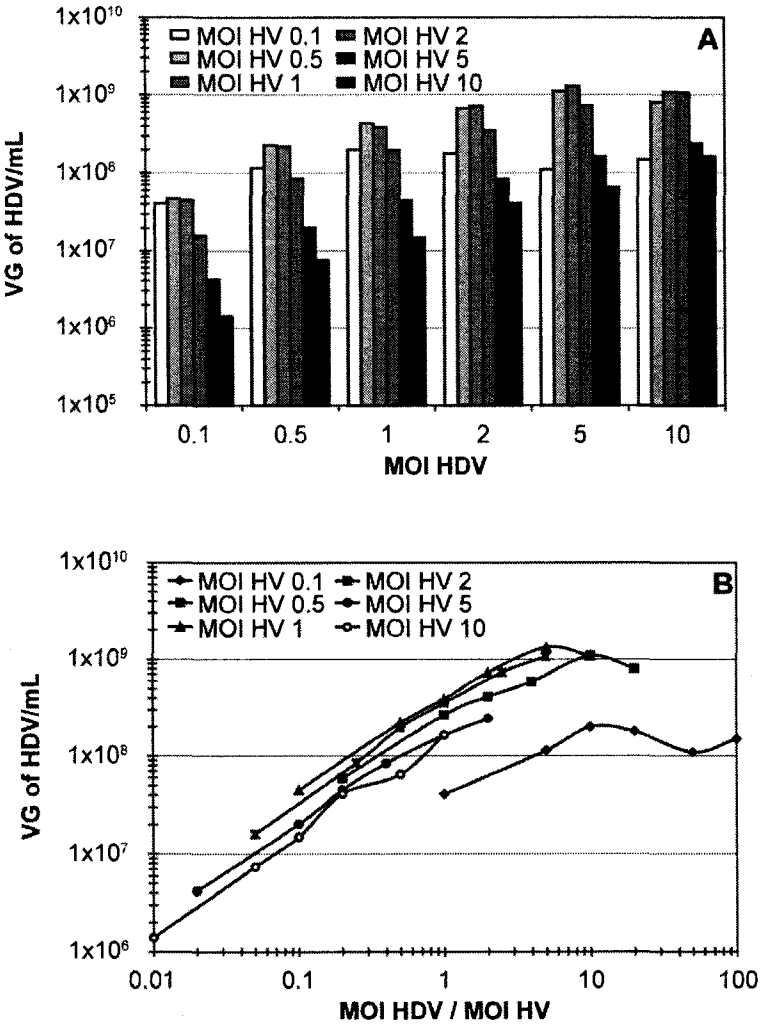

Figure 3.2 HDV concentration in VG of HDV/mL at 48 hpi. (A) HDV concentration at various MOI of HDV and HV. (B) Correlation between HDV concentration and MOI ratios

HV

The HV concentration ranged over ~2 log from 4×10^6 to 1×10^8 VG/mL as shown in Figure 3.3 A. In contrast to the titer and concentration of HDV previously shown in Figures 3.1 B and 3.2 B, Figure 3.3 B highlights the independence of the HV concentration from the MOI ratios. However, a broader VG concentration distribution was noticeable for an MOI of HV equal to 0.1. Figure 3.3 C shows the dependence of the HV concentration from the sole parameter MOI of HV. Again, at a MOI of HV equal to 0.1, the HV concentration varied with the MOI of HDV, i.e the lower the MOI of HDV, the higher the HV concentration.

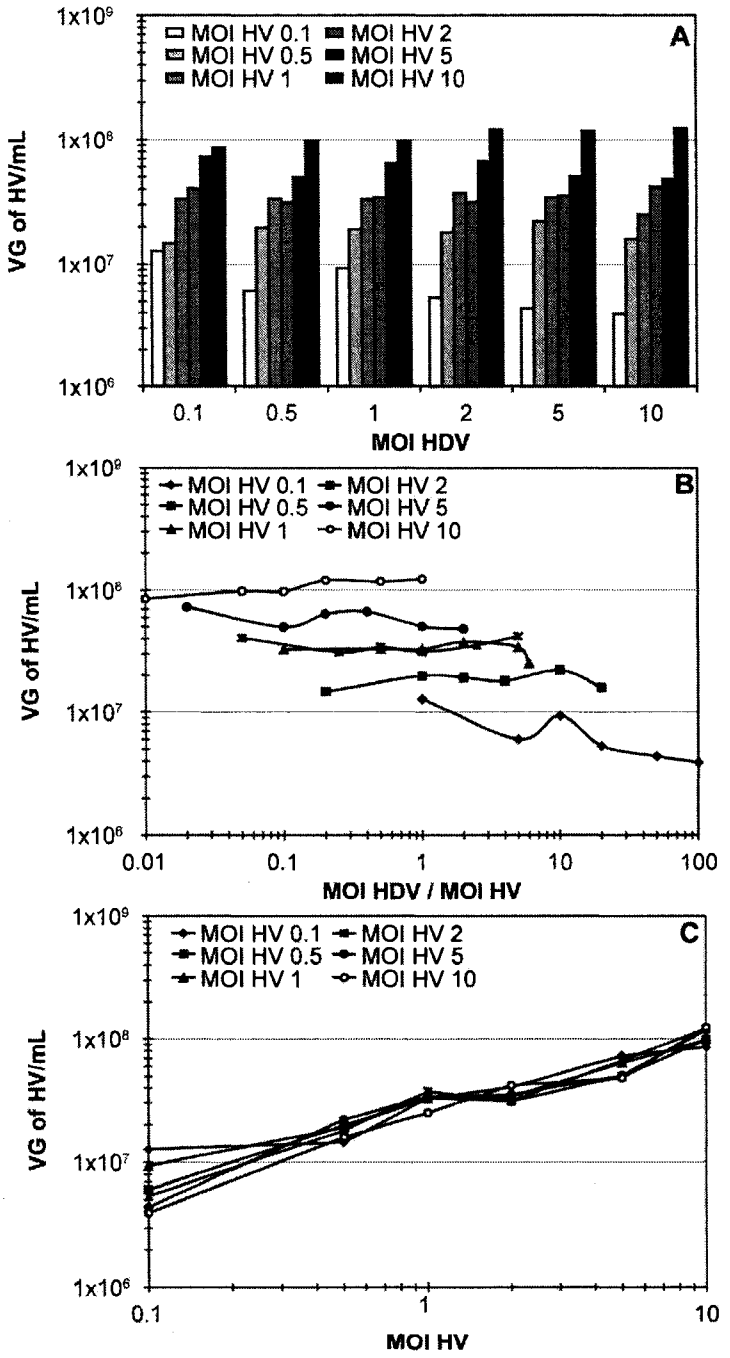

Figure 3.3 HV concentration in VG of HV/mL at 48 hpi. (A) HV concentration at various MOI of HDV and HV. (B) Correlation between HV concentration and MOI ratios. (C) Correlation between HV concentration and MOI of HV

Contamination by HV

Considering that the contamination by HV represents HV concentration over HDV concentration, and that the former concentration depends on the MOI of HV whereas the latter concentration depends on the MOIs ratio, the contamination by HV is a complex function of second order term. The contamination by HV is reported in Figure 3.4. The level varied between ~2% and 6000% (Figure 3.4 A). The contamination is dependent on the MOI of HV: at low MOI of HV, the level and contamination ranges are smaller (4 to 40%) than at high MOI of HV (68 to 6244%) (Figure 3.4 A). The MOI of HDV had an inverse effect on the contamination: the level and contamination ranges at low MOI of HDV were higher (21 to 6244%) than at high MOI of HDV (3 to 68%) (Figure 3.4 A). Summarizing, at high MOI ratios, the contamination was low and at low MOI ratios, the contamination was high (Figure 3.4 B). Moreover, for the highest HDV yield (MOI of HDV 5, MOI of HV 0.5, 2×10^8 IU/mL), the contamination was the lowest. For the lowest HDV yield (MOI of HDV 0.1, MOI of HV 10), the contamination was dramatically high.

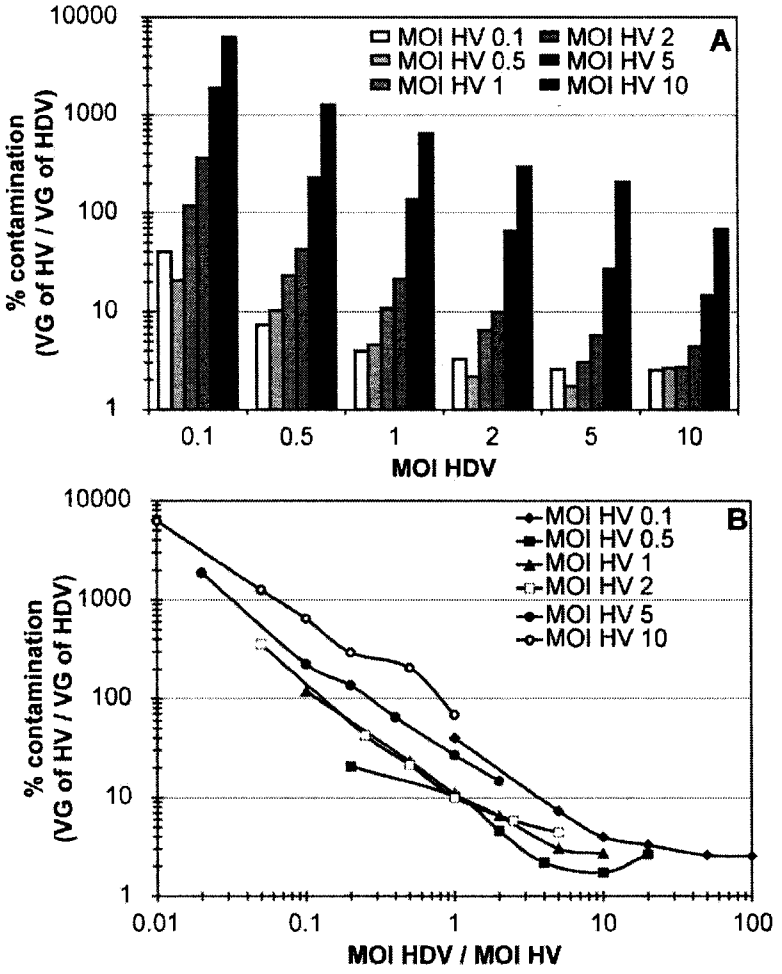

Figure 3.4 Contamination by HV expressed in term of the ratio between VG of HV and VG of HDV at 48 hpi. (A) Contamination by HV at various MOI of HDV and HV. (B) Correlation between contamination by HV and MOI ratios

3.2.4.3 ΔTOI Experiment

Co-Infection Kinetics

In Figure 3.5 A and B, ~1 log increase was observed for the HDV titer and concentration between 24 and 48 hpi. At the standard harvest time (48 hpi) (Meneses-Acosta et al. 2007) the HDV yield was 8×10^8 VG/mL (910 VG/cell) and 3×10^8 IU/mL (380 IU/cell). Between 48 and 96 hpi, HDV concentrations and titers were constant (Figure 3.5 A). Comparatively, the HV concentration was doubling from 2.3×10^7 VG/mL (23 VG/cell) at 24 hpi to 5.3×10^7 VG/mL (54 VG/cell) at 48 hpi (Figure 3.5 A). Constant volumetric and specific HV concentrations were noted after 48 hpi.

The kinetics of HDV infectivity, contamination by HV and cell viability are shown in Figure 3.5 B. The level of contamination by HV decreased rapidly between 24 and 48 hpi from 55 to 3% as a consequence of sudden rise in HDV concentration and slow rise in the HV concentration. Thereafter, the level of contamination remained ~3-6% over time. The HDV infectivity was reduced between 24 hpi (50%) to 96 hpi (25%). Also the cell viability declined between 24 to 96 hpi to reach ~25%.

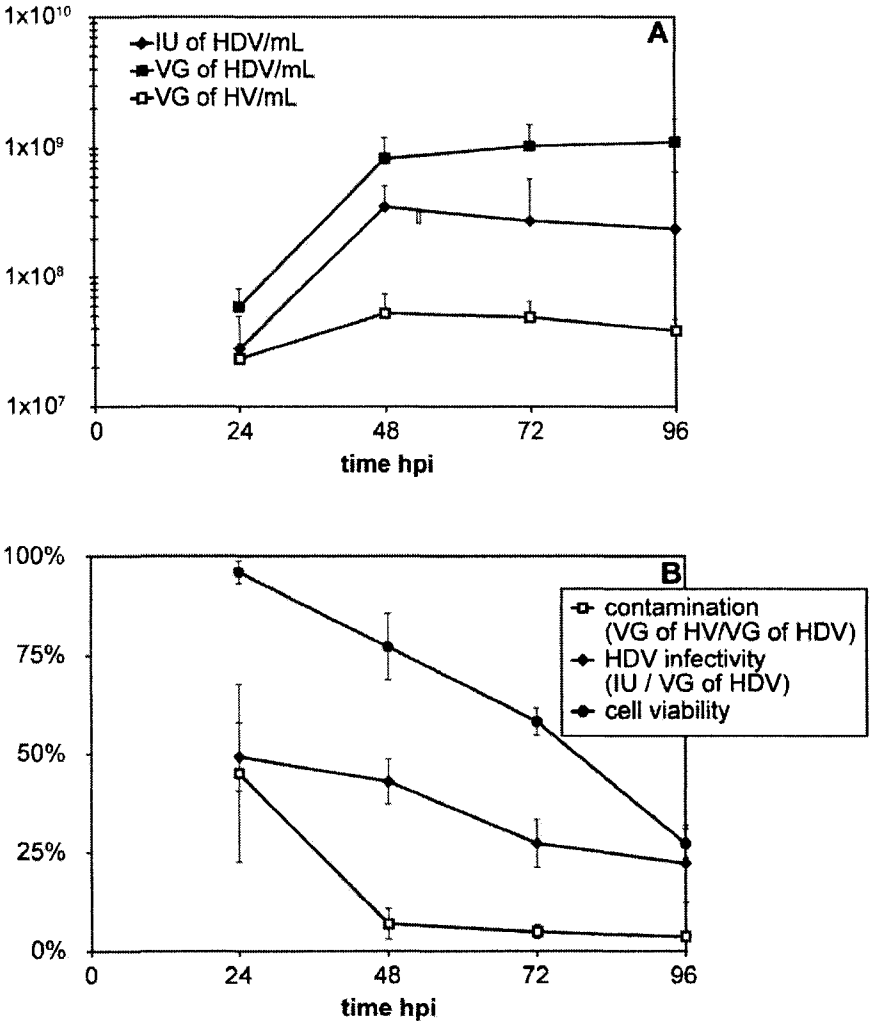

Figure 3.5 Viral kinetics of HDV and HV from 24 to 96 hpi. (A) HDV and HV amplification kinetics through titer and concentrations. (B) Cell viability, HDV infectivity and HV contamination evolution over time

Delay in Infection

The efficiency of the HDV infection measured by the relative level of GFP was assessed from the delayed infection time point (Figure 3.6 A). At 24 hpi, a

downshift in the level of GFP expression was noted with the differential time of infection ranging from 1.5 to 6 h. After 24 hpi, the level of GFP expression was above the level of the co-infected control. The cells infected with only HDV represented 25% of the GFP-positive co-infected control. Also the level of GFP expression represented only 12% of the control. However, at the time of maximum yield, i.e 48 hpi, the HDV titer was not enhanced by applying an infection delay (Figure 3.6 B).

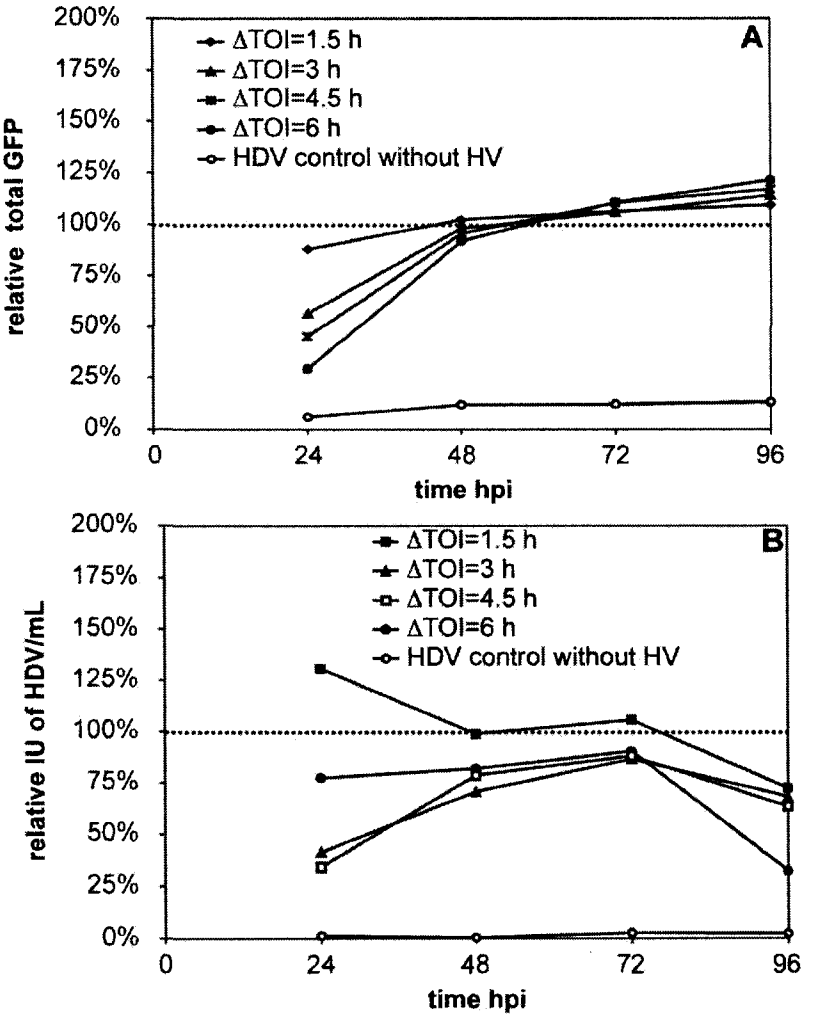

Figure 3.6 Effect of delaying HV infection from 0 to 6 hpi on kinetics from 24 to 96 hpi for (A) Relative total GFP expression. (B). Relative HDV titer

3.2.5 Discussion

Currently, HDV production by infection is highly empirical and thus, impossible to translate into a robust process for large-scale production following basic bioengineering principles. To overcome these drawbacks, we evaluated a

strategy of co-infection based on the MOI, a widely approved parameter to control infection processes. The MOI-strategy provides several important improvements to the technology of HDV production that should help to take plain advantage of the system in terms of HDV yield and contamination by HV.

We first evaluated various assays to accurately characterize the HDV production. For low HV contaminated samples, a good correlation between TVP and TVG values has been obtained by correcting the TVP concentration for the difference in DNA size. The difference observed in the HV titers of the HDV stocks is attributable to the high variability of the CPE (Mittereder et al. 1996). Moreover, it is known that titers assessed by CPE are below titers determined by GTA assays (Mittereder et al. 1996). However, to assess accurately the contamination by HV as well as the HDV and the HV concentrations allowing a reliable comparison, these assays are not suitable. To remediate this shortcoming, a highly specific duplex qPCR assay has been developed. Using highly specific primer design, we implemented an affordable SYBR Green I qPCR technique suitable for routine analysis. As expected, the contamination by HV determined by qPCR showed less variability between the HDV stocks and was also higher than the contamination determined by infectivity assays. Taken together the HDV infectivity assay and the SYBR Green I qPCR assay constitute a set of appropriate assays for the routine characterization of the HDV production in terms of quantity (HDV titer and concentration, HV concentration) and quality (contamination by HV, HDV infectivity). Therefore, their use was

further extended to evaluate the results of the infection experiments. To produce HDV, the use of stocks in a lysate form has been shown to be as efficient as the use of HDV purified material. Not only is the preparation of lysates less time-consuming, but also results in less waste of precious material (Meneses-Acosta et al. 2007). Moreover, the high HDV titers, the high infectivity and the low HV contamination of the HDV stocks ensure that the lysate will not affect the cell culture during production by substantial addition of cell-toxic material.

The stoichiometry of the viral constituents, i.e the viral genome and the viral proteins, is a defined property of an adenoviral particle (Schenk 2001). In the case of production of HDV, the HDV components are provided by two viruses harboring distinct growth properties dictated by their respective constructions. Also, the HDV functions are complemented by the HV and the cell system. Consequently, the production of HDV consists of a highly complex cascade of events involving concomitant participation of the HDV, the HV and the cell system through complex kinetics. Previously, Park et al. (2004) and Schoofs et al. (1998) did not report any differences for the AdV yields of first generation when a MOI between 1 and 125 and between 5 and 100 was used, respectively. Here, the HDV yield has shown to be dependent on both the MOI of HDV and the MOI of HV on a small range of values. At the single cell level, both the HDV and the HV have to be present to form a new HDV particle. During early amplification steps for which the MOI of HDV<1, HDV produced at a too low level cannot be supplied to every individual cells. However, the HV which

provides all the necessary transcriptional elements (except E1 function) for the HDV production, is required in cells that already contain the HDV. Therefore, the synchronous infection of the cells with the HV is a prerequisite for efficient HDV production. This requirement is easy to meet considering that the HV quantity is not a limited factor compared to the HDV quantity. The synchronous infection of cells with the HV is thought to occur at a MOI of 0.5, considering that the maximum HDV titer and concentration was always obtained using this condition regardless of the MOI of HDV. The use of a higher MOI of HV was detrimental to the HDV yield and generated a higher HV concentration. For non-limiting HV infection conditions, the HV titer has been shown to be strictly dependent on the MOI of HV. This observation is not surprising considering that the HDV does not contribute to the formation of HV particles. However, we observed that under limiting HV infection conditions, the MOI of HDV had a direct impact on the HV titer. In Ng et al. (2002a), Sato et al. (2002), Ng et al. (2001) and Sandig et al. (2000), the authors have suggested that the difference in yields observed for the first generation AdV and the HDV resulted from a competition for replication and packaging factors between the two vectors. This competition largely favors the HV propagation because of its genome structure (Sandig et al. 2000). Here, at a low MOI of HV, the impact of HDV competition can be observed. Based on this observation, we believe that the relative amounts of input viruses balance the competition between HDV and HV for their respective amplification, even if this competition is mainly driven by the genome structures. At the optimal MOI of

HV, infecting with HDV at MOI>5 corresponded to a small decrease in the HDV yield. This result has also been observed by Ng et al. (2001) and Meneses-Acosta et al. (2007). In the former study, a one-log decrease in the HDV titer was observed when MOI of HDV~100-200 were used in a volume-based protocol of infection. In the latter study, a small decrease in HDV titer was seen above a MOI of 5. Optimizing the MOI ratio to improve the HDV titer probably enhanced the relative amplification efficiency of the HDV by balancing the competitiveness between the two vectors. Finally, in non-limiting conditions for HV, we have shown that the HDV yield was optimized at a MOI ratio of 10. Comparatively, MOI ratio of 0.25 have shown to generate higher HDV yields than MOI ratio of 1 (Barjot et al. 2002). Major differences in the HV construction through the deletion of the E2B region could probably explain the lower MOI ratios compared to our optimal MOI ratio. The contamination by HV is highly dependent on the co-infection conditions. To our knowledge, this is the first article reporting that co-infection conditions are determinant of the contamination by HV. Ng et al. (2002a) pointed out the importance of the recombinase level in the efficiency of HV removal. Also, the regulation of recombinase expression has been shown to improve the HDV yield (Hartigan-O'Connor et al. 2002a). Interestingly, the higher HDV yield has been correlated to the lower contamination by HV. A MOI strategy of co-infection to control production by limiting the number of amplification passages is an approach that appears quite relevant, considering that less amplification passages diminishes the process

duration (Dormond et al., to be published) and the possibility of vector rearrangement in the system (Hardy et al. 1997; Hartigan-O'Connor et al. 2002a; Sandig et al. 2000). At the rescue and the first amplification steps, the strategy of co-infection should focus on increasing the HDV yield by infecting the cell culture synchronously at the lowest MOI of HV. When the HDV titer is sufficiently high to perform a synchronous co-infection of cells, the HDV and the HV input should be controlled to avoid over-infection of cells with HDV. By co-infecting with the optimal MOI, a high HDV yield and a low HV contamination are ensured while the waste of input viral material is limited.

Usually during the amplification steps, HDV titer increases 10 to 100-fold per passage whereas during the amplification of the first generation AdV, titer increases more than 100-fold. We observed that the maximum rate of HDV propagation occurred between 24 to 48 hpi with more than a 10-fold increase in the HDV titer. Generally, the propagation rate of the first generation AdV peaks between 0 and 24 hpi with a 100-fold increase in titer. A 2- to 6-fold increase in titer is usually observed between 24 and 48 hpi (Altaras et al. 2005; Kamen and Henry 2004). The major differences in amplification efficiency mentioned previously are also time dependent. Downstream processing constraints should be taken into account when choosing the harvesting period (Altaras et al. 2005). Therefore, a good harvest time is based on a compromise between both high HDV titers and infectivity, good cell viability and low contamination by HV. At 48 hpi, a maximum HDV titer with a minimal HV contamination was obtained. Cell

viability of 77% is high enough to recover the viral material in the cell fraction. Because the HDV has been shown to induce an *in vivo* toxicity associated to the capsid proteins (reviewed in Palmer and Ng 2005), a highly infective stock is desirable to limit the amount of non-active that remain toxic. The infectivity ratio at 48 hpi is sufficiently high that it will not compromise HDV infectivity. The HDV replication was previously identified to limit the HDV yield (Hartigan-O'Connor et al. 2002a; Sandig et al. 2000; Sato et al. 2002). Moreover, we think that the HDV amplification is delayed compared to the first generation AdV. The hypothesis behind the delay in the HV infection experiments was that HDV replication could be enhanced by providing the cellular machinery earlier with the HDV genome. The HDV pre-infection does not allow the onset of DNA replication which is known to start with expression of the E2 region (reviewed in Liu et al. 2003), the latter being provided by the HV. Indeed, the HDV pre-infection was carried out to prepare the HDV genome for replication. Therefore, a small time frame for the second infection was chosen (0 to 6 hpi) so as to limit compromising the cascade of viral events. Although the delay in the HV infection resulted in an increase of relative reporter gene expression, enhancement of the HDV yield was not achieved. Consequently, the optimal time for HV infection consists of infecting simultaneously the cells with the HDV and the HV. The co-infection situation is closer to the wild-type AdV infection: the viral promoters are still wild-type and the viral cascade of events are probably best orchestrated when components are brought at the same time if not in the same construction.

A reliable bioprocess for HDV production was established by the identification of critical parameters allowing prediction of the overall production performances. Because of the ease for implementation in a scalable production environment, infection strategies involving the MOI and delay of infection were evaluated for the manipulation of stoichiometry of HDV components. For the first time, this article relates the concomitant importance of the MOI of HDV and HV for production of HDV lots of high quality. These findings support and define a rational infection strategy based on minimizing the amount of viral stock employed for infection, enhancing the HDV yield and limiting the contamination by HV. These results are relevant for the development of a robust large-scale process to support the evaluation of the HDV for the delivery of therapeutic genes in clinical protocols.

3.2.6 Acknowledgements

The authors thank the Swiss National Funds for providing E.D with a prospective research fellowship (PBSK2—115853). Dr. V. Sandig and Dr. P. Lowenstein are gratefully acknowledged for the HDV and the HV constructs. The authors thank also Dr. Y. Durocher, Alice Bernier, Gilles St-Laurent and Lucie Bourget for pertinent advices. Julie Shareck is acknowledged for editorial review.

3.3 Résultats Complémentaires

3.3.1 Introduction

Dans une cellule où la présence du HDV et du HV est requise pour produire de nouvelles particules de HDV, les deux vecteurs sont en compétition pour leur réplication et leur encapsidation respective. L'efficacité d'amplification du HDV est faible comparée à l'efficacité d'amplification de l'AdV de première génération. Une stratégie d'infection différée pour le HV a été précédemment testée dans l'idée d'avantager l'amplification du HDV par rapport à celle du HV. Cette stratégie n'a cependant pas permis d'améliorer le titre de HDV à 48 hpi.

La réponse cellulaire au choc thermique est un phénomène physiologique bien documenté. Elle se caractérise par l'augmentation et la relocalisation des protéines de choc thermique (heat shock protein, hsp) dans la cellule hôte suite à l'infection. Cette réponse a été observée pour de nombreux vecteurs viraux comme l'AdV, le virus herpes simplex, le cytomégalovirus, le virus de la poliomyélite et le virus de la rubéole (Burch and Weller 2005; Chromy et al. 2003; Glotzer et al. 2000; Haviv et al. 2001; Kao and Nevins 1983; Lopez et al. 2006; Phillips et al. 1991; Santomenna and Colberg-Poley 1990; Vasconcelos et al. 1998). Glotzer et al. (2000) ont montré que la réponse cellulaire au choc thermique était essentielle pour la réplication de l'AdV CELO. Un AdV CELO incapable d'augmenter et de relocaliser certaines hsp retrouvait partiellement

ses fonctions réplicatives si son hôte cellulaire subissait un choc thermique. Lopez et al. (2006) ont montré qu'un choc thermique facilitait la réplication du rotavirus dans les cellules de rein de jeune hamster (Baby Hamster Kidney, BHK). Plus spécifiquement pour l'AdV de type 5, certains auteurs ont découvert que le produit du gène E1A induit la réponse cellulaire au choc thermique (Kao and Nevins 1983; Nevins 1982). De plus, les niveaux d'expression des produits de E1A sont reliés aux niveaux d'expression des hsp (Imperiale et al. 1984).

L'hypothèse de travail est qu'un choc thermique augmente la réponse de choc thermique ce qui améliore l'amplification de l'AdV de type 5 par une augmentation de la réplication virale. L'objectif est de favoriser spécifiquement l'amplification du HDV par une stratégie qui consiste à appliquer un choc thermique à la culture cellulaire avant d'infecter celle-ci en apposant un délai entre l'infection par le HDV et le HV.

3.3.2 Matériels et Méthodes

Les cellules, milieu et conditions de culture sont identiques à celles décrites précédement au *Chapitre 2, 2.2.3 Materials and Methods*. L'expérience de délai d'infection est celle décrite au *Chapitre 3, 3.2.3 Materials and Methods*. L'expérience de choc thermique et délai d'infection est présentée au Tableau 3.3. Après inoculation, les flacons agités sont placés pendant 30 min dans l'incubateur humidifié à 37°C, 5% CO_2. Le choc thermique s'effectue pendant 1

h : les flacons, placés sur une plaque rotative, sont submergés au-dessus du niveau liquide de la culture dans un bain-marie préchauffé à 41°C ou 45°C pour assurer efficacement le transfert de chaleur. Les bouchons des flacons sont maintenus fermés pour éviter l'échange gazeux. Les flacons contrôles sont maintenus bouchons fermés dans l'incubateur à 37°C. Les flacons sont ensuite replacés dans l'incubateur à 37°C pendant 30 min avant de procéder à l'infection. Une partie des flacons est co-infectée avec le HDV et le HV à une MOI de 5 et 0.5, respectivement. L'autre partie des flacons est infectée successivement avec le HDV puis avec le HV 3 h après, selon les mêmes MOI. L'échantillonnage des flacons a lieu à 24, 48, 72 et 96 hpi, relativement au moment de l'infection par le HV. Les comptes cellulaires, les analyses au cytomètre en flux (GFP total) et la détermination des titres infectieux à 48 hpi sont effectués tel que décrit précédemment au *Chapitre 2, 2.2.3 Materials and Methods.*

Tableau 3.3 Description des conditions expérimentales pour le choc thermique et l'infection

Température de choc thermique	Délai d'infection entre HDV et le HV (ΔTOI)	Nombre de flacons (n)
37°C	0 h	4
	3 h	4
41°C	0 h	2
	3 h	2
45°C	0 h	2
	3 h	2

3.3.3 Résultats et Discussion

La Figure 3.7 indique les densités et les viabilités cellulaires en fonction du temps. Les cultures ayant uniquement subit un délai entre l'infection du HDV et du HV voient leur densité cellulaire augmenter après infection et ce, d'autant plus lorsque le délai d'infection est grand (Figure 3.7). L'infection du HV induit l'arrêt du métabolisme cellulaire par l'expression de la région E2 (revue dans Liu et al. 2003). L'application d'un délai retarde donc l'arrêt du métabolisme cellulaire et permet à la culture de continuer à croître après l'infection par le HDV. Les viabilités cellulaires sont cependant relativement comparables dans le temps.

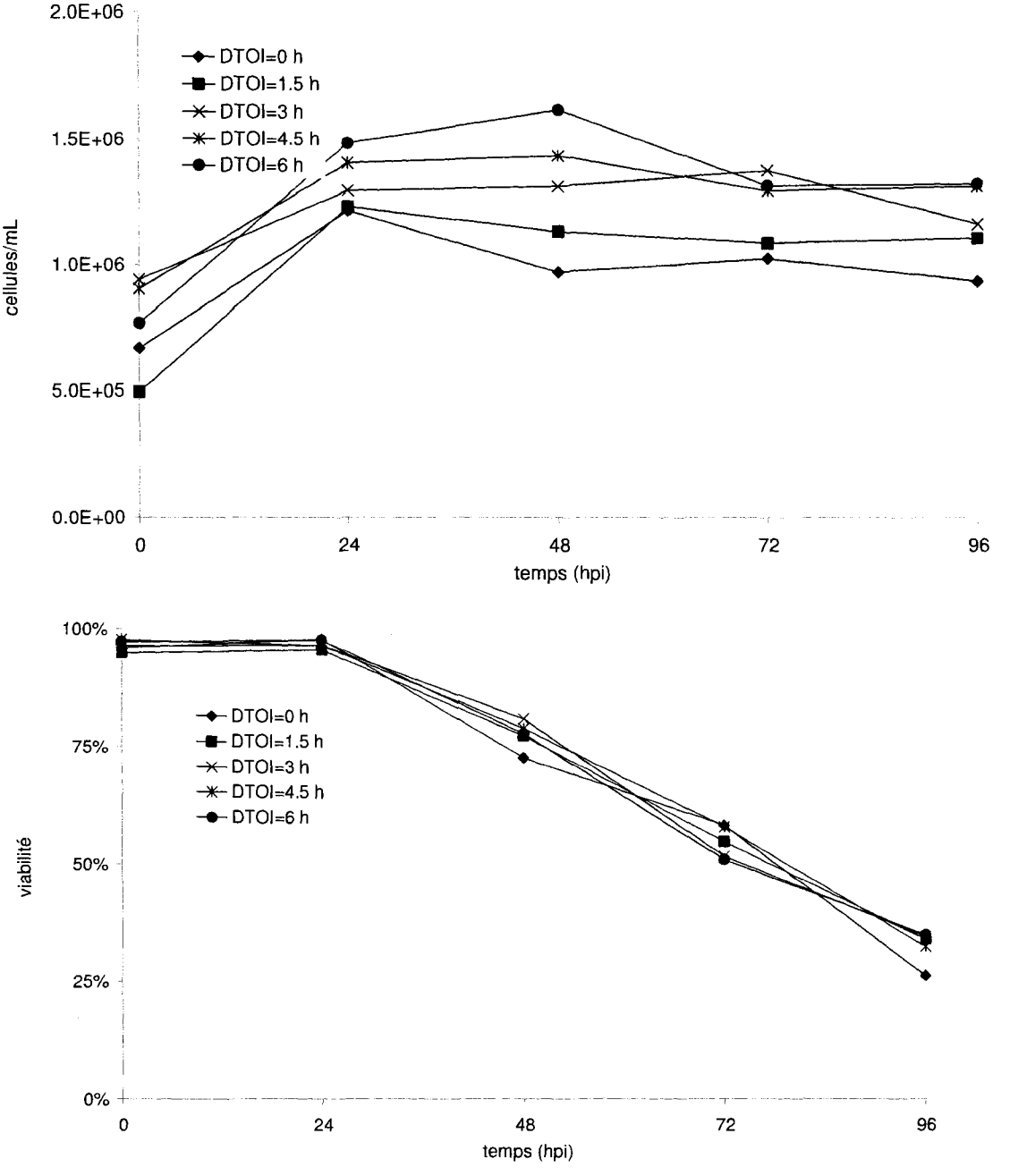

Figure 3.7 Cinétique de viabilité et de densité cellulaire. Les cultures sont maintenues à 37°C et l'infection du HV a lieu avec ou sans délai (ΔTOI = 0 h, 1.5 h, 3 h, 4.5 h, 6h) (n = 2)

Afin de limiter le nombre de flacons, un délai de 3 h est choisit dans l'expérience de choc thermique et correspond au délai moyen utilisé au *Chapitre 3, 3.2 Identification of critical infection parameters to control helper-dependent adenoviral vector production using qPCR.* Pour les cultures ayant subit un choc thermique à 41°C et ayant été infectées par le HV avec un délai de 3 h (Figure 3.8), la densité cellulaire est plus élevée que pour le traitement sans délai d'infection. Cependant, le traitement thermique à 45°C est néfaste pour les cultures: d'une part la densité cellulaire dans le temps est égale ou inférieure à la densité cellulaire d'inoculation et d'autre part, à 24 hpi la viabilité cellulaire est basse comparée au traitement thermique à 41°C. A partir de 48 hpi, les viabilités cellulaires sont relativement comparables indiquant la capacité de récupération de la cellule après ce choc thermique.

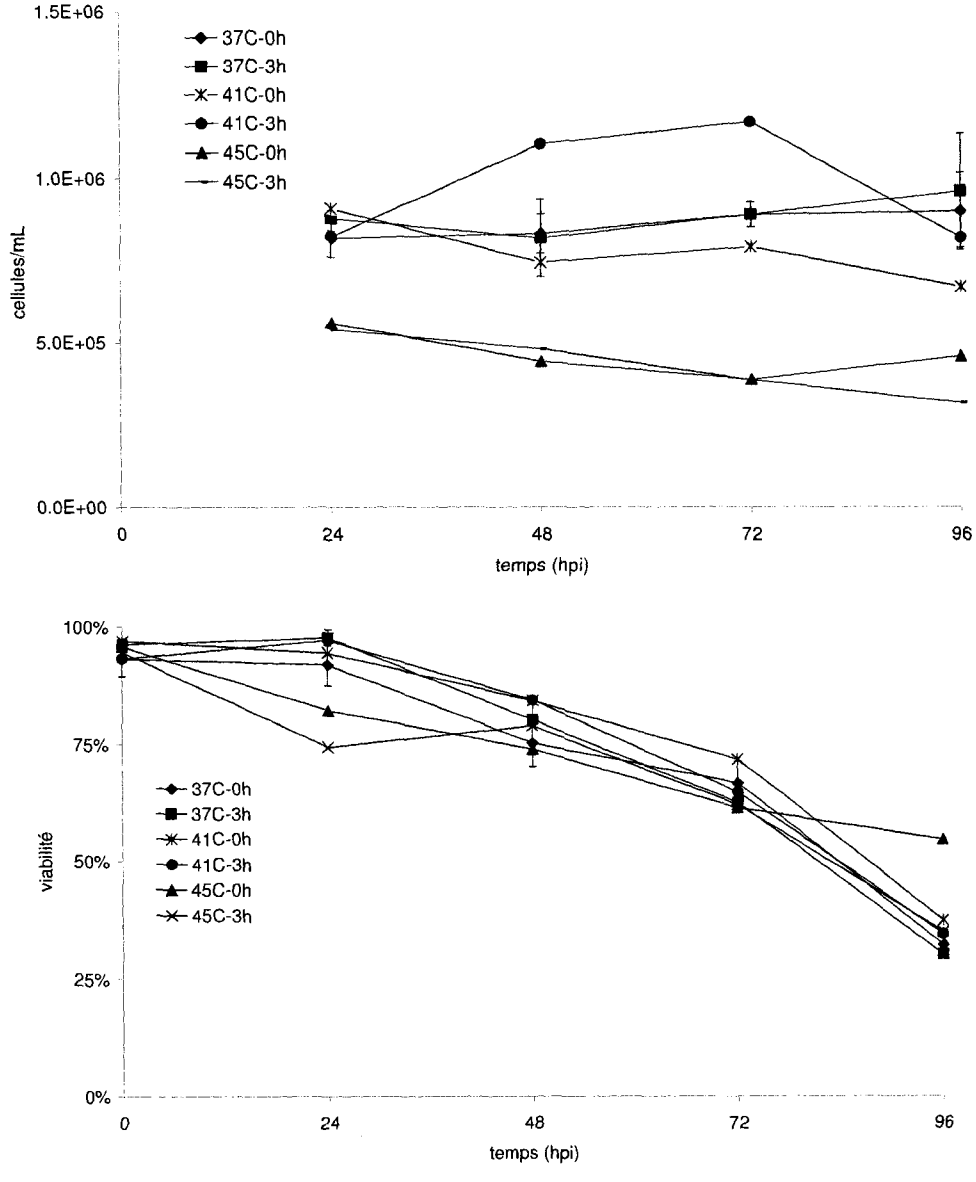

Figure 3.8 Cinétique de viabilité et de densité cellulaire. Les cultures ont subit un choc thermique (41 °C et 45 °C) avec ou sans délai d'infection du HV (ΔTOI = 0 h ou 3 h) (n = 2). Les cultures contrôles sont maintenues à 37 °C et l'infection du HV a lieu avec ou sans délai (0 h ou 3 h) (n = 4)

L'expression de la GFP n'est certes pas corrélée à la production de HDV. Elle est cependant reportée afin d'obtenir d'avantage d'information (Figure 3.9). Les niveaux d'expression sont similaires à 48, 72 et 96 hpi pour les cultures traitées à 41°C et les cultures contrôles. Les niveaux d'expression sont bas pour les cultures traitées à 45°C, suggérant à nouveau l'effet néfaste d'un traitement thermique à cette température sur la culture cellulaire.

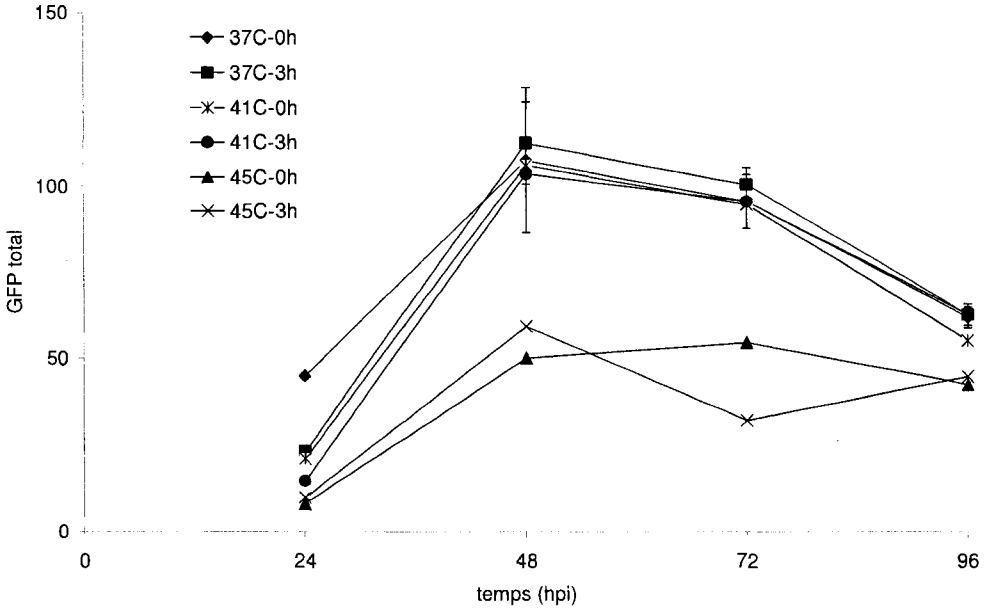

Figure 3.9 Cinétique d'expression de la GFP. Les cultures ont subit un choc thermique (41 °C et 45°C) avec ou sans délai d'infection du HV (ΔTOI = 0 h ou 3 h) (n = 2). Les cultures contrôles sont maintenues à 37°C et l'infection du HV a lieu avec ou sans délai (ΔTOI = 0 h ou 3 h) (n = 4)

L'analyse de la production volumétrique et spécifique indique que le traitement thermique couplé au délai d'infection ne permet pas d'améliorer l'amplification

du HDV à 48 hpi (Figure 3.10). Les résultats concernant le délai d'infection corroborent les résultats obtenus antécédemment au *Chapitre 3, 3.2 Identification of Critical Infection Parameters to Control Helper-Dependent Adenoviral Vector Production using QPCR*. Ceux-ci montraient que l'application d'un délai d'infection n'améliore pas l'amplification du HDV. De même, l'amplification du HDV ne peut être améliorée par l'application d'un choc thermique. Probablement, la réponse de choc thermique induite par la région E1A présente dans le génome de la cellule HEK293 n'est pas un facteur limitant pour la réplication virale. Eventuellement, l'amplification du HDV dans des cellules non-permissives caractérisées par l'absence de la région E1 (exemple : lignée cellulaire A549) pourrait être efficace si le choc thermique permet d'induire l'expression des gènes viraux précoces. Cette avenue, qui présenterait l'avantage d'éviter la génération de RCA reste cependant à évaluer.

Les analyses des expériences (analyse de l'efficacité de réplication virale par qPCR, analyse des niveaux d'expression des hsp40 et hsp70) n'a pas été réalisée puisque l'objectif à atteindre n'a pas été rencontré.

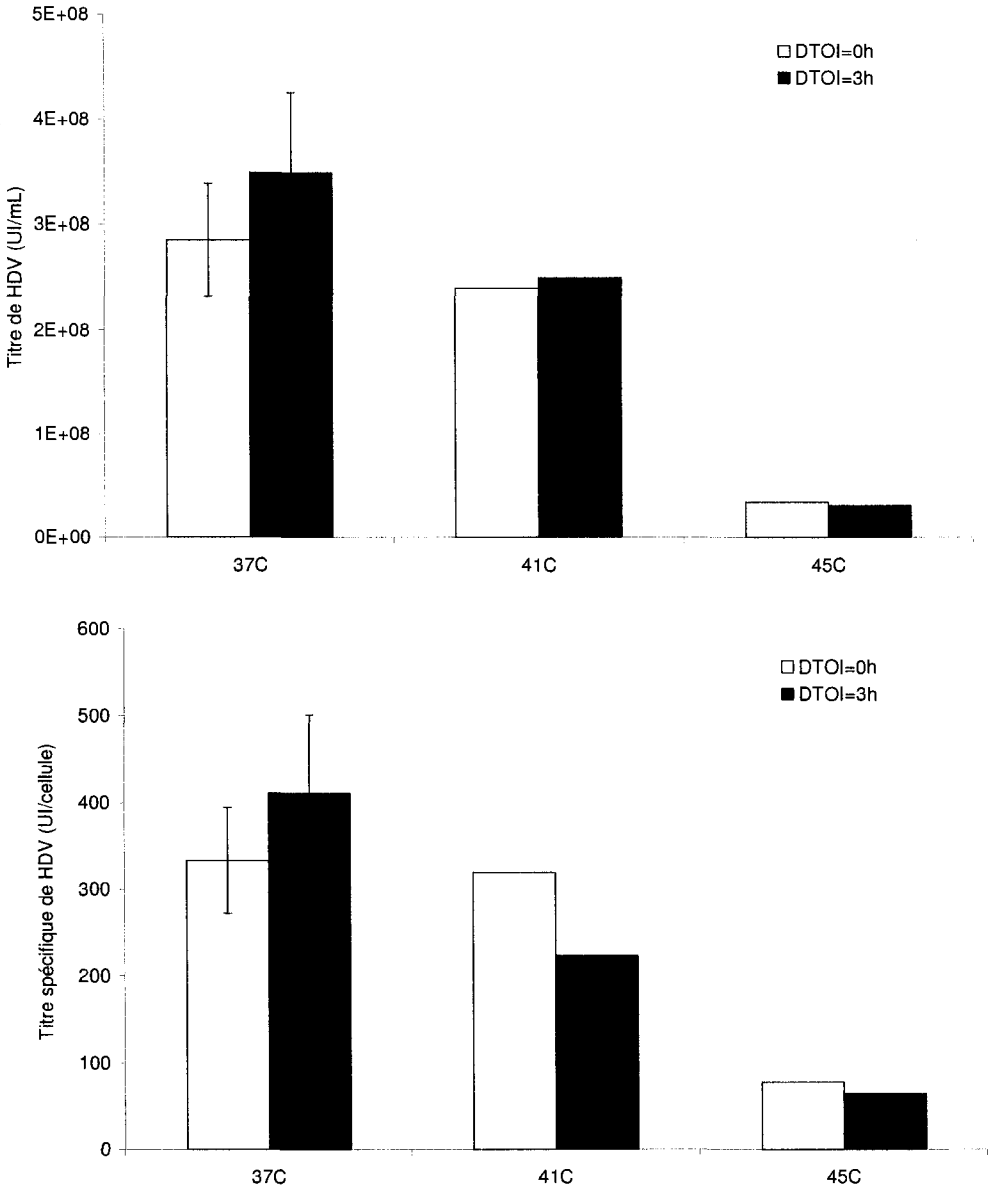

Figure 3.10 Titres infectieux volumétriques et spécifiques de HDV à 48 hpi. Les cultures ont subit un choc thermique (41 °C et 45°C) avec ou sans délai d'infection du HV (ΔTOI = 0 h ou 3 h) (n = 2). Les cultures contrôles sont maintenues à 37°C et l'infection du HV a lieu avec ou sans délai (ΔTOI = 0 h ou 3 h) (n = 4)

3.3.4 Conclusion

L'amplification du HDV est restreinte par la compétition pour les facteurs de réplication provenant de la nécessité de supplémenter la production par le HV (Ng et al. 2001; Ng et al. 2002a; Sandig et al. 2000; Sato et al. 2002). Dans cette courte étude, l'application d'un choc thermique suivit d'un délai d'infection entre les deux vecteurs n'a pas permis d'améliorer la production du HDV. La méthode de co-infection à 37°C par le contrôle de la MOI et du ratio des deux vecteurs reste donc actuellement le meilleur moyen d'optimiser la production du HDV.

3.4 Références

Alba R, Hearing P, Bosch A, Chillon M. 2007. Differential amplification of adenovirus vectors by flanking the packaging signal with attB/attP-PhiC31 sequences: implications for helper-dependent adenovirus production. *Virology* 367(1):51-8.

Altaras NE, Aunins JG, Evans RK, Kamen A, Konz JO, Wolf JJ. 2005. Production and formulation of adenovirus vectors. *Adv Biochem Eng Biotechnol* 99:193-260.

Aucoin MG, Perrier M, Kamen AA. 2006. Production of adeno-associated viral vectors in insect cells using triple infection: optimization of baculovirus concentration ratios. *Biotechnol Bioeng* 95(6):1081-92.

Barjot C, Hartigan-O'Connor D, Salvatori G, Scott JM, Chamberlain JS. 2002. Gutted adenoviral vector growth using E1/E2b/E3-deleted helper viruses. *J Gene Med* 4(5):480-9.

Brunetti-Pierri N, Ng P. 2008. Progress and prospects: gene therapy for genetic diseases with helper-dependent adenoviral vectors. *Gene Ther* 15(8):553-60.

Burch AD, Weller SK. 2005. Herpes simplex virus type 1 DNA polymerase requires the mammalian chaperone hsp90 for proper localization to the nucleus. *J Virol* 79(16):10740-9.

Chromy LR, Pipas JM, Garcea RL. 2003. Chaperone-mediated in vitro assembly of Polyomavirus capsids. *Proc Natl Acad Sci U S A* 100(18):10477-82.

Côté J, Bourget L, Garnier A, Kamen A. 1997. Study of adenovirus production in serum-free 293SF suspension culture by GFP-expression monitoring. *Biotechnol Prog* 13(6):709-14.

Côté J, Garnier A, Massie B, Kamen A. 1998. Serum-free production of recombinant proteins and adenoviral vectors by 293SF-3F6 cells. *Biotechnol Bioeng* 59:567-65.

Durocher Y, Perret S, Kamen A. 2002. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res* 30(2):E9.

Glotzer JB, Saltik M, Chiocca S, Michou AI, Moseley P, Cotten M. 2000. Activation of heat-shock response by an adenovirus is essential for virus replication. *Nature* 407(6801):207-11.

Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps ML. 1997. Construction of adenovirus vectors through Cre-lox recombination. *J Virol* 71(3):1842-9.

Hartigan-O'Connor D, Amalfitano A, Chamberlain JS. 1999. Improved production of gutted adenovirus in cells expressing adenovirus preterminal protein and DNA polymerase. *J Virol* 73(9):7835-41.

Hartigan-O'Connor D, Barjot C, Crawford R, Chamberlain JS. 2002. Efficient rescue of gutted adenovirus genomes allows rapid production of concentrated stocks without negative selection. *Hum Gene Ther* 13(4):519-31.

Haviv YS, Blackwell JL, Li H, Wang M, Lei X, Curiel DT. 2001. Heat shock and heat shock protein 70i enhance the oncolytic effect of replicative adenovirus. *Cancer Res* 61(23):8361-5.

Imperiale MJ, Kao HT, Feldman LT, Nevins JR, Strickland S. 1984. Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1A-like activity. *Mol Cell Biol* 4(5):867-74.

Kamen A, Henry O. 2004. Development and optimization of an adenovirus production process. *J Gene Med* 6 Suppl 1:S184-92.

Kao HT, Nevins JR. 1983. Transcriptional activation and subsequent control of the human heat shock gene during adenovirus infection. *Mol Cell Biol* 3(11):2058-65.

Klyushnichenko V, Bernier A, Kamen A, Harmsen E. 2001. Improved high-performance liquid chromatographic method in the analysis of adenovirus particles. *Journal of Chromatography B. Biomedical Sciences and Applications* 755(1-2):27-36.

Kreppel F, Biermann V, Kochanek S, Schiedner G. 2002. A DNA-based method to assay total and infectious particle contents and helper virus contamination in high-capacity adenoviral vector preparations. *Hum Gene Ther* 13(10):1151-6.

Liu H, Naismith JH, Hay RT. 2003. Adenovirus DNA replication. *Curr Top Microbiol Immunol* 272:131-64.

Lopez T, Lopez S, Arias CF. 2006. Heat shock enhances the susceptibility of BHK cells to rotavirus infection through the facilitation of entry and post-entry virus replication steps. *Virus Res* 121(1):74-83.

Meghrous J, Aucoin MG, Jacob D, Chahal PS, Arcand N, Kamen AA. 2005. Production of recombinant adeno-associated viral vectors using a baculovirus/insect cell suspension culture system: from shake flasks to a 20-L bioreactor. *Biotechnol Prog* 21(1):154-60.

Meneses-Acosta A, Dormond E, Jacob D, Tom R, Bernier A, Perret S, St-Laurent G, Durocher Y, Gilbert R, Kamen A. 2007. Development of a suspension serum-free helper-dependent adenovirus production system and assessment of co-infection conditions. *J Virol Methods.*

Mittereder N, March KL, Trapnell BC. 1996. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J Virol* 70(11):7498-509.

Nadeau I, Gilbert PA, Jacob D, Perrier M, Kamen A. 2002. Low-protein medium affects the 293SF central metabolism during growth and infection with adenovirus. *Biotechnol Bioeng* 77(1):91-104.

Nevins JR. 1982. Induction of the synthesis of a 70,000 dalton mammalian heat shock protein by the adenovirus E1A gene product. *Cell* 29(3):913-9.

Ng P, Beauchamp C, Evelegh C, Parks R, Graham FL. 2001. Development of a FLP/frt system for generating helper-dependent adenoviral vectors. *Mol Ther* 3(5 Pt 1):809-15.

Ng P, Evelegh C, Cummings D, Graham FL. 2002a. Cre levels limit packaging signal excision efficiency in the Cre/loxP helper-dependent adenoviral vector system. *J Virol* 76(9):4181-9.

Ng P, Parks RJ, Graham FL. 2002b. Preparation of helper-dependent adenoviral vectors. *Methods Mol Med* 69:371-88.

O'Reilly DR, Miller LK, Luckow VA. 1994. *Baculovirus Expression Vectors: A Laboratory Manual.* New York: Oxford University Press.

Palmer DJ, Ng P. 2004. Physical and infectious titers of helper-dependent adenoviral vectors: a method of direct comparison to the adenovirus reference material. *Mol Ther* 10(4):792-8.

Palmer DJ, Ng P. 2005. Helper-dependent adenoviral vectors for gene therapy. *Hum Gene Ther* 16(1):1-16.

Palomares LA, Lopez S, Ramirez OT. 2002. Strategies for manipulating the relative concentration of recombinant rotavirus structural proteins during simultaneous production by insect cells. *Biotechnology and Bioengineering* 78(6):635-44.

Park MT, Lee MS, Kim SH, Jo EC, Lee GM. 2004. Influence of culture passages on growth kinetics and adenovirus vector production for gene therapy in monolayer and suspension cultures of HEK 293 cells. *Appl Microbiol Biotechnol* 65(5):553-8.

Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A* 93(24):13565-70.

Phillips B, Abravaya K, Morimoto RI. 1991. Analysis of the specificity and mechanism of transcriptional activation of the human hsp70 gene during infection by DNA viruses. *J Virol* 65(11):5680-92.

Puntel M, Curtin JF, Zirger JM, Muhammad AK, Xiong W, Liu C, Hu J, Kroeger KM, Czer P, Sciascia S and others. 2006. Quantification of high-capacity helper-dependent adenoviral vector genomes in vitro and in vivo, using quantitative TaqMan real-time polymerase chain reaction. *Hum Gene Ther* 17(5):531-44.

Sandig V, Youil R, Bett AJ, Franlin LL, Oshima M, Maione D, Wang F, Metzker ML, Savino R, Caskey CT. 2000. Optimization of the helper-dependent adenovirus system for production and potency in vivo. *Proc Natl Acad Sci U S A* 97(3):1002-7.

Santomenna LD, Colberg-Poley AM. 1990. Induction of cellular hsp70 expression by human cytomegalovirus. *J Virol* 64(5):2033-40.

Sato M, Suzuki S, Kubo S, Mitani K. 2002. Replication and packaging of helper-dependent adenoviral vectors. *Gene Ther* 9(7):472-6.

Schenk TE. 2001. *Adenoviridae: The Viruses and Their Replication.* In: Knipe DM, Howley PM, editors. Fundamental Virology. Lippincott Williams & Wilkins ed. p 979-1016.

Schoofs G, Monica TJ, Ayala J, Howtitz J, Montgomery T, Roth G, Castillo FJ. 1998. A High-Yielding Serum-Free, Suspension culture Process to Manufacture Recombinant Adenoviral Vectors for Gene Therapy. *Cytotechnology* 28:81-89.

Shabram PW, Giroux DD, Goudreau AM, Gregory RJ, Horn MT, Huyghe BG, Liu X, Nunnally MH, Sugarman BJ, Sutjipto S. 1997. Analytical anion-exchange HPLC of recombinant type-5 adenoviral particles. *Hum Gene Ther* 8(4):453-65.

Transfiguracion J, Bernier A, Arcand N, Chahal P, Kamen A. 2001. Validation of a high-performance liquid chromatographic assay for the quantification of adenovirus type 5 particles. *J Chromatogr B Biomed Sci Appl* 761(2):187-94.

Umana P, Gerdes CA, Stone D, Davis JR, Ward D, Castro MG, Lowenstein PR. 2001. Efficient FLPe recombinase enables scalable production of helper-dependent adenoviral vectors with negligible helper-virus contamination. *Nat Biotechnol* 19(6):582-5.

Vasconcelos D, Norrby E, Oglesbee M. 1998. The cellular stress response increases measles virus-induced cytopathic effect. *J Gen Virol* 79 (Pt 7):1769-73.

Zhou H, Zhao T, Pastore L, Nageh M, Zheng W, Rao XM, Beaudet AL. 2001. A Cre-expressing cell line and an E1/E2a double-deleted virus for preparation of helper-dependent adenovirus vector. *Mol Ther* 3(4):613-22.

CHAPITRE 4: PRODUCTION ET PURIFICATION DU VECTEUR ADÉNOVIRAL DÉPENDANT D'UN VIRUS AUXILIAIRE : COMPARAISON ENTRE LES LIGNÉES PARENTALE ET RECOMBINASE HEK293

4.1 Présentation de l'Article

La purification du HDV et la diminution de la contamination par le HV est préférable avant l'utilisation *in vivo* du HDV. Les méthodologies conventionnelles utilisant un gradient de densité de chlorure de césium sont couramment employées pour à la fois purifier les préparations virales des impuretés provenant de la culture et séparer les vecteurs sur la base de leur infime différence de densité. Plusieurs étapes d'ultracentrifugation pendant de longues heures sont nécessaires. Les rendements sont génerlement très faibles.

L'article ci-dessous reporte l'élaboration d'un procédé de purification pour le HDV et constitue la deuxième partie de ce chapitre. Cet article intitulé *An Efficient Process for the Purification of Helper-Dependent Adenoviral Vector and Removal of Helper Virus by Iodixanol Ultracentrifugation* a été soumis à la revue *Analytical Biochemistry*. Un procédé de purification complet utilisant Des méthodes de chromatographies couplées à une méthode d'ultracentrifugation par gradient de densité d'iodixanol est présenté. Ce procédé documente pour la première fois la purification et la séparation des particules infectieuses de HDV

de l'ensemble des contaminants incluant les particules non-infectieuses et les particules de HV. Bien que restreint dans la capacité de mise à l'échelle par l'utilisation d'une méthode d'ultracentrifugation, ce procédé se révèle efficace et rapide. D'excellents taux de recouvrement sont obtenus.

4.2 An Efficient Process for the Purification of Helper-Dependent Adenoviral Vector and Removal of Helper Virus by Iodixanol Ultracentrifugation

Edwige Dormond[1,2], Alice Bernier[1], Parminder Chahal[1], Michel Perrier[2] and Amine Kamen[1]

[1]Animal Cell Technology Group, Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montréal, Québec, Canada H4P 2R2

[2]Chemical Engineering Department, École Polytechnique de Montréal, Campus de l'Université de Montréal, 2500, chemin de Polytechnique, Montréal, Québec, Canada, H3T 1J4

Keywords : Helper-dependent adenoviral vector; Purification; Iodixanol, Ultracentrifugation; Chromatography.

4.2.1 Abstract

The preparation of large amount of purified helper-dependent adenoviral vector (HDV) material is hampered by the lack of development of downstream processes with proven records on separation and recovery efficiencies. In order to facilitate the use of clinical-grade HDV material for large-scale *in vivo* studies, we developed a three-step purification scheme consisting of (1) an anion exchange chromatography, (2) an iodixanol density ultracentrifugation and (3) a size exclusion chromatography. The novel and fast iodixanol density ultracentrifugation step was highly effective in separating infectious HDV from contaminating helper virus (HV) and non-infectious viral vectors. The overall downstream processing scheme gave up to 75% infectious particle yield with about 100 % infectivity. Moreover, the contamination by HV was reduced by a factor of 7 and a high level of purity was achieved.

4.2.2 Introduction

Adenoviral vector (AdV) of third generation also known as helper-dependent adenoviral vector (HDV) are attracting number of investigators for gene therapy delivery (reviewed in Brunetti-Pierri and Ng 2008). The production of HDV has been proven to be amenable to large-scale volume with reduced process duration and optimized infection conditions (Meneses-Acosta et al. 2007). However, the efficient downstream processing of HDV has not been yet reported

with proven records on material purity and recovery. Obtaining high quality-grade HDV in sufficient amount is a critical task that hampers the characterization of this vector in pre-clinical studies.

Large-scale downstream processing of first generation AdV is well established (reviewed in Burova and Ioffe 2005); however the purification of HDV suffers from separation difficulties arising from the production methods. The HDV is produced in human embyo kidney 293 (HEK293)-derived cell line and requires the help of a first generation AdV called helper vector (HV) to provide all the HDV viral missing functions. In order to limit the concomitant production of HV, a recombinase-expressing HEK293 cell line is usually used (Alba et al. 2007; Ng et al. 2001; Parks et al. 1996; Umana et al. 2001). Recombinase recognition sites are placed besides the HV packaging signal, rendering the latter non-packageable following recombinase action. However, such a virus-cell system is not fully efficient and the HV arises as a contaminant during HDV production. Although the capacity to limit the HV contamination is diminished by the AdV-mediated host cell shutoff (Ng et al. 2002a), the HV contamination could also be leveled by modulating the infection parameters during production (Dormond et al., to be published) or by improving the recombinase expression level (Hartigan-O'Connor et al. 2002a; Ng et al. 2001). Using our HEK293SF-FLPe cell system under the best infection conditions, the HV contamination is ~2% HV/HDV (Dormond et al., to be published). Another group reports 0.4 to 0.1% HV/HDV contamination on semi-purified HDV with their improved cell line

(Palmer and Ng 2003). Nevertheless, further reduction of the HV contamination is often desirable (Palmer and Ng 2005).

Because of same capsid characteristics, the only technique allowing separation of HV from HDV rely on the minimal density difference between the viruses. Separation is claimed to be achieved with time-consuming ultracentrifugation run using CsCl density medium (Hartigan-O'Connor et al. 2002b; Ng et al. 2001; Ng et al. 2002b; Oka and Chan 2005). However, reports do not provide information regarding proof of separation and recovery yield. CsCl medium has slow sedimentation rates and therefore requires long separation run. Moreover, because it is hyperosmotic and cytotoxic at the density used to band the virus, the viral recoveries are low and a medium exchange by dialysis should be performed before *in vivo* injection. Iodixanol, an iodinated compound was originally used as a nontoxic X-ray contrast medium (Nossen et al. 1990; Svaland et al. 1992). The use of iodixanol for viral vector separation has largely proven to solve the aforementioned limitations encountered with CsCl (Dettenhofer and Yu 1999; Gias et al. 2008; Nielsen et al. 2006; Peng et al. 2006; Segura et al. 2006; Zolotukhin et al. 1999).

In this work, an overall purification scheme is described and documented. We combined a well-established large-scale downstream processing strategy with an improved iodixanol ultracentrifugation procedure designed to rapidly separate

components harboring low density differences. This high yield purification scheme resulted in the generation of highly purified HDV material.

4.2.3 Materials and Methods

4.2.3.1 Adenoviral Vectors, Cell Lines, Viral Stocks Production

The HDV is a kind gift from Dr. V. Sandig. This 30 kb vector carries a CMV-GFP expression cassette. The HV is a E1/E3 deleted adenoviral vector kindly provided by Dr. P. Lowenstein and described elsewhere (Umana et al. 2001). This 36 kb vector carries a CMV-luciferase expression cassette.

The HEK293SF-derived cell lines (Côté et al. 1997) were used to amplify the HDV and HV. The HV infecting stock was produced by the original HEK293SF-3F6 cell line cultivated in a 3 L controlled bioreactor in NSFM13 medium (Nadeau et al. 2002). Cells seeded at 0.3×10^6 cells/mL were infected with HV after 24 h at a multiplicity of infection (MOI) of 10. Cells were harvested at 48 h post-infection (hpi), centrifuged at $290 \times g$ for 15 min and concentrated 20 times in the spent medium. This raw lysate containing HV was further employed for the HDV production.

The HDV infecting stock was produced in a 3 L bioreactor following HDV rescue and amplification in the HEK293SF-FLPe cell line (Meneses-Acosta et al. 2007).

The HDV purification stocks were prepared by infecting the HEK293SF and HEK293SF-FLPe cell lines cultivated in two separate 3 L bioreactors. Cell lines cultivated in HSFM (Gibco Invitrogen, Grand Island, NY) supplemented with 10 mM HEPES, 0.1% Pluronic F-68 (Sigma), 1% BCS and 0.75 µg/mL puromycin (HEK293SF-FLPe only) (Durocher et al. 2002) were seeded at 0.25×10^6 cells/mL. Infection was carried out after 24 h with HDV (MOI of 5) and HV (MOI of 0.5) without medium exchange.

4.2.3.2 Purification Process

Figure 4.1 shows the downstream processing scheme. This scheme involves release of virus particles prior to the purification steps. The virus is then captured on anion-exchange chromatography, HV contaminants are isolated and final buffer exchange and other small molecule contaminants are removed by size exclusion chromatography. AdV were captured by anion-exchange (AEX) chromatography. HDV were separated from HV in a 38.6% self-forming iodixanol ultracentrifugation run at $180,000 \times g_{av}$ for 3 h. AdV were further purified by size-exclusion chromatography (SEC) allowing also iodixanol removal. Two independent purification runs were performed. They correspond to the purification of material coming from viral productions using HEK293SF cell line (SF run) and HEK293SF-FLP cell line (FLP run). Major difference of those productions concerns the level of the HV contamination (see Results and Discussion).

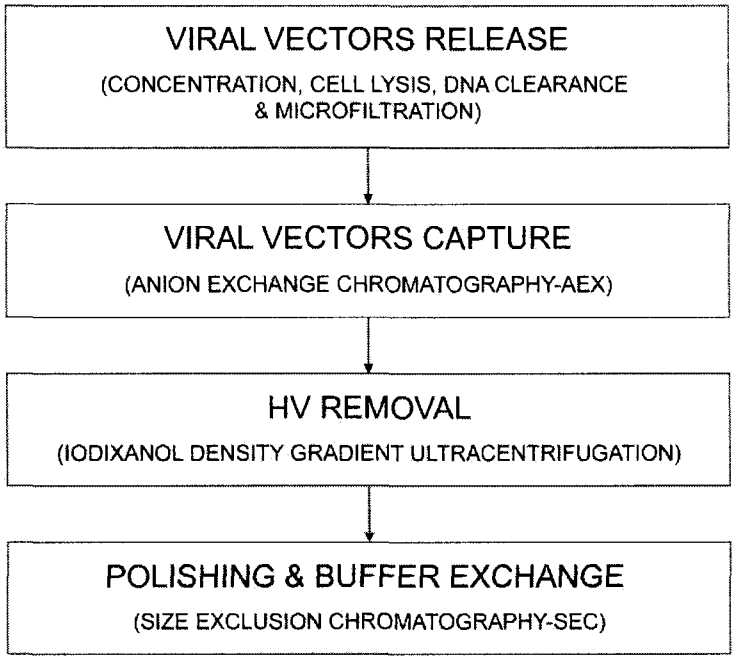

Figure 4.1 Dowstream processing strategy. From the 1× cell lysate, AdV were released by low speed centrifugation concentration, by cells lysis using freeze/thaw cycle in lysis buffer, DNA clearing using Benzonase®, 300 mM NaCl conditioning and by centrifugation and 0.45 µm microfiltration clarification

Cell Concentration, Cell Lysis, DNA Clearance and Microfiltration

Cells were harvested at 48 hpi, centrifuged for 15 min at 290 × g and resuspended in lysis buffer containing 10 mM HEPES, 2 mM $MgCl_2$ pH 7.5 in one-tenth of their culture volume. The 10× cell lysates were aliquoted and stored at –80°C until further use. The 10× cell lysates were thawed at 37°C and homogenized by pipette triturating. Benzonase® (Merck KGaA, Darmstadt, Germany) was added at a concentration of 100 units/mL to digest the

contaminating nucleic acids. The lysates were incubated at room temperature for 1 h with gentle shaking and centrifuged at 4700 × g for 10 min. Concentrated NaCl solution was added dropwise to the supernatants to obtain a final concentration of 300 mM NaCl. The conditioned supernatants were filtered using a 0.45 μm cellulose acetate membrane in a vacuum filtration unit with a glass fiber pre-filter (Corning Life Sciences, Lowell, MA).

Anion-Exchange (AEX) Chromatography

The AEX chromatography was used to selectively capture the AdV and remove the majority of protein contaminants (Figure 4.1). The AEX purification was performed at room temperature using the low-pressure GradiFrac system (Amersham Biosciences, Uppsala, Sweden) with UV elution monitoring at 280 nm and linear flow rate of 2 mL/min. The mobile phase was made by mixing Buffer A (50 mM HEPES, 2 mM MgCl2, 2% sucrose pH 7.5) and Buffer B (50 mM HEPES, 2 mM $MgCl_2$, 2% sucrose, 1 M NaCl pH 7.5) as required. The Fractogel ® DEAE beads (Merck KGaA) were packed in HR 5/5 glass column (Amersham Bioscences) with a 3.6 mL bead volume and equilibrated with 30% of Buffer B. The clarified-conditioned supernatants were loaded onto the column. The loaded material was washed with 30% Buffer B until a base line was obtained. The elution was carried out by a step gradient formed with 45% of Buffer B in 7 column volumes (CV). All unbound proteins were removed by 1 M NaCl. After each run, the column was cleaned with 0.5 M NaOH and 1 M NaCl

solution at 1 mL/min for 1 h, rinsed with 10 CV of water at 2mL/min and stored in 20% ethanol and 150 mM NaCl solution. The collected AEX-AdV peaks were processed immediately by iodixanol gradient ultracentrifugation step.

Iodixanol Gradient Ultracentrifugation

The AEX-AdV peaks were subjected to iodixanol gradient ultracentrifugation to isolate HDV from HV (Figure 4.1). OptiPrep iodixanol 60% stock solution (Axis-Shield, Oslo, Norway) was diluted in 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA pH 7.9. The self-forming gradients were made in 13.5 mL PA Ultracrimp tube (Thermo Scientific, Milford, MA) with final 38.7% iodixanol solution containing AEX-AdV peak materials. The ultracentrifugation was carried out in a Sorvall discovery ultracentrifuge (Thermo Scientific) using a Stepsaver 50V39 vertical rotor and running at 180,000 × g for 3 h at 4℃. A total of six tubes per run were processed and 16 fractions per tube were collected by puncturing at the bottom. The refractive index was measured for each fraction and correlated to the iodixanol content (% w/v) and density (g/mL) using OptiPrep Table (http://www.axis-shield-density-gradient-media.com/Applic/V01.pdf). Fractions were immediately analyzed and kept overnight at 4℃. The HDV iodixanol-containing fractions were pooled, sampled and subjected to size exclusion chromatography the following day.

Size Exclusion Chromatography (SEC)

SEC was used to remove iodixanol and remaining protein contaminants (Figure 4.1). The SEC step was carried with a Sepharose 4FF resin (Amersham Bioscences, Pistacaway, NJ) packed in a XK 16/70 glass column with a bead volume of 29 mL. The SEC step was performed at room temperature using the low-pressure GradiFrac system (GE Healthcare, Uppsala, Sweden) with elution monitoring at 280 nm and linear flow rate of 2 mL/min for load and 3 mL/min for elution. The mobile phase was 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2% sucrose pH 7.9. A maximum of 15% of the CV was loaded. The SEC-AdV peaks were collected, pooled, aliquoted and stored at –80℃ or were analyzed immediately.

4.2.3.3 Viral Quantification

Total Viral Particle Concentration

The total viral particle (TVP) concentrations were determined using AEX high performance liquid chromatography (HPLC) coupled to UV detection spectroscopy (Klyushnichenko et al. 2001). The cell lysates (thawed if stored at –80℃) were centrifuged at 4,500 × g for 2 min. The supernatants were filtered through a 0.45 μm GHP membrane syringe filter before injection. To prevent interference during UV detection, the iodixanol-containing fractions were loaded

in an enhanced time to completely elute iodixanol before the virus elution step. The TVP concentration was corrected for the difference in vector size and amount of vectors between the wild type AdV (36 kb) used to make the standard curve, the HDV (30 kb) and the HV (36 kb) using the VG ratio (HDV to HDV+HV) (determined following the formula described below).

$$\text{HDV VG ratio} = \frac{\text{HDV VG concentration}}{(\text{HDV VG concentration} + \text{HV VG concentration})}$$

$$\text{corrected TVP concentration} = \frac{\text{TVP concentration}}{\text{wild type AdV DNA size}} \times (\text{HDV VG ratio} \times \text{HDV DNA size} + (1 - \text{VG ratio of HDV}) \times \text{HV DNA size})$$

HDV and HV Viral Genome Concentration, HV Contamination Ratio

The viral genomes (VG) concentration of HDV and HV were determined by a highly specific and duplex SYBR-GreenI quantitative PCR assay (qPCR) against HDV and HV sequences. Details of qPCR run are available upon request. The HV contamination ratio is calculated by dividing the HV over the HDV concentration.

Infectious HDV Concentration

The HDV carries the GFP reporter gene allowing the quantification of infectious particles using target cells and flow cytometry analysis. For this purpose, the

HEK293E cells cultivated in suspension were seeded at 0.5×10^6 cells/mL in 12 well plates with HSFM medium, 10 mM HEPES, 1% BCS, 50 µg/mL G-418 (Durocher et al. 2002). Dilution of unknown (100 µL) was applied on cells. At 24 hpi, cells were counted and resuspended in 2% *p*-formaldehyde in PBS. After 1 h of fixation at 4°C, at least 10,000 events were analyzed using the Coulter EPICS^{TM} XL-MCL cytometer and EXPO32 software to determine the percentage of GFP-positive cells and the mean GFP fluorescence. A minimum of two dilutions showing 3 to 30% GFP-positive cells were taken into account for the titer calculation as previously described (Côté et al. 1997). Values are reported in infectious units (IU).

4.2.3.4 GFP and Luciferase Quantification

In order to select the iodixanol fractions of interest, all fractions were tested for the relative presence of HDV and HV. HDV expresses the GFP reporter gene whereas HV expresses the luciferase reporter gene in target cells. A 1:1,000 dilution of iodixanol-containing fractions was used to infect HEK293 6E cells cultivated as described in the Infectious HDV particles concentration section. At 20 h post-infection, one half volume of the cell culture was analyzed for the ratio of GFP-positive cells while the other half was analyzed for luciferase (HV reporter gene) expression. The ratio of GFP-positive cells was determined as described in the Infectious HDV particles concentration section. The level of luciferase was assessed by the Luminoskan Ascent (Thermo Scientific) using

the Luciferase Assay System (Promega, Madison, WI). The relative level of luciferase expression was calculated by dividing the luciferase expression level in the fraction to the maximum observed level for all fractions.

4.2.3.5 Protein Analysis

Total protein concentration was determined by the Bradford Protein Assay (Bio-Rad, Hercules, CA) using BSA as a standard. Electrophoresis samples were diluted 2:1 in sample buffer containing 50 mM DTT, heated 5 min in boiling water and centrifuged 2 min at 16,000 × g. Samples were loaded on 4-20% SDS Tris-HCl Ready Gels (Bio-Rad) and run with the Mini Protean II system (Bio-Rad). A molecular weight marker (broad molecular weight standard for SDS-PAGE, GE Healthcare; pre-stained low molecular weight standard for Western Blot, Bio-Rad) and the ARM standard (ATCC VR-1516) were applied to each gel. Protein bands were visualized by silver staining (Silver Staining Plus, Bio-Rad). Western transfer was carried out on a Hybond ECL nitrocellulose membrane (Amersham Biosciences) using a Tran-Blot SD semidry transfer cell (Bio-Rad). The primary antibody against adenovirus type 5 (rabbit polyclonal anti-AdV type 5 antibody, Access Biomedicals, San Diego, CA) (dilution 1/5,000) was incubated overnight. Proteins were visualized following the secondary antibody coupling (protein A HRP conjugated, Bio-Rad) (dilution 1/3,000) and revelation was done using the BM Chemiluminescence Blotting Substrate (Roche Diagnostics, Indianapolis, IN).

4.2.4 Results and Discussion

4.2.4.1 Purification by AEX Chromatography

Mediated by the strong ion exchange properties of the hexon protein, the AEX chromatography is the most suitable purification step of all scalable downstream processing strategies for AdV (Altaras et al. 2005). From the variety of resins that have been tested for now, Fractogel® DEAE has been widely approved and was used in our laboratory to purify up to 20 L of AdV-containing suspension cell culture (Arcand et al. 2003; Kamen and Henry 2004).

The knowledge of the elution profile of AdV for this resin allowed us to directly apply a NaCl step gradient to ensure a high separation efficiency between viral particles and protein contaminants with a reduce process time and an increased concentration factor (Arcand et al. 2003). Following viral load at 300 mM NaCl, the column was washed with 300 mM NaCl and the AEX-AdV peak was eluted at 450 mM NaCl step change. The column was further washed with 1 M NaCl to remove the rest of the bound material (Figure 4.2).

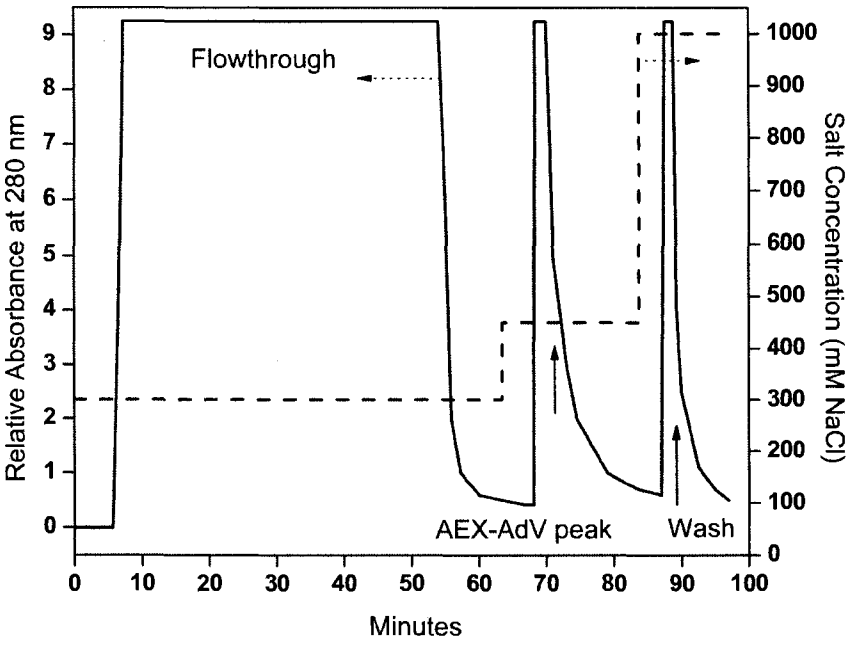

Figure 4.2 AEX chromatography profile. Conditionned-clarified lysates (43.3 and 97.6 mL containing 1.72×10^{12} TVP and 1.19×10^{12} TVP for SF and FLP runs, respectively) were loaded on a 3.6 mL-DEAE Fractogel column. Viral load was carried out at 300 mM NaCl, and column wash at 1 M NaCl. AdV-AEX peaks were eluted using a 450 mM NaCl step gradient (22.4 and 22.7 mL containing 1.01×10^{12} TVP and 1.03×10^{12} TVP, for SF and FLP runs, respectively)

The targeted product was the infectious HDV for the whole purification scheme, therefore same amount of IU of HDV for both stocks determined the volume to be processed. The initial clarification-conditioning procedure consisting of cell concentration and lysis, Benzonase® treatment, NaCl conditioning, centrifugation and microfiltration were highly efficient to prepare the load for an

AEX as suggested by the high recovery yields in TVP (116 and 109% for SF and FLP runs, respectively). The AEX column was loaded at 35 to 50% of its maximum TVP capacity which permitted to obtain high infectious recovery yields of about 80% for both stocks (Table 4.1). The AEX chromatography step was an excellent mean for concentrating the virus by a factor 18 to 40. The recovery yields were in accordance with previous results mostly obtained for first generation AdV (reviewed in Burova and Ioffe 2005). Blanche et al. (2000) reported a 50% overall recovery in TVP for the purification of HDV using AEX chromatography followed by SEC and ultrafiltration.

Table 4.1 Overall purification results

	1× cell lysate		Clarified-conditionned lysate		AEX-AdV peak		HDV-pooled iodixanol fraction		SEC-HDV peak	
Run	SF[a]	FLP[b]	SF	FLP	SF	FLP	SF	FLP	SF[c]	FLP[c]
IU ($\times 10^{11}$)	3.74	3.74	2.65	6.65	2.87	5.11	2.17	5.33	1.15	3.27
VP ($\times 10^{12}$)	1.88	1.09	1.72	1.19	1.01	1.03	0.236	0.525	0.144	0.269
IU/TVP	20	34	15	56	28	50	92	101	80	122
HV contamination	120.2	2.6	112.4	1.5	108.7	1.3	17.9	0.3	15.6	0.2
IU Yield (cumulative)	-	-	100	100	108	77	82	80	69	80
TVP Yield (cumulative)	-	-	100	100	59	87	14	44	13	37
Concentration factor (cumulative)	1	1	9	9	18	40	19	44	12	22

Notes : [a] 407 mL of 1× cell lysate were processed. [b] 920 mL of 1× cell lysate were processed. [c] Only 12.73 mL of 21.31 mL of HDV pooled iodixanol fraction were processed.

The examination of viral purity by SDS-PAGE profile indicated that the clarification-conditioning procedure was not modifying the protein content (lane 1 to 4, Figure 4.3). The comparison of the electrophoretic profiles of lane 3 to 4 showed that most of the protein contaminants passed through the column. The SDS profile for AEX-AdV peak displayed a high content of high molecular weight proteins (lane 5), whereas the low proteins were found in the 1 M NaCl wash fraction (lane 6). A high purification factor and a high recovery yield of TVP were therefore achieved with the AEX chromatographic procedure.

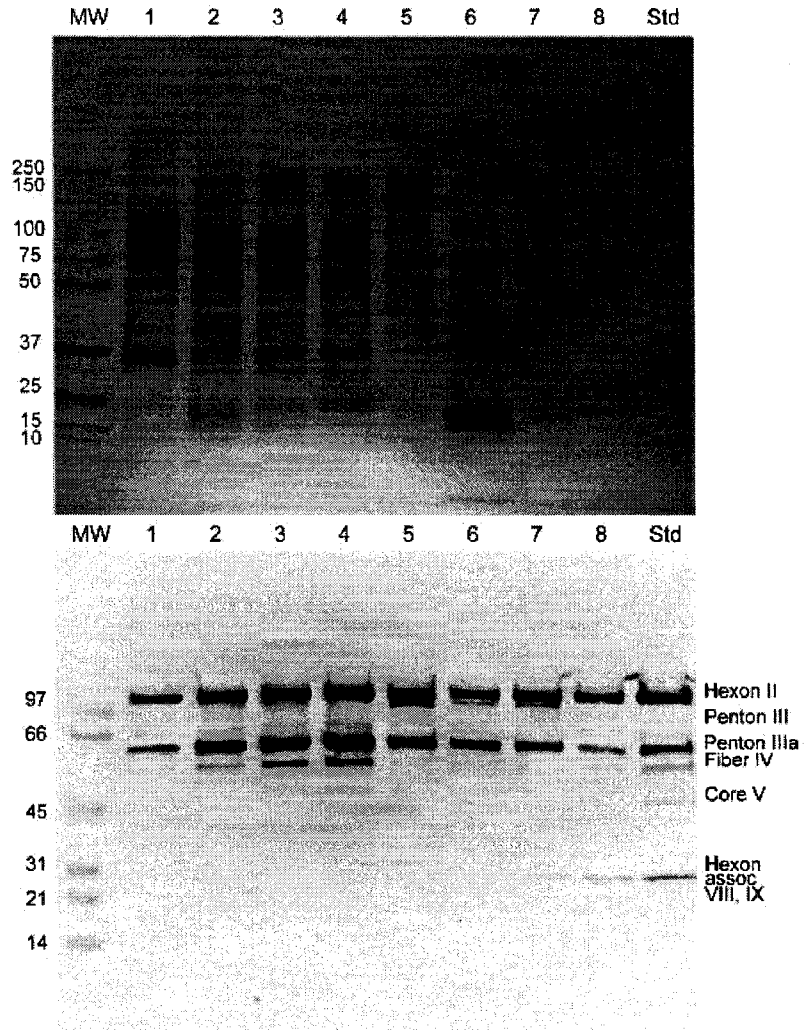

Figure 4.3 Electrophoretic analysis of the overall downstream processing scheme on 4-20% SDS-PAGE. (A) Silver-stained SDS-PAGE. (B) Western blot of SDS-PAGE revealing adenoviral proteins.

Lane 1: 1× cell lysate ; lane 2: 10× cell lysate ; lane 3: conditioned-clarified lysate ; lane 4: AEX flowthrough ; lane 5: AEX-AdV peak ; lane 6: AEX wash ; lane 7: HDV-iodixanol pooled fractions ; lane 8: SEC-AdV peak. Molecular weight marker (MW) and AdV type 5 standard (Std) were loaded at the gel extremities. Protein loaded: 500 ng/lane; lane 8 loaded undiluted (40 ng). Separation of Viruses by Self-Forming Iodixanol Gradient

Separation by ultracentrifugation is difficult when components have a small difference in their densities. Ultracentrifugation runs were performed in screening experiments using swinging bucket rotor (SW28 Beckman rotor, sedimentation path length (SPL) 85.7 mm) with step or continuous gradient or using fixed angle rotor (70 Ti Beckman, SPL 52.4 mm) with self-forming gradient (data not shown). Using these rotor types, separation of HV and HDV was never achieved; the two viruses usually settled in the same fraction. The large SPL of swinging bucket rotors and fixed angle rotors with large volume tubes generated steep gradients from the top to the bottom of the tube. In these gradients, the density difference between fractions was too high to allow a good separation between HDV and HV. Thus, in order to decrease the SPL, samples were run in a small volume vertical angle rotor as suggested in Ford et al. (1994) (Sorvall Stepsaver 50V39 vertical rotor, SPL 16 mm). The purpose of this part was to highlight the reliability and reproducibility of the iodixanol run with no regard to the HDV stock characteristics. The use of two viral stocks with highly different HV contamination levels was thought to provide the reader with some limitation aspect concerning the use of density gradient separation.

The gradients profiles observed in Figure 4.4 are sigmoidal, steep at both ends and shallow at the middle. Iodixanol content in fractions were 66% to 18% corresponding to 1.35 to 1.09 g/mL, respectively. The shallow part of the gradient (fractions 4 to 13) contained from 43.5 to 37.6% of iodixanol corresponding to density of 1.23 to 1.20 g/mL, respectively. The fractions of

interest were found at the same fraction number for both runs and the range of iodixanol content/densities were similar (Table 4.2). The HV banded at 40.8% or 1.22 g/mL iodixanol and the HDV banded at 38.7% or 1.21 g/mL iodixanol. Although HDV and HV settled at an infinitely small difference in densities (2.1% iodixanol or 0.01 g/mL in iodixanol medium), the fractions expected to contain a maximum of HV and HDV were distant by at least two fractions allowing a good separation of the HDV and the HV. The data indicated that the separation method was giving a high reproducibility.

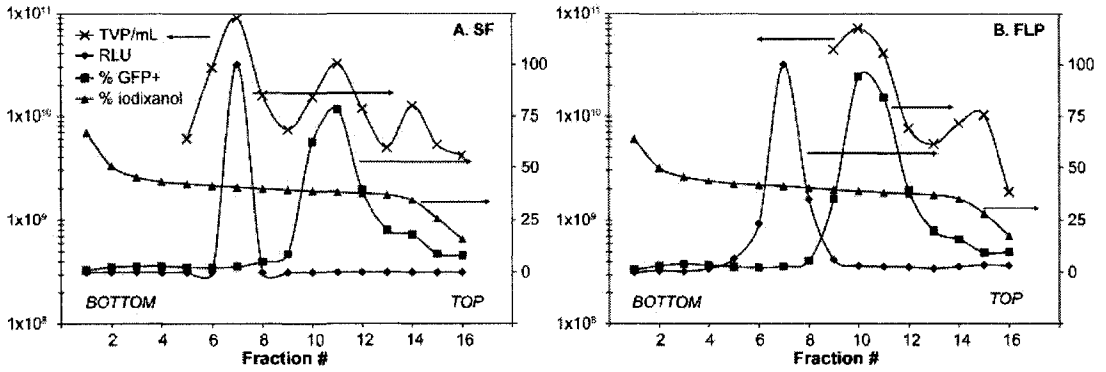

Figure 4.4 Iodixanol gradient profile at the end of the ultracentrifugation runs for SF run (A) and FLP run (B). For both runs, 16 fractions representing 0.5 cm of tube height were collected and characterized. Levels of relative luciferase unit (RLU) and % of GFP+ in target cells were assessed to separate the infectious HDV containing-fraction (9-12) from the infectious HV-containing fractions (6-8). Iodixanol content of individual fractions was determined by refractive index measurement and TVP concentration was assessed by HPLC as described in *Material and Methods*

Table 4.2 Characteristics of the self-formed iodixanol density gradients obtained following 3 h run at 180,000 × gav

		SF	FLP
HV	Fraction number	6-8	6-8
	Iodixanol content (% w/v)	41.2-39.3	41.6-40.3
	Density (g/mL)	1.222-1.215	1.224-1.217
HDV	Fraction number	9-12	9-12
	Iodixanol content (% w/v)	39.3-37.6	39.4-37.6
	Density (g/mL)	1.212-1.203	1.212-1.203

The profile of TVP concentrations assessed for all fractions showed three peaks for the SF and two peaks for the FLP run. The first peak only visible for the SF run (between fractions 5 to 9) coincided with the luciferase peak and therefore corresponded to the HV TVP particles (Figure 4.4 A). This peak was observed in the FLP run for which HV production had been hindered by the recombinase action (Figure 4.4 B). The peak appearing between fractions 9 and 13 overlaid the GFP-positive target cells peak, identified the location of the infectious HDV. The third peak occurring after fraction 13 did not coincide with any other peak therefore could be linked to the location of the non-infectious HDV (Figure 4.4 A and 4.4 B). When comparing the SF and the FLP iodixanol profiles, although similar level of GFP-positive cells were noted, the absolute level for luciferase expression was greatly higher for the SF run for all fractions (data not shown).

This indicated that the HDV fraction of interest was still highly contaminated with HV compared to FLP run.

A high cumulative IU yield was obtained for both stocks from these four fractions (82% and 80% for SF and FLP runs, respectively). It corresponded to a substantial loss of TVP for the SF run which had shown to be initially highly contaminated with the HV (Table 4.1). The absolute value of the infectivity ratio should be carefully interpreted considering that the quantification methods for IU and TVP differ. Nevertheless, the run permitted to greatly enhance the infectivity ratio of HDV from 28 and 50% (for AEX-AdV peak) to 92 and 101% (HDV iodixanol pooled fractions) for SF and FLP runs, respectively. The iodixanol separation step reduced the HV contamination by a factor of 7. An enhancement of HV separation is not possible by a longer ultracentrifugation run. Longer run would have steeped the gradient and let the HDV and HV fractions overlay. Only successive runs would help to further reduce the HV contamination. The iodixanol run did not dilute or concentrate the virus.

The electrophoretic analysis of the fractions indicated a similar protein profile for all fractions, even faint bands of high molecular weight and low molecular weight of non-viral proteins were observed in fraction 1 and 16 (Figure 4.5 A). A low molecular weight non-viral protein contaminated the fraction 4 (<10 kDa). A similar observation could be done for fraction 11 to 13, although the contamination was predominant in fraction 12 (~20 kDa). Moreover, an overall

protein contamination remained in all fractions. In the overall purification SDS profile (Figure 4.3 A), a similar pattern for lane 5 and lane 7 suggested that the iodixanol run was not further reducing the non-viral protein content. These results suggest that the contaminants that elute in AEX were of the same density as dictated by iodixanol fractions from 1 to 16. These contaminants will be removed by polishing step comprised of size exclusion chromatography.

The western blot profile highlighted the presence of virus in all the fractions although higher levels of viral proteins were observed for fractions 9 to 14 (Figure 4.5 B). Interestingly, a high hexon to penton band intensity ratio was seen for fractions 9 to 16, whereas the ratio seemed to be lower for fractions 1 to 8. The reason of such a difference remains to be elucidated.

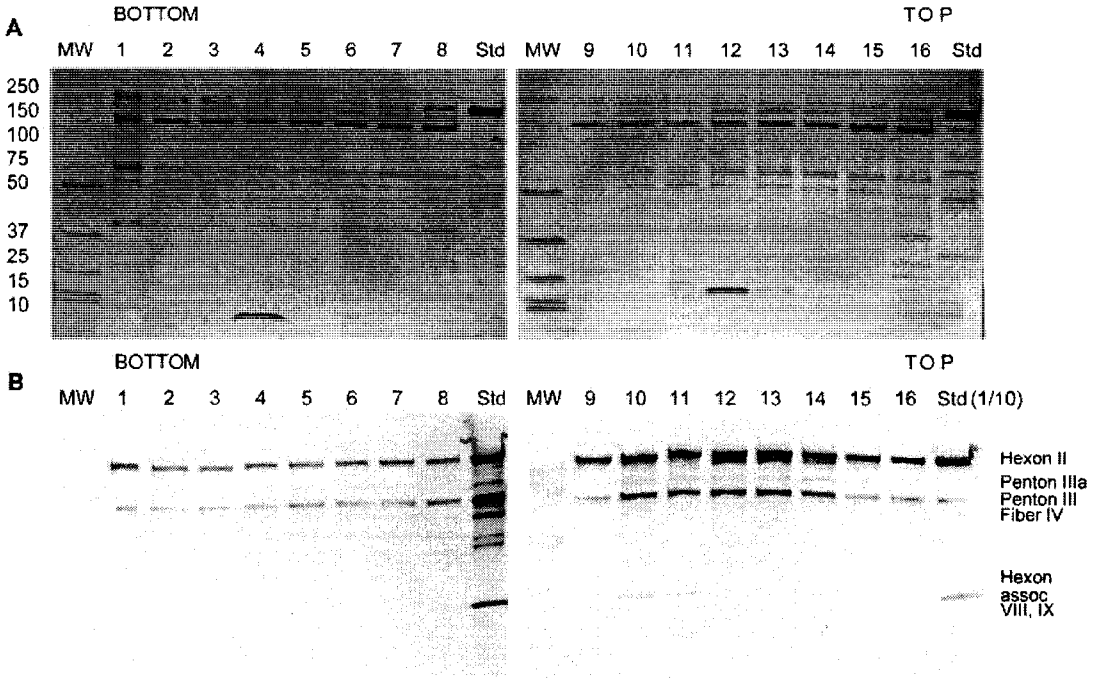

Figure 4.5 Electrophoretic analysis of iodixanol fractions 1 to 16 on 4-20% SDS-PAGE. (A) Silver-stained SDS-PAGE. (B) Western blot of SDS-PAGE revealing AdV proteins. Molecular weight marker (MW) and AdV type 5 standard (Std) were loaded at the gel extremities. Protein loaded: 500 ng/lane

The iodixanol run is a simple, fast and efficient procedure to separate infectious HDV from non-infectious and HV. This procedure was designed to antecede the AEX chromatographic process where the separation of AdV from most of the cell culture protein contaminants takes place and to precede the size exclusion chromatography step to remove additional protein contaminant (Figure 4.1).

4.2.4.2 Polishing by Size-Exclusion Chromatography

Size-exclusion chromatography was previously used as a combined polishing and iodixanol removal step (Segura et al. 2006). The chromatographic elution profile showed good separation efficiency between the HDV and the iodixanol (Figure 4.6). The 100,000 kDa AdV was eluted in the excluded volume whereas the small iodixanol molecules along with other protein contaminants were retained in the resin and eluted thereafter.

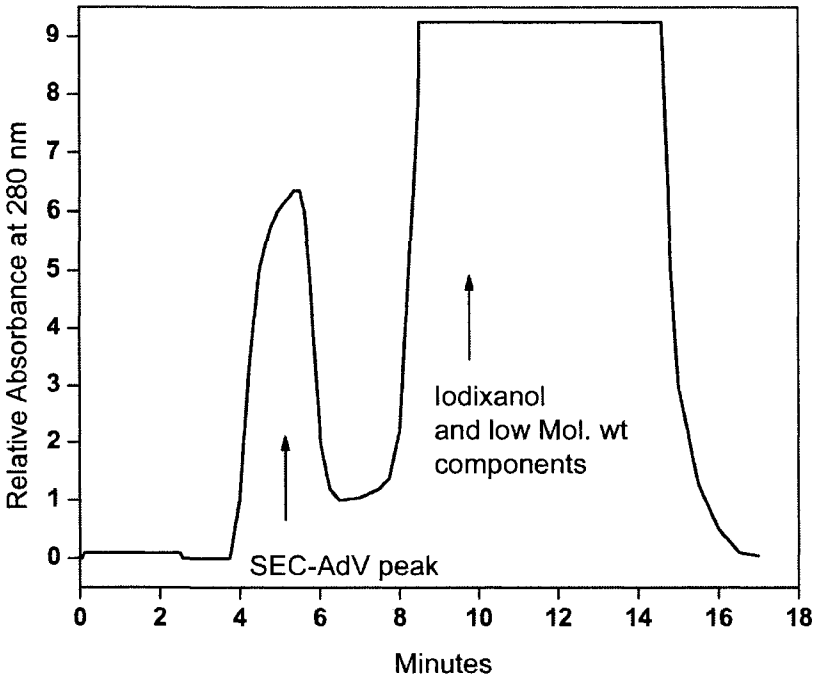

Figure 4.6 SEC profile. HDV-iodixanol pooled fractions (4.24 mL per run) were loaded onto a 29 mL Sepharose 4FF column. AdV-SEC peak was recovered in the flowthrough (6.75 mL and 7.70 mL corresponding to 4.8×10^{10} TVP and 8.97×10^{10} TVP, per run for SF and FLP run respectively). For SF and FLP runs, 3 SEC runs were performed and SEC-AdV peaks were pooled

The recoveries were 69 and 80% of IU for SF and FLP runs, respectively (13 and 37% of TVP, respectively) (Table 4.1). The concentrations of final purified materials were 7.11×10^9 TVP/mL with 80% IU/TVP and 1.17×10^{10} TVP/mL with 120% IU/TVP for SF and FLP runs, respectively. The contamination ratio remained similar to what was assessed in the HDV-iodixanol pooled fractions

(15.6% and 0.2% for SF and FLP runs, respectively). The concordance of the TVP concentrations and the HV contamination ratio for the HDV-iodixanol pooled fraction and the SEC-AdV peak indicated that the HPLC method and the purification of AdV particles for qPCR were not affected by the iodixanol presence in samples. Moreover, the reduction of HV contamination was once more documented with similar results for both runs. Depending on the starting production concentration, a concentration factor of 12 to 22 was achieved.

A 100-fold reduction in protein content has been observed following the SEC step (from 0.32 mg/mL in iodixanol pooled fractions to 0.003 mg/mL in SEC-AdV peak). Moreover, on the SDS-PAGE pattern, the predominant however faint band probably corresponded to the viral hexon (II) protein (Figure 4.3 A, lane 8) as suggested by the AdV standard profile. This band was not the predominant one in the HDV-iodixanol pooled fraction (Figure 4.3 A, lane7) and therefore constituted, with the 100-fold reduction in protein content, another proof of the reduction in non-viral protein contaminants following the SEC step. In the SEC-AdV Western blot pattern, hexon (II), penton (IIIa) and hexon associated (VIII, IX) were visualized. Faint bands corresponding to the penton (III) and fiber (IV) were also observed whereas they were less obvious for the other purification steps (Figure 4.3 B, lane 8 compared to other lanes). It further confirmed the purification enhancement achieved with this SEC step.

Although the iodixanol medium might be *in vivo* injected (Nossen et al. 1990; Svaland et al. 1992), the iodixanol removal by SEC alleviated the problems associated to the use of viscous medium (homogenization, sterile filtration). Moreover, the SEC step contributed to polish the final HDV material by further reducing the non-viral protein content.

4.2.5 Conclusion

For the first time here, an efficient processing scheme for the purification of HDV is reported. An easy, fast, and high recovery separation procedure was described and fully documented. An iso-osmotic and non-toxic ultracentrifugation medium, iodixanol, was used to form a shallow density gradient and has proven to reliably and reproducibly separate infectious HDV from HV and non-infectious AdV with high recovery. This ultracentrifugation run was integrated in an overall chromatographic purification scheme with proven efficiency and scale-up potential. Overall, high infectious recovery yields (69 to 80%) and purity have been reported. Therefore, this efficient processing scheme might be employed to purify HDV for gene therapy applications where high amounts of viral material with optimal purity, potency and safety are required.

4.2.6 Acknowledgements

The authors would like to acknowledge the Swiss National Science Foundation for the prospective researcher fellowship to E.D (PBSK2—115853). Dr. Volker Sandig and Dr. Pedro Lowenstein are also gratefully acknowledged for providing the authors with the HDV and the HV constructs. Also, we would like to thank Danielle Jacob for its support during productions in 3 L bioreactors, Lucie Bourget for help with the cytometry flux analysis and André Migneault for artwork.

4.3 Résultats Complémentaires

4.3.1 Introduction

Les résultats complémentaires constituent la première partie de ce chapitre. Une comparaison entre les lignées cellulaires parentales et recombinase pour produire le HDV y est présentée. La production du HDV est réalisée à partir de l'ADN du HDV ou à partir d'un stock viral de HDV.

Comme conclu au *Chapitre 3,* la contamination par le HV est réduite à la fois grâce au contrôle de la MOI du HV et à l'utilisation d'un système recombinase empêchant l'encapsidation du HV. L'utilisation du système recombinase nécessite la génération d'une lignée cellulaire exprimant de façon constitutive cette enzyme. La sélection d'un clone efficace doit à la fois prendre en compte

la capacité de la lignée à produire le HDV et à restreindre l'encapsidation du HV. C'est bien souvent une première étape dans le développement de la production du HDV. A ce stade, un manque de connaissance du système de production restreint l'évaluation adéquate des clones. Si la contamination est dépendante des niveaux d'expression de la recombinase (Hartigan-O'Connor et al. 2002a; Ng et al. 2002a), elle est aussi dépendante des paramètres d'infection comme démontré au *Chapitre 3, 3.2 Identification of Critical Infection Parameters to Control Helper-Dependent Adenoviral Vector Production using QPCR*. La caractérisation de la lignée cellulaire nécessite des méthodes de quantification adaptée comme la qPCR en temps réel développée au *Chapitre 3, 3.2 Identification of Critical Infection Parameters to Control Helper-Dependent Adenoviral Vector Production using QPCR*. Ajouté à ces contraintes, l'obtention de clones stables est un processus laborieux qui requiert du temps et de la main d'œuvre qualifiée.

L'étude au *Chapitre 3* a révélé que l'apport de HDV et de HV contrôle la production de HDV. A priori, l'avantage d'utiliser une lignée cellulaire recombinase réside uniquement dans la capacité à limiter la contamination par le HV. D'autre part, la production dans cette lignée est systématiquement suivie d'une méthode d'ultracentrifugation réduisant d'avantage la contamination par le HV. L'hypothèse de travail est que la lignée cellulaire parentale HEK293SF-3F6 développée spécifiquement pour la production d'AdV en suspension (Côté et al. 1998) produit le HDV à un titre similaire grâce au contrôle de la MOI et du ratio

entre les vecteurs HDV et HV. Par la suite, la contamination par le HV peut être réduite par l'utilisation d'un procédé de purification efficace comme celui décrit dans ce Chapitre (*4.2 An Efficient Process for the Purification of Helper-Dependent Adenoviral Vector and Removal of Helper Virus by Iodixanol Ultracentrifugation*).

4.3.2 Matériels et Méthodes

Les lignées cellulaires HEK293SF et HEK293SF-FLPe sont décrites dans Côté et al. (1998) et Meneses-Acosta et al. (2007). Les cellules sont maintenues dans le milieu HSFM supplémenté avec 10 mM HEPES, 1% BCS et 0.75 µg/mL puromycine pour la lignée HEK293SF-FLPe uniquement. La production du HDV est effectuée en flacon agité inoculé avec 25 mL de culture cellulaire à 0.5×10^6 cellules/mL dans du milieu frais. Dans le cas de la production en flacon agité sans changement de milieu, l'inoculation est effectuée à 0.25×10^6 cellules/mL 24 h avant l'infection.

Le secours et l'amplification du HDV à partir de l'adénofection est détaillé au *Chapitre 2, 2.2.3 Materials and Methods*. L'addition de HV est omise pour les passages d'amplification avec la lignée HEK293SF. L'amplification du HDV à partir du stock viral est réalisé avec les stocks viraux de HDV et HV provenant des productions en bioréacteur de 3 L *Chapitre 2, 2.2.3 Materials and Methods*. Les cultures sont infectées avec une MOI de HDV de 5 et une MOI de HV de

0.5. La récolte virale est effectuée à 48 hpi. Les productions en bioréacteurs sont réalisées comme décrit au *Chapitre 4, 4.2.3 Materials and Methods*. Les productions sont analysées comme décrit au *Chapitre 3, 3.2.3 Materials and Methods*.

4.3.3 Résultats et Discussion

La capacité des lignées cellulaires à générer le HDV à partir du DNA du HDV est comparée. L'utilisation de la lignée parentale nécessite deux passages d'amplification supplémentaires (Figure 4.7). Avec la lignée recombinase, la faible contamination obtenue à chaque passage permet de réajuster de manière optimale la MOI du HV. La production du HDV qui dépend partiellement de la MOI du HV (*Chapitre 3, 3.2 Identification of Critical Infection Parameters to Control Helper-Dependent Adenoviral Vector Production using QPCR)* est ainsi plus efficace lorsque la MOI du HV est contrôlée. Ce contrôle est impossible avec la lignée parentale qui ne limite pas l'encapsidation du HV. A chaque passage d'amplification, les cultures cellulaires de HEK293SF sont assurément infectées à des MOI de HV supérieures à la MOI optimale (0.5) [1]. Cependant, le titre maximum de HDV est similaire pour les deux lignées et de l'ordre de 1-2 ×

[1] L'adénofection est réalisée avec une MOI de 5 et le facteur d'amplification du HV est de l'ordre de 50 - valeur exporté de Sato M, Suzuki S, Kubo S, Mitani K. 2002. Replication and packaging of helper-dependent adenoviral vectors. Gene Ther 9(7):472-6. : le HDV et le HV répliquent dans HEK293SF avec une efficacité similaire et l'augmentation en BTU du HDV est de l'ordre de 50 - la MOI du HV au P1 est alors de 25.

10^8 UI/mL. Le secours et l'amplification du HDV, étapes nécessaires pour la génération d'un stock viral d'infection, peuvent être réalisés avec la lignée cellulaire parentale. Pour atteindre cependant un titre infectieux comparable, quelques passages supplémentaires d'amplification sont nécessaires.

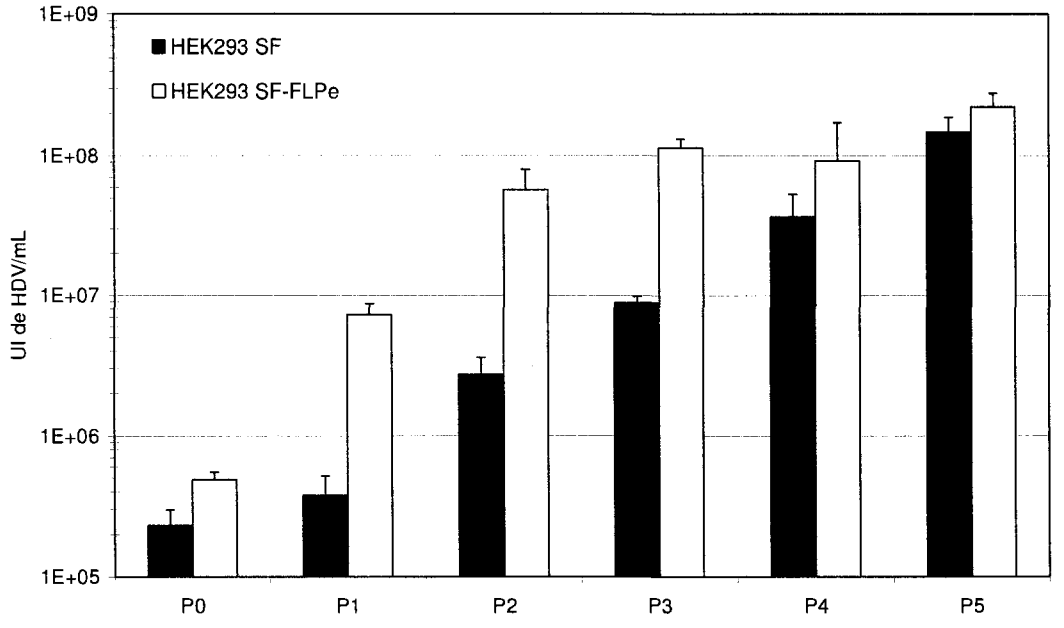

Figure 4.7 Secours et amplification du HDV à partir de l'adénofection (n = 4)

La production de HDV à partir d'un stock viral est analysée au Tableau 4.3. Les titres de HDV sont similaires pour les productions avec les lignées parentale et recombinase. Avec la lignée parentale, les concentrations en génomes viraux sont relativemenet semblables, indiquant une efficacité d'amplification similaire pour le HDV et le HV voire légérement avantagée pour le HV. L'infectivité du HDV est d'environ 37% pour les deux lignées. La contamination par le HV

atteint cependant des niveaux en moyenne 23 fois plus élevés avec la lignée parentale. La production de HDV n'est pas affectée par l'utilisation de la lignée recombinase ou parentale. Seul le niveau de contamination par le HV est affecté.

Tableau 4.3 Production du HDV à partir de stock viraux (n = 4)

	Titre de HDV UI de HDV/mL	Concentration		Infectivité du HDV UI/VG (%)	Contamination par HV GV de HV/GV de HDV (%)
		HDV GV de HDV/mL	HV GV de HV/mL		
HEK293SF (x 10^8)	5.2 (± 3.2)	12 (± 6.5)	14 (± 5.3)	36.7 (± 14.0)	125.8 (± 18.5)
HEK293SF-FLPe (x 10^8)	4.0 (± 1.1)	11 (± 4.4)	0.52 (± 0.041)	38.2 (± 4.6)	5.4 (± 1.7)

Afin de simplifier le processus de production à grande échelle, le HDV est produit sans changement de milieu à l'infection. Les différences de production sont peu significatives à cette densité cellulaire d'infection (Tableau 4.4). Les productions en bioréacteur de 3 L sont donc réalisées sans changement de milieu évitant ainsi la consommation supplémentaire de milieu et un risque de contamination accru par des manipulations extensives (vidange du bioréacteur, centrifugation de la culture, suspension dans du milieu frais et à nouveau inoculation du bioréacteur). Ces résultats indiquent aussi la capacité de mise à l'échelle du procédé à des volumes plus importants pour lesquels le changement de milieu n'est pas techniquement et économiquement réalisable.

Tableau 4.4 Production du HDV avec ou sans changement de milieu à l'infection (n = 4)

	Titre de HDV UI de HDV/mL	
	Avec changement	Sans changement
HEK293 SF (x 10^8)	6.5 (±1.9)	2.9 (± 1.1)
HEK 293SF-FLPe (x 10^8)	3.8 (±1.5)	2.9 (± 0.52)

Quelques détails des deux productions à l'échelle de 3 L sont présentées à la Tableau 4.5. D'autres données pertinentes sont présentées au *Chapitre* 4 *4.2 An Efficient Process for the Purification of Helper-Dependent Adenoviral vector and Removal of Helper Virus by Iodixanol Ultracentrifugation*, Table 4.1.

Tableau 4.5 Production du HDV en bioreacteur de 3 L sans changement de milieu à l'infection (n = 1)

	Densité cellulaire cellules/mL	Viabilité %	Titre de HDV HDV UI/mL	Titre spécifique de HDV UI de HDV/cellule
HEK293 SF	8.2 x 10^5	86	9.2 x 10^8	1115
HEK293SF-FLPe	9.0 x 10^5	77	4.1 x 10^8	457

4.3.4 Conclusions

L'amplification du HDV n'est pas dépendante de la sélection par recombinase. Les titres maxima de HDV sont similaires avec les deux lignées. La production du HDV dans la lignée parentale est donc une solution envisageable. La difficulté liée à la génération d'une lignée stable et efficace et l'efficacité de la production du HDV dans la lignée parentale rendent cette solution particulièrement attractive. La production du HDV dans la lignée parentale peut aussi constituer un excellent point de départ dans le développement d'une

lignée cellulaire capable de produire le HDV et de réduire la contamination par le HV de manière efficace. Entre autre, la production du HDV dans la lignée parentale permet d'établir des méthodes de caractérisation adéquate des lignées stables grâce à la génération de matériel viral.

Le procédé de purification décrit dans l'article de ce chapitre n'a pas permis de diminuer la contamination par le HV à des niveaux proche de ceux obtenus avec la lignée recombinase. Éventuellement, une étape supplémentaire d'ultracentrifugation permettrait d'atteindre des niveaux de contamination comparable.

4.4 Références

Alba R, Hearing P, Bosch A, Chillon M. 2007. Differential amplification of adenovirus vectors by flanking the packaging signal with attB/attP-PhiC31 sequences: implications for helper-dependent adenovirus production. *Virology* 367(1):51-8.

Altaras NE, Aunins JG, Evans RK, Kamen A, Konz JO, Wolf JJ. 2005. Production and formulation of adenovirus vectors. *Adv Biochem Eng Biotechnol* 99:193-260.

Arcand N, Bernier A, Transfiguracion J, Jacob D, Coehlo H, Kamen A. 2003. Adenovirus Type 5 (Ad5) chromatographic purification process at the 20 L scale. *Bioprocess J* 2:72-75.

Blanche F, Cameron B, Barbot A, Ferrero L, Guillemin T, Guyot S, Somarriba S, Bisch D. 2000. An improved anion-exchange HPLC method for the detection and purification of adenoviral particles. *Gene Ther* 7(12):1055-62.

Brunetti-Pierri N, Ng P. 2008. Progress and prospects: gene therapy for genetic diseases with helper-dependent adenoviral vectors. *Gene Ther* 15(8):553-60.

Burova E, Ioffe E. 2005. Chromatographic purification of recombinant adenoviral and adeno-associated viral vectors: methods and implications. *Gene Ther* 12 Suppl 1:S5-17.

Côté J, Bourget L, Garnier A, Kamen A. 1997. Study of adenovirus production in serum-free 293SF suspension culture by GFP-expression monitoring. *Biotechnol Prog* 13(6):709-14.

Côté J, Garnier A, Massie B, Kamen A. 1998. Serum-free production of recombinant proteins and adenoviral vectors by 293SF-3F6 cells. *Biotechnol Bioeng* 59:567-65.

Dettenhofer M, Yu XF. 1999. Highly purified human immunodeficiency virus type 1 reveals a virtual absence of Vif in virions. *J Virol* 73(2):1460-7.

Durocher Y, Perret S, Kamen A. 2002. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res* 30(2):E9.

Ford T, Graham J, Rickwood D. 1994. Iodixanol: a nonionic iso-osmotic centrifugation medium for the formation of self-generated gradients. *Anal Biochem* 220(2):360-6.

Gias E, Nielsen SU, Morgan LA, Toms GL. 2008. Purification of human respiratory syncytial virus by ultracentrifugation in iodixanol density gradient. *J Virol Methods* 147(2):328-32.

Hartigan-O'Connor D, Barjot C, Crawford R, Chamberlain JS. 2002a. Efficient rescue of gutted adenovirus genomes allows rapid production of concentrated stocks without negative selection. *Hum Gene Ther* 13(4):519-31.

Hartigan-O'Connor D, Barjot C, Salvatori G, Chamberlain JS. 2002b. Generation and growth of gutted adenoviral vectors. *Methods Enzymol* 346:224-46.

Ishii-Watabe A, Uchida E, Iwata A, Nagata R, Satoh K, Fan K, Murata M, Mizuguchi H, Kawasaki N, Kawanishi T and others. 2003. Detection of replication-competent adenoviruses spiked into recombinant adenovirus vector products by infectivity PCR. *Mol Ther* 8(6):1009-16.

Kamen A, Henry O. 2004. Development and optimization of an adenovirus production process. *J Gene Med* 6 Suppl 1:S184-92.

Klyushnichenko V, Bernier A, Kamen A, Harmsen E. 2001. Improved high-performance liquid chromatographic method in the analysis of adenovirus particles. *Journal of Chromatography B. Biomedical Sciences and Applications* 755(1-2):27-36.

Meneses-Acosta A, Dormond E, Jacob D, Tom R, Bernier A, Perret S, St-Laurent G, Durocher Y, Gilbert R, Kamen A. 2007. Development of a suspension serum-free helper-dependent adenovirus production system and assessment of co-infection conditions. *J Virol Methods*.

Nadeau I, Gilbert PA, Jacob D, Perrier M, Kamen A. 2002. Low-protein medium affects the 293SF central metabolism during growth and infection with adenovirus. *Biotechnol Bioeng* 77(1):91-104.

Ng P, Beauchamp C, Evelegh C, Parks R, Graham FL. 2001. Development of a FLP/frt system for generating helper-dependent adenoviral vectors. *Mol Ther* 3(5 Pt 1):809-15.

Ng P, Evelegh C, Cummings D, Graham FL. 2002a. Cre levels limit packaging signal excision efficiency in the Cre/loxP helper-dependent adenoviral vector system. *J Virol* 76(9):4181-9.

Ng P, Parks RJ, Graham FL. 2002b. Preparation of helper-dependent adenoviral vectors. *Methods Mol Med* 69:371-88.

Nielsen SU, Bassendine MF, Burt AD, Martin C, Pumeechockchai W, Toms GL. 2006. Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. *J Virol* 80(5):2418-28.

Nossen JO, Aakhus T, Berg KJ, Jorgensen NP, Andrew E. 1990. Experience with iodixanol, a new nonionic dimeric contrast medium. Preliminary results from the human phase I study. *Invest Radiol* 25 Suppl 1:S113-4.

Oka K, Chan L. 2005. Construction and characterization of helper-dependent adenoviral vectors for sustained in vivo gene therapy. *Methods Mol Med* 108:329-50.

Palmer D, Ng P. 2003. Improved system for helper-dependent adenoviral vector production. *Mol Ther* 8(5):846-52.

Palmer DJ, Ng P. 2005. Helper-dependent adenoviral vectors for gene therapy. *Hum Gene Ther* 16(1):1-16.

Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A* 93(24):13565-70.

Peng HH, Wu S, Davis JJ, Wang L, Roth JA, Marini FC, 3rd, Fang B. 2006. A rapid and efficient method for purification of recombinant adenovirus with arginine-glycine-aspartic acid-modified fibers. *Anal Biochem* 354(1):140-7.

Sato M, Suzuki S, Kubo S, Mitani K. 2002. Replication and packaging of helper-dependent adenoviral vectors. *Gene Ther* 9(7):472-6.

Segura MM, Garnier A, Kamen A. 2006. Purification and characterization of retrovirus vector particles by rate zonal ultracentrifugation. *J Virol Methods* 133(1):82-91.

Svaland MG, Haider T, Langseth-Manrique K, Andrew E, Hals PA. 1992. Human pharmacokinetics of iodixanol. *Invest Radiol* 27(2):130-3.

Umana P, Gerdes CA, Stone D, Davis JR, Ward D, Castro MG, Lowenstein PR. 2001. Efficient FLPe recombinase enables scalable production of helper-dependent adenoviral vectors with negligible helper-virus contamination. *Nat Biotechnol* 19(6):582-5.

Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, Chesnut K, Summerford C, Samulski RJ, Muzyczka N. 1999. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther* 6(6):973-85.

DISCUSSION GÉNÉRALE, CONCLUSIONS ET RECOMMANDATIONS

L'échec d'un protocole clinique utilisant l'AdV de première génération a gravement miné la confiance du public quant à la sûreté des thérapies virales. Ce revers a fait l'objet d'un examen approfondi de la FDA. Celle-ci a, d'une part, identifié le besoin d'élaborer un standard de référence (ARM) pour pallier le manque de caractérisation des stocks viraux et, d'autre part, défini le niveau d'infectivité minimum et le niveau de RCA maximum admissibles dans les lots cliniques. Par ailleurs, l'implication de la réponse immunitaire dans le décès du patient sujet à un traitement thérapeutique adénoviral a accéléré le développement de l'AdV de troisième génération, le HDV.

Le standard de référence permet d'exploiter de manière adéquate les différentes méthodes de quantification des particules virales totales et infectieuses. Le bénéfice apporté par le standard de référence dépasse la simple caractérisation du matériel final. La quantification d'un stock viral permet de définir des procédés de productions reproductibles grâce à des paramètres d'infection prédictifs des performances de production, ce qui limite la variabilité du produit final et garanti l'optimisation de la production. Les procédés de production établis selon des principes non-empiriques ont l'avantage d'être plus facilement adapté à grande échelle. Grâce au standard de référence, les procédés de production et leurs performances peuvent être comparés.

L'objectif final étant de définir un processus intégré pour la production du HDV à grande échelle, la caractérisation de la production revêt une importance cruciale dans ce travail. Cependant, les méthodes de quantification standardisées sont inadaptés pour l'AdV de troisième génération. Elles ne peuvent servir ni à la quantification des particules virales totales et infectieuses de HDV ni à l'évaluation de la contamination par le HV. De plus, le développement de nombreuses méthodologies de quantification permettant la caractérisation des productions de HDV rend difficile les comparaisons entre les différentes études. L'établissement d'un standard de référence pour l'AdV de troisième génération permettrait de comparer adéquatement les productions. Dans cette étude, la quantification des particules virales totales de HDV et de HV est réalisée par qPCR. L'unité de mesure étant la même pour le HDV et le HV, le niveau de contamination par le HV est évalué avec précision. Les productions sont rapportées en terme d'UI de HDV, d'UI de HV, de TVP, de GV de HDV et de GV de HV, d'infectivité du HDV et de contamination par le HV permettant ainsi une évaluation très complète des expériences.

Dans ce travail, trois procédés utilisant des méthodes innovatrices sont présentés. La définition de ces procédés repose sur l'emploi de paramètres de production faciles d'utilisation, corrélés aux performances. Ainsi, les procédés permettent de produire le HDV à grande échelle avec fiabilité et efficacité pour limiter à la fois le temps de production et garantir une utilisation efficace du matériel initial. Dans le premier procédé, le complexe d'adénofection est identifié

comme un complexe très efficace pour transférer le matériel viral à la cellule et produire le HDV. La composition des complexes est corrélée au titre de HDV. A partir de l'ADN du HDV, la génération d'un stock viral à grande échelle et caractérisé par un titre maximum de HDV nécessite 6 jours. L'avantage de ce procédé réside dans sa capacité de mise à l'échelle et son faible coût grâce à l'utilisation de l'agent complexant PEI. Le second procédé utilise le stock viral généré par le premier procédé. La production du HDV est contrôlée par la MOI et le ratio entre les deux vecteurs. Ces deux paramètres sont corrélés au titre de HDV et à la contamination par le HV. A partir d'un seul stock viral caractérisé, ce procédé permettrait d'effectuer de manière reproductible de multiples productions à grande échelle en l'espace de 2 jours. La stratégie de purification est réalisée en 1 jour grâce à une nouvelle méthode d'ultracentrifugation. La possibilité de mise à l'échelle du procédé de purification dépend de la disponibilité d'équipements d'ultracentrifugation de grande capacité. Il est cependant important de noter que la séparation du HDV et du HV, s'il est nécessaire, ne peut être réalisée que grâce à une méthode d'ultracentrifugation.

L'étude des méthodes d'adénofection et d'infection conduit à mieux comprendre la formation de HDV. La production du HDV est généralement diminuée d'un facteur 10 à 100 comparé à la production d'AdV de première génération. Le transfert du HDV par adénofection (sous forme d'ADN) ou par infection (sous forme de vecteur viral) est le facteur le plus critique de la production du HDV. Le HDV possède une capacité d'amplification différente suivant la forme dans

laquelle il se trouve. Contrairement au génome viral, l'ADN du HDV n'est pas lié à la protéine terminale laquelle a une grande affinité pour les facteurs de réplication. En conséquence, la capacité de réplication de l'ADN du HDV est faible relativement à celle du génome viral du HDV. Au-delà d'avantager le transfert de l'ADN du HDV, la présence simultanée du HDV et du HV favorise la réplication du HDV lors de de l'infection et vraisemblablement lors l'adénofection. Cependant puisque la production du HDV par adénofection dépend du HV à la fois pour le transfert de l'ADN et pour la production du HDV, son rôle dans ce contexte est difficile à déterminer. La différence de production entre l'AdV de première et troisième génération est aussi due à la présence de deux vecteurs distincts dans le même hôte cellulaire. Cette présence simultanée induit une compétition entre les deux vecteurs pour leur amplification respective. Le HDV présente un avantage réplicatif grâce à la taille de son génome (30 kb pour le HDV versus 36 kb pour le HV) et un avantage en terme d'encapsidation grâce à son signal non-modifié (contrairement au signal d'encapsidation du HV bordé de sites de reconnaissance de la recombinase). Cependant, la nature des séquences (non-codantes pour le HDV versus virales pour le HV) avantage vraisemblablement l'amplification du HV. En effet, dans des conditions d'infection optimales pour la production du HDV, l'amplification dans la lignée cellulaire parentale avantage légèrement le HV. Il est postulé que les quantités relatives de chacun des vecteurs apportés au moment de l'infection (dictées par la MOI et le ratio des virus) ont un effet sur la compétition entre les vecteurs

même si cette compétition est d'avantage contrôlée par les structures virales. En revanche, le délai d'infection, le choc de température et le système de sélection par recombinase ne modifient pas la compétition entre les deux vecteurs. En résumé, la présence des deux vecteurs dans le même système cellulaire de production induit à la fois une compétition pour les facteurs de réplications et d'encapsidation et une aide pour la réplication.

A l'heure actuelle, le développement d'un standard de référence pour l'AdV de troisième génération fait défaut et les niveaux acceptables de contamination par le HV ne sont pas encore établis par la FDA. Ceci empêche la définition de conditions précises pour la production du HDV. Précédemment, il a été noté que la capacité de mise à l'échelle de la production du HDV est restreinte par l'utilisation d'une méthode d'ultracentrifugation pour diminuer la contamination par le HV. Cependant, la méthode d'ultracentrifugation sépare aussi les particules virales infectieuses des non-infectieuses. Une partie de la réponse immunitaire est corrélée à la quantité de particules virales totales, mais seules les particules virales infectieuses ont un intérêt thérapeutique. En vue de diminuer cette réponse immunitaire, il convient éventuellement d'utiliser une méthode d'ultracentrifugation. La méthode d'infection peut servir à contrôler efficacement la contamination par le HV. Cependant, elle est limitée par les caractéristiques intrinsèques de la lignée cellulaire recombinase. La lignée recombinase est généralement sélectionnée pour sa capacité à exciser le signal d'encapsidation du HV. L'efficacité d'excision n'est pas totale et certains

génomes de HV sont encapsidés générant un certain niveau de contamination par le HV. Le niveau de contamination semble dépendre du niveau d'expression de la recombinase. Le phénomène d'arrêt du métabolisme cellulaire dû à l'AdV exacerbe l'excision incomplète du signal d'encapsidation du HV. Actuellement, aucune étude n'a corrélé le niveau d'expression de la recombinase à l'efficacité d'excision et à l'efficacité d'amplification du HDV. Des recherches en ce sens permettraient d'établir les caractéristiques requises pour qu'une lignée cellulaire soit à la fois capable d'amplifier efficacement le HDV et de limiter la contamination par le HV. Des stratégies visant à limiter le phénomène d'arrêt du métabolisme cellulaire par l'AdV pourraient être explorées. Une avenue très intéressante consisterait à empêcher totalement la contamination par le HV au moyen de nouveaux systèmes de production. Ces nouveaux systèmes ont déjà fait l'objet d'étude mais nécessitent des améliorations pour limiter la génération de HV non-infectieux ou de RCA. L'utilisation finale du matériel, le niveau de contamination jugé acceptable et l'efficacité du système cellulaire à réduire la contamination par le HV définiront en dernier lieu la capacité de mise à l'échelle du procédé par le besoin ou non de séparer les deux vecteurs. Cependant, la volonté de réduire la contamination par le HV ne doit pas occulter les points suivants. D'une part, le HV est présent à des faibles niveaux de contamination avec les systèmes cellulaires actuels et ceci avant même l'étape de purification. D'autre part, le HV, un AdV de première génération a été utilisé dans de nombreux protocoles cliniques sans aucun effet néfaste.

La production du HDV peut très certainement être améliorée par une modification des structures virales. Par exemple, pour le HDV, la nature des séquences de remplissages et l'insertion d'origine de réplication de séquences virales ont démontré avoir une influence positive sur l'amplification du HDV. Pour le HV, la présence de la région E3, le positionnement des sites de reconnaissance de la recombinase ont une influence sur le titre de HDV. D'autres recherches visant la modification des séquences en lien étroit avec l'étude de la biologie du virus permettraient d'augmenter le titre de HDV. D'un point de vue procédé, des améliorations substantielles de production du HDV peuvent être obtenues. Il s'agit d'appliquer le bagage impressionnant de connaissances déjà acquises pour la production d'AdV de première génération. Dans cette thèse, le choix du milieu de culture est dicté pour accommoder initialement la méthode d'adénofection. Le choix de la densité cellulaire est réalisé en vue d'obtenir les meilleures efficacités de transfection ainsi que les meilleures productions spécifiques dans des conditions de cultures en cuvée. La production du HDV à de plus grandes densités cellulaires avec des milieux optimisés sans sérum dans des conditions maintenant un état physiologique propice à la production d'AdV doit être étudiée. A cette fin, l'utilisation d'outils comme l'analyse de flux métabolique est recommandée.

RÉFÉRENCES

Alba R, Hearing P, Bosch A, Chillon M. 2007. Differential amplification of adenovirus vectors by flanking the packaging signal with attB/attP-PhiC31 sequences: implications for helper-dependent adenovirus production. *Virology* 367(1):51-8.

Altaras NE, Aunins JG, Evans RK, Kamen A, Konz JO, Wolf JJ. 2005. Production and formulation of adenovirus vectors. *Adv Biochem Eng Biotechnol* 99:193-260.

Amalfitano A, Begy CR, Chamberlain JS. 1996. Improved adenovirus packaging cell lines to support the growth of replication-defective gene-delivery vectors. *Proc Natl Acad Sci U S A* 93(8):3352-6.

Amalfitano A, Chamberlain JS. 1997. Isolation and characterization of packaging cell lines that coexpress the adenovirus E1, DNA polymerase, and preterminal proteins: implications for gene therapy. *Gene Ther* 4(3):258-63.

Andrews JL, Kadan MJ, Gorziglia MI, Kaleko M, Connelly S. 2001. Generation and characterization of E1/E2a/E3/E4-deficient adenoviral vectors encoding human factor VIII. *Mol Ther* 3(3):329-36.

Arcand N, Bernier A, Transfiguracion J, Jacob D, Coehlo H, Kamen A. 2003. Adenovirus Type 5 (Ad5) chromatographic purification process at the 20 L scale. *Bioprocess J* 2:72-75.

Aucoin MG, Perrier M, Kamen AA. 2006. Production of adeno-associated viral vectors in insect cells using triple infection: optimization of baculovirus concentration ratios. *Biotechnol Bioeng* 95(6):1081-92.

Baker A, Cotten M. 1997. Delivery of bacterial artificial chromosomes into mammalian cells with psoralen-inactivated adenovirus carrier. *Nucleic Acids Res* 25(10):1950-6.

Baker A, Saltik M, Lehrmann H, Killisch I, Mautner V, Lamm G, Christofori G, Cotten M. 1997. Polyethylenimine (PEI) is a simple, inexpensive and effective reagent for condensing and linking plasmid DNA to adenovirus for gene delivery. *Gene Ther* 4(8):773-82.

Barjot C, Hartigan-O'Connor D, Salvatori G, Scott JM, Chamberlain JS. 2002. Gutted adenoviral vector growth using E1/E2b/E3-deleted helper viruses. *J Gene Med* 4(5):480-9.

Berdichevsky M, Gentile MP, Hughes B, Meis P, Peltier J, Blumentals I, Aunins J, Altaras NE. 2008. Establishment of higher passage PER.C6 cells for adenovirus manufacture. *Biotechnol Prog* 24(1):158-65.

Bett AJ, Prevec L, Graham FL. 1993. Packaging capacity and stability of human adenovirus type 5 vectors. *J Virol* 67(10):5911-21.

Blanche F, Cameron B, Barbot A, Ferrero L, Guillemin T, Guyot S, Somarriba S, Bisch D. 2000. An improved anion-exchange HPLC method for the detection and purification of adenoviral particles. *Gene Ther* 7(12):1055-62.

Branca MA. 2005. Gene therapy: cursed or inching towards credibility? *Nat Biotechnol* 23(5):519-21.

Brunetti-Pierri N, Ng P. 2006. Progress towards the clinical application of helper-dependent adenoviral vectors for liver and lung gene therapy. *Curr Opin Mol Ther* 8(5):446-54.

Brunetti-Pierri N, Ng P. 2008. Progress and prospects: gene therapy for genetic diseases with helper-dependent adenoviral vectors. *Gene Ther* 15(8):553-60.

Burch AD, Weller SK. 2005. Herpes simplex virus type 1 DNA polymerase requires the mammalian chaperone hsp90 for proper localization to the nucleus. *J Virol* 79(16):10740-9.

Burova E, Ioffe E. 2005. Chromatographic purification of recombinant adenoviral and adeno-associated viral vectors: methods and implications. *Gene Ther* 12 Suppl 1:S5-17.

Callahan JD. 2002. A Statistical Analysis of Adenovirus Reference Material Assay Results. *Bioprocessing J* 1(3):43-7.

Campeau P, Chapdelaine P, Seigneurin-Venin S, Massie B, Tremblay JP. 2001. Transfection of large plasmids in primary human myoblasts. *Gene Ther* 8(18):1387-94.

Catalucci D, Sporeno E, Cirillo A, Ciliberto G, Nicosia A, Colloca S. 2005. An adenovirus type 5 (Ad5) amplicon-based packaging cell line for production of high-capacity helper-independent deltaE1-E2-E3-E4 Ad5 vectors. *J Virol* 79(10):6400-9.

Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M. 1996. Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. *J Virol* 70(7):4805-10.

Cherng JY, Schuurmans-Nieuwenbroek NM, Jiskoot W, Talsma H, Zuidam NJ, Hennink WE, Crommelin DJ. 1999. Effect of DNA topology on the transfection efficiency of poly((2-dimethylamino)ethyl methacrylate)-plasmid complexes. *J Control Release* 60(2-3):343-53.

Cheshenko N, Krougliak N, Eisensmith RC, Krougliak VA. 2001. A novel system for the production of fully deleted adenovirus vectors that does not require helper adenovirus. *Gene Ther* 8(11):846-54.

Chromy LR, Pipas JM, Garcea RL. 2003. Chaperone-mediated in vitro assembly of Polyomavirus capsids. *Proc Natl Acad Sci U S A* 100(18):10477-82.

Cortin V, Thibault J, Jacob D, Garnier A. 2004. High-titer adenovirus vector production in 293S cell perfusion culture. *Biotechnol Prog* 20(3):858-63.

Côté J, Bourget L, Garnier A, Kamen A. 1997. Study of adenovirus production in serum-free 293SF suspension culture by GFP-expression monitoring. *Biotechnol Prog* 13(6):709-14.

Côté J, Garnier A, Massie B, Kamen A. 1998. Serum-free production of recombinant proteins and adenoviral vectors by 293SF-3F6 cells. *Biotechnol Bioeng* 59:567-65.

Cotten M, Wagner E, Zatloukal K, Phillips S, Curiel DT, Birnstiel ML. 1992. High-efficiency receptor-mediated delivery of small and large (48 kilobase gene constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles. *Proc Natl Acad Sci U S A* 89(13):6094-8.

Curiel DT, Agarwal S, Wagner E, Cotten M. 1991. Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. *Proc Natl Acad Sci U S A* 88(19):8850-4.

Danthinne X, Imperiale MJ. 2000. Production of first generation adenovirus vectors: a review. *Gene Ther* 7(20):1707-14.

Dettenhofer M, Yu XF. 1999. Highly purified human immunodeficiency virus type 1 reveals a virtual absence of Vif in virions. *J Virol* 73(2):1460-7.

Diebold SS, Lehrmann H, Kursa M, Wagner E, Cotten M, Zenke M. 1999. Efficient gene delivery into human dendritic cells by adenovirus polyethylenimine and mannose polyethylenimine transfection. *Hum Gene Ther* 10(5):775-86.

Dunphy EJ, Redman RA, Herweijer H, Cripe TP. 1999. Reciprocal enhancement of gene transfer by combinatorial adenovirus transduction and plasmid DNA transfection in vitro and in vivo. *Hum Gene Ther* 10(14):2407-17.

Durocher Y, Perret S, Kamen A. 2002. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res* 30(2):E9.

Durocher Y, Pham PL, St-Laurent G, Jacob D, Cass B, Chahal P, Lau CJ, Nalbantoglu J, Kamen A. 2007. Scalable serum-free production of recombinant adeno-associated virus type 2 by transfection of 293 suspension cells. *J Virol Methods*.

Engelhardt JF, Ye X, Doranz B, Wilson JM. 1994. Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. *Proc Natl Acad Sci U S A* 91(13):6196-200.

Everts B, van der Poel HG. 2005. Replication-selective oncolytic viruses in the treatment of cancer. *Cancer Gene Ther* 12(2):141-61.

Fallaux FJ, Bout A, van der Velde I, van den Wollenberg DJ, Hehir KM, Keegan J, Auger C, Cramer SJ, van Ormondt H, van der Eb AJ and others. 1998. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum Gene Ther* 9(13):1909-17.

Fallaux FJ, Kranenburg O, Cramer SJ, Houweling A, Van Ormondt H, Hoeben RC, Van Der Eb AJ. 1996. Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum Gene Ther* 7(2):215-22.

Ferreira TB, Ferreira AL, Carrondo MJ, Alves PM. 2005. Effect of re-feed strategies and non-ammoniagenic medium on adenovirus production at high cell densities. *J Biotechnol* 119(3):272-80.

Ford T, Graham J, Rickwood D. 1994. Iodixanol: a nonionic iso-osmotic centrifugation medium for the formation of self-generated gradients. *Anal Biochem* 220(2):360-6.

Gao GP, Engdahl RK, Wilson JM. 2000. A cell line for high-yield production of E1-deleted adenovirus vectors without the emergence of replication-competent virus. *Hum Gene Ther* 11(1):213-9.

Garnier A, Cote J, Nadeau I, Kamen A, Massie B. 1994. Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells. *Cytotechnology* 15(1-3):145-55.

Gias E, Nielsen SU, Morgan LA, Toms GL. 2008. Purification of human respiratory syncytial virus by ultracentrifugation in iodixanol density gradient. *J Virol Methods* 147(2):328-32.

Gilbert PA, Garnier A, Jacob D, Kamen A. 2000. On-line measurement of GFP fluorescence for the monitoring of recombinant adenovirus production. *Biotechnol Lett* 22:561-7.

Gilbert PA, Kamen A, Bernier A, Garnier A. 2007. A simple macroscopic model for the diffusion and adsorption kinetics of r-adenovirus. *Biotechnol Bioeng* 98(1):239-51.

Glotzer JB, Saltik M, Chiocca S, Michou AI, Moseley P, Cotten M. 2000. Activation of heat-shock response by an adenovirus is essential for virus replication. *Nature* 407(6801):207-11.

Gorziglia MI, Kadan MJ, Yei S, Lim J, Lee GM, Luthra R, Trapnell BC. 1996. Elimination of both E1 and E2 from adenovirus vectors further improves prospects for in vivo human gene therapy. *J Virol* 70(6):4173-8.

Gorziglia MI, Lapcevich C, Roy S, Kang Q, Kadan M, Wu V, Pechan P, Kaleko M. 1999. Generation of an adenovirus vector lacking E1, e2a, E3, and all of E4 except open reading frame 3. *J Virol* 73(7):6048-55.

Grable M, Hearing P. 1992. cis and trans requirements for the selective packaging of adenovirus type 5 DNA. *J Virol* 66(2):723-31.

Graham FL, Smiley J, Russell WC, Nairn R. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 36(1):59-74.

Graham FL, van der Eb AJ. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52(2):456-67.

Gueret V, Negrete-Virgen JA, Lyddiatt A, Al-Rubeai M. 2002. Rapid titration of adenoviral infectivity by flow cytometry in batch culture of infected HEK293 cells. *Cytotechnology* 38(1-3):87-97.

Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps ML. 1997. Construction of adenovirus vectors through Cre-lox recombination. *J Virol* 71(3):1842-9.

Hartigan-O'Connor D, Amalfitano A, Chamberlain JS. 1999. Improved production of gutted adenovirus in cells expressing adenovirus preterminal protein and DNA polymerase. *J Virol* 73(9):7835-41.

Hartigan-O'Connor D, Barjot C, Crawford R, Chamberlain JS. 2002a. Efficient rescue of gutted adenovirus genomes allows rapid production of concentrated stocks without negative selection. *Hum Gene Ther* 13(4):519-31.

Hartigan-O'Connor D, Barjot C, Salvatori G, Chamberlain JS. 2002b. Generation and growth of gutted adenoviral vectors. *Methods Enzymol* 346:224-46.

Haviv YS, Blackwell JL, Li H, Wang M, Lei X, Curiel DT. 2001. Heat shock and heat shock protein 70i enhance the oncolytic effect of replicative adenovirus. *Cancer Res* 61(23):8361-5.

Hehir KM, Armentano D, Cardoza LM, Choquette TL, Berthelette PB, White GA, Couture LA, Everton MB, Keegan J, Martin JM and others. 1996. Molecular characterization of replication-competent variants of adenovirus vectors and genome modifications to prevent their occurrence. *J Virol* 70(12):8459-67.

Henry O, Dormond E, Perrier M, Kamen A. 2004. Insights into adenoviral vector production kinetics in acoustic filter-based perfusion cultures. *Biotechnol Bioeng* 86(7):765-74.

Henry O, Perrier M, Kamen A. 2005. Metabolic flux analysis of HEK-293 cells in perfusion cultures for the production of adenoviral vectors. *Metab Eng* 7(5-6):467-76.

Hutchins B. 2002. Development of a Reference Material for Characterizing Adenovirus Vectors. *Bioprocessing J* 1(1):25-8.

Hutchins B, Sajjadi N, Seaver S, Shepherd A, Bauer SR, Simek S, Carson K, Aguilar-Cordova E. 2000. Working toward an adenoviral vector testing standard. *Mol Ther* 2(6):532-4.

Imler JL, Bout A, Dreyer D, Dieterle A, Schultz H, Valerio D, Mehtali M, Pavirani A. 1995. Trans-complementation of E1-deleted adenovirus: a new vector to reduce the possibility of codissemination of wild-type and recombinant adenoviruses. *Hum Gene Ther* 6(6):711-21.

Imperiale MJ, Kao HT, Feldman LT, Nevins JR, Strickland S. 1984. Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1A-like activity. *Mol Cell Biol* 4(5):867-74.

Ishii-Watabe A, Uchida E, Iwata A, Nagata R, Satoh K, Fan K, Murata M, Mizuguchi H, Kawasaki N, Kawanishi T and others. 2003. Detection of replication-competent adenoviruses spiked into recombinant adenovirus vector products by infectivity PCR. *Mol Ther* 8(6):1009-16.

Iyer P, Ostrove JM, Vacante D. 1999. Comparaison of manufacturing techniques for adenovirus production. *Cytotechnology* 30:169-72.

Jardon M, Garnier A. 2003. PH, pCO2, and temperature effect on R-adenovirus production. *Biotechnol Prog* 19(1):202-8.

Kamen A, Henry O. 2004. Development and optimization of an adenovirus production process. *J Gene Med* 6 Suppl 1:S184-92.

Kao HT, Nevins JR. 1983. Transcriptional activation and subsequent control of the human heat shock gene during adenovirus infection. *Mol Cell Biol* 3(11):2058-65.

Klyushnichenko V, Bernier A, Kamen A, Harmsen E. 2001. Improved high-performance liquid chromatographic method in the analysis of adenovirus particles. *Journal of Chromatography B. Biomedical Sciences and Applications* 755(1-2):27-36.

Kochanek S. 1999. High-capacity adenoviral vectors for gene transfer and somatic gene therapy. *Hum Gene Ther* 10(15):2451-9.

Kreppel F, Biermann V, Kochanek S, Schiedner G. 2002. A DNA-based method to assay total and infectious particle contents and helper virus contamination in high-capacity adenoviral vector preparations. *Hum Gene Ther* 13(10):1151-6.

Kumar-Singh R, Chamberlain JS. 1996. Encapsidated adenovirus minichromosomes allow delivery and expression of a 14 kb dystrophin cDNA to muscle cells. *Hum Mol Genet* 5(7):913-21.

Lehmberg E, Traina JA, Chakel JA, Chang RJ, Parkman M, McCaman MT, Murakami PK, Lahidji V, Nelson JW, Hancock WS and others. 1999. Reversed-phase high-performance liquid chromatographic assay for the adenovirus type 5 proteome. *J Chromatogr B Biomed Sci Appl* 732(2):411-23.

Lewis AM, Jr., Prigge KO, Rowe WP. 1966. Studies of adenovirus-SV40 hybrid viruses. IV. An adenovirus type 2 strain carrying the infectious SV40 genome. *Proc Natl Acad Sci U S A* 55(3):526-31.

Lewis AM, Jr., Rowe WP. 1970. Isolation of two plaque variants from the adenovirus type 2-simian virus 40 hybrid population which differ in their efficiency in yielding simian virus 40. *J Virol* 5(4):413-20.

Lieber A, He CY, Kirillova I, Kay MA. 1996. Recombinant adenoviruses with large deletions generated by Cre-mediated excision exhibit different biological properties compared with first-generation vectors in vitro and in vivo. *J Virol* 70(12):8944-60.

Liu CH, Wu PS. 2007. Optimization of adenoviral production in human embryonic kidney cells using response surface methodology. *J Biosci Bioeng* 103(5):406-11.

Liu H, Naismith JH, Hay RT. 2003. Adenovirus DNA replication. *Curr Top Microbiol Immunol* 272:131-64.

Lochmuller H, Jani A, Huard J, Prescott S, Simoneau M, Massie B, Karpati G, Acsadi G. 1994. Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants (delta E1 + delta E3) during multiple passages in 293 cells. *Hum Gene Ther* 5(12):1485-91.

Longley R, Radzniak L, Santoro M, Tsao YS, Condon RGG, Lio P, Voloch M, Liu Z. 2005. Development of a Serum-free Suspension Process for the Production of a Conditionally Replicating Adenovirus using A549 Cells *Cytotechnology* 49(2-3):161-71.

Lopez T, Lopez S, Arias CF. 2006. Heat shock enhances the susceptibility of BHK cells to rotavirus infection through the facilitation of entry and post-entry virus replication steps. *Virus Res* 121(1):74-83.

Louis N, Evelegh C, Graham FL. 1997. Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line. *Virology* 233(2):423-9.

Lusky M. 2005. Good manufacturing practice production of adenoviral vectors for clinical trials. *Hum Gene Ther* 16(3):281-91.

Lusky M, Christ M, Rittner K, Dieterle A, Dreyer D, Mourot B, Schultz H, Stoeckel F, Pavirani A, Mehtali M. 1998. In vitro and in vivo biology of recombinant adenovirus vectors with E1, E1/E2A, or E1/E4 deleted. *J Virol* 72(3):2022-32.

Lusky M, Grave L, Dieterle A, Dreyer D, Christ M, Ziller C, Furstenberger P, Kintz J, Hadji DA, Pavirani A and others. 1999. Regulation of adenovirus-mediated transgene expression by the viral E4 gene products: requirement for E4 ORF3. *J Virol* 73(10):8308-19.

Ma L, Bluyssen HA, De Raeymaeker M, Laurysens V, van der Beek N, Pavliska H, van Zonneveld AJ, Tomme P, van Es HH. 2001. Rapid determination of adenoviral vector titers by quantitative real-time PCR. *J Virol Methods* 93(1-2):181-8.

Maizel JVJ, White DO, Scharff MD. 1968. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 36(1):115-25.

Maranga L, Aunins JG, Zhou W. 2005. Characterization of changes in PER.C6 cellular metabolism during growth and propagation of a replication-deficient adenovirus vector. *Biotechnol Bioeng* 90(5):645-55.

McConnell MJ, Imperiale MJ. 2004. Biology of adenovirus and its use as a vector for gene therapy. *Hum Gene Ther* 15(11):1022-33.

Meghrous J, Aucoin MG, Jacob D, Chahal PS, Arcand N, Kamen AA. 2005. Production of recombinant adeno-associated viral vectors using a baculovirus/insect cell suspension culture system: from shake flasks to a 20-L bioreactor. *Biotechnol Prog* 21(1):154-60.

Meier O, Greber UF. 2003. Adenovirus endocytosis. *J Gene Med* 5(6):451-62.

Meneses-Acosta A, Dormond E, Jacob D, Tom R, Bernier A, Perret S, St-Laurent G, Durocher Y, Gilbert R, Kamen A. 2007. Development of a suspension serum-free helper-dependent adenovirus production system and assessment of co-infection conditions. *J Virol Methods*.

Meunier-Durmort C, Grimal H, Sachs LM, Demeneix BA, Forest C. 1997. Adenovirus enhancement of polyethylenimine-mediated transfer of regulated genes in differentiated cells. *Gene Ther* 4(8):808-14.

Mitani K, Graham FL, Caskey CT, Kochanek S. 1995. Rescue, propagation, and partial purification of a helper virus-dependent adenovirus vector. *Proc Natl Acad Sci U S A* 92(9):3854-8.

Mittereder N, March KL, Trapnell BC. 1996. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J Virol* 70(11):7498-509.

Monica TJ, Montgomery T, Ayala JL, Schoofs GM, Whiteley EM, Roth G, Garbutt JJ, Harvey S, Castillo FJ. 2000. Monitoring adenovirus infections with on-line and off-line methods. *Biotechnol Prog* 16(5):866-71.

Montgomery DC. 2005. *Design and analysis of experiments*. In: Wiley, editor. New York. p 431-2.

Morsy MA, Caskey CT. 1999. Expanded-capacity adenoviral vectors--the helper-dependent vectors. *Mol Med Today* 5(1):18-24.

Murakami P, McCaman MT. 1999. Quantitation of adenovirus DNA and virus particles with the PicoGreen fluorescent Dye. *Anal Biochem* 274(2):283-8.

Nadeau I, Garnier A, Côté J, Massie B, Chavarie C, Kamen A. 1996. Improvement of recombinant protein production with the human adenovirus/293S expression system using fed-batch strategies. *Biotechnol Bioeng* 51:613-23.

Nadeau I, Gilbert PA, Jacob D, Perrier M, Kamen A. 2002. Low-protein medium affects the 293SF central metabolism during growth and infection with adenovirus. *Biotechnol Bioeng* 77(1):91-104.

Nadeau I, Jacob D, Perrier M, Kamen A. 2000. 293SF metabolic flux analysis during cell growth and infection with an adenoviral vector. *Biotechnol Prog* 16(5):872-84.

Nadeau I, Kamen A. 2003. Production of adenovirus vector for gene therapy. *Biotechnology Advances* 20(7-8):475-489.

National Institute of Health. 2002. Assessement of adenoviral vectors safety and toxicity: Report of the National Institutes of Health Recombinant DNA Advisory Committee. *Hum Gene Ther* 13(1):1-13.

Nevins JR. 1982. Induction of the synthesis of a 70,000 dalton mammalian heat shock protein by the adenovirus E1A gene product. *Cell* 29(3):913-9.

Ng P, Beauchamp C, Evelegh C, Parks R, Graham FL. 2001. Development of a FLP/frt system for generating helper-dependent adenoviral vectors. *Mol Ther* 3(5 Pt 1):809-15.

Ng P, Evelegh C, Cummings D, Graham FL. 2002a. Cre levels limit packaging signal excision efficiency in the Cre/loxP helper-dependent adenoviral vector system. *J Virol* 76(9):4181-9.

Ng P, Parks RJ, Graham FL. 2002b. Preparation of helper-dependent adenoviral vectors. *Methods Mol Med* 69:371-88.

Nielsen SU, Bassendine MF, Burt AD, Martin C, Pumeechockchai W, Toms GL. 2006. Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. *J Virol* 80(5):2418-28.

Nossen JO, Aakhus T, Berg KJ, Jorgensen NP, Andrew E. 1990. Experience with iodixanol, a new nonionic dimeric contrast medium. Preliminary results from the human phase I study. *Invest Radiol* 25 Suppl 1:S113-4.

Nyberg-Hoffman C, Shabram P, Li W, Giroux D, Aguilar-Cordova E. 1997. Sensitivity and reproducibility in adenoviral infectious titer determination. *Nat Med* 3(7):808-11.

O'Reilly DR, Miller LK, Luckow VA. 1994. *Baculovirus Expression Vectors: A Laboratory Manual.* New York: Oxford University Press.

Oka K, Chan L. 2005. Construction and characterization of helper-dependent adenoviral vectors for sustained in vivo gene therapy. *Methods Mol Med* 108:329-50.

Okada T, Nomoto T, Yoshioka T, Nonaka-Sarukawa M, Ito T, Ogura T, Iwata-Okada M, Uchibori R, Shimazaki K, Mizukami H and others. 2005. Large-scale production of recombinant viruses by use of a large culture vessel with active gassing. *Hum Gene Ther* 16(10):1212-8.

Palmer D, Ng P. 2003. Improved system for helper-dependent adenoviral vector production. *Mol Ther* 8(5):846-52.

Palmer DJ, Ng P. 2004. Physical and infectious titers of helper-dependent adenoviral vectors: a method of direct comparison to the adenovirus reference material. *Mol Ther* 10(4):792-8.

Palmer DJ, Ng P. 2005. Helper-dependent adenoviral vectors for gene therapy. *Hum Gene Ther* 16(1):1-16.

Palomares LA, Lopez S, Ramirez OT. 2002. Strategies for manipulating the relative concentration of recombinant rotavirus structural proteins during simultaneous production by insect cells. *Biotechnology and Bioengineering* 78(6):635-44.

Park MT, Lee GM. 2000. Rapid titer assay of adenovirus containing green fluorescent protein gene using flow cytometry analysis. *Bioprocess Engineering* 22:403-6.

Park MT, Lee MS, Kim SH, Jo EC, Lee GM. 2004. Influence of culture passages on growth kinetics and adenovirus vector production for gene therapy in monolayer and suspension cultures of HEK 293 cells. *Appl Microbiol Biotechnol* 65(5):553-8.

Parks RJ, Bramson JL, Wan Y, Addison CL, Graham FL. 1999. Effects of stuffer DNA on transgene expression from helper-dependent adenovirus vectors. *J Virol* 73(10):8027-34.

Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A* 93(24):13565-70.

Parks RJ, Graham FL. 1997. A helper-dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging. *J Virol* 71(4):3293-8.

Pearson S, Jia H, Kandachi K. 2004. China approves first gene therapy. *Nat Biotechnol* 22(1):3-4.

Peng HH, Wu S, Davis JJ, Wang L, Roth JA, Marini FC, 3rd, Fang B. 2006. A rapid and efficient method for purification of recombinant adenovirus with arginine-glycine-aspartic acid-modified fibers. *Anal Biochem* 354(1):140-7.

Peng Z. 2005. Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. *Hum Gene Ther* 16(9):1016-27.

Phillips B, Abravaya K, Morimoto RI. 1991. Analysis of the specificity and mechanism of transcriptional activation of the human hsp70 gene during infection by DNA viruses. *J Virol* 65(11):5680-92.

Pierce WE, Rosenbaum MJ, Edwards EA, Peckinpaugh RO, Jackson GG. 1968. Live and inactivated adenovirus vaccines for the prevention of acute respiratory illness in naval recruits. *Am J Epidemiol* 87(1):237-46.

Puntel M, Curtin JF, Zirger JM, Muhammad AK, Xiong W, Liu C, Hu J, Kroeger KM, Czer P, Sciascia S and others. 2006. Quantification of high-capacity helper-dependent adenoviral vector genomes in vitro and in vivo, using quantitative TaqMan real-time polymerase chain reaction. *Hum Gene Ther* 17(5):531-44.

Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, Wilson JM, Batshaw ML. 2003. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* 80(1-2):148-58.

Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG. 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med* 84(3):570-3.

Sakhuja K, Reddy PS, Ganesh S, Cantaniag F, Pattison S, Limbach P, Kayda DB, Kadan MJ, Kaleko M, Connelly S. 2003. Optimization of the generation and propagation of gutless adenoviral vectors. *Hum Gene Ther* 14(3):243-54.

Sandhu KS, Al-Rubeai M. 2008. Monitoring of the adenovirus production process by flow cytometry. *Biotechnol Prog* 24(1):250-61.

Sandig V, Youil R, Bett AJ, Franlin LL, Oshima M, Maione D, Wang F, Metzker ML, Savino R, Caskey CT. 2000. Optimization of the helper-dependent adenovirus system for production and potency in vivo. *Proc Natl Acad Sci U S A* 97(3):1002-7.

Santomenna LD, Colberg-Poley AM. 1990. Induction of cellular hsp70 expression by human cytomegalovirus. *J Virol* 64(5):2033-40.

Sargent KL, Ng P, Evelegh C, Graham FL, Parks RJ. 2004. Development of a size-restricted pIX-deleted helper virus for amplification of helper-dependent adenovirus vectors. *Gene Ther* 11(6):504-11.

Sato M, Suzuki S, Kubo S, Mitani K. 2002. Replication and packaging of helper-dependent adenoviral vectors. *Gene Ther* 9(7):472-6.

Schalk JA, de Vries CG, Orzechowski TJ, Rots MG. 2007. A rapid and sensitive assay for detection of replication-competent adenoviruses by a combination of microcarrier cell culture and quantitative PCR. *J Virol Methods* 145(2):89-95.

Schenk TE. 2001. *Adenoviridae: The Viruses and Their Replication.* In: Knipe DM, Howley PM, editors. Fundamental Virology. Lippincott Williams & Wilkins ed. p 979-1016.

Schiedner G, Hertel S, Johnston M, Biermann V, Dries V, Kochanek S. 2002. Variables affecting in vivo performance of high-capacity adenovirus vectors. *J Virol* 76(4):1600-9.

Schiedner G, Hertel S, Kochanek S. 2000. Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production. *Hum Gene Ther* 11(15):2105-16.

Schoofs G, Monica TJ, Ayala J, Howtitz J, Montgomery T, Roth G, Castillo FJ. 1998. A High-Yielding Serum-Free, Suspension culture Process to Manufacture Recombinant Adenoviral Vectors for Gene Therapy. *Cytotechnology* 28:81-89.

Segura MM, Garnier A, Kamen A. 2006. Purification and characterization of retrovirus vector particles by rate zonal ultracentrifugation. *J Virol Methods* 133(1):82-91.

Shabram P, Aguilar-Cordova E. 2000. Multiplicity of infection/multiplicity of confusion. *Mol Ther* 2(5):420-1.

Shabram PW, Giroux DD, Goudreau AM, Gregory RJ, Horn MT, Huyghe BG, Liu X, Nunnally MH, Sugarman BJ, Sutjipto S. 1997. Analytical anion-exchange HPLC of recombinant type-5 adenoviral particles. *Hum Gene Ther* 8(4):453-65.

Simek S, Byrne BJ, Bauer SR. 2002. FDA perspectives on the use of the adenovirus reference material. *Bioprocessing J* 1(3):40-42.

Subramanian S, Kim JJ, Harding F, Altaras GM, Aunins JG, Zhou W. 2008. Scaleable production of adenoviral vectors by transfection of adherent PER.C6 cells. (8756-7938 (Print)).

Svaland MG, Haider T, Langseth-Manrique K, Andrew E, Hals PA. 1992. Human pharmacokinetics of iodixanol. *Invest Radiol* 27(2):130-3.

Tamanoi F, Stillman BW. 1982. Function of adenovirus terminal protein in the initiation of DNA replication. *Proc Natl Acad Sci U S A* 79(7):2221-5.

Tatsis N, Ertl HC. 2004. Adenoviruses as vaccine vectors. *Mol Ther* 10(4):616-29.

Transfiguracion J, Bernier A, Arcand N, Chahal P, Kamen A. 2001. Validation of a high-performance liquid chromatographic assay for the quantification of adenovirus type 5 particles. *J Chromatogr B Biomed Sci Appl* 761(2):187-94.

Umana P, Gerdes CA, Stone D, Davis JR, Ward D, Castro MG, Lowenstein PR. 2001. Efficient FLPe recombinase enables scalable production of helper-dependent adenoviral vectors with negligible helper-virus contamination. *Nat Biotechnol* 19(6):582-5.

van Bergen BG, van der Ley PA, van Driel W, van Mansfeld AD, van der Vliet PC. 1983. Replication of origin containing adenovirus DNA fragments that do not carry the terminal protein. *Nucleic Acids Res* 11(7):1975-89.

Varga CM, Tedford NC, Thomas M, Klibanov AM, Griffith LG, Lauffenburger DA. 2005. Quantitative comparison of polyethylenimine formulations and adenoviral vectors in terms of intracellular gene delivery processes. *Gene Ther* 12(13):1023-32.

Varga CM, Wickham TJ, Lauffenburger DA. 2000. Receptor-mediated targeting of gene delivery vectors: insights from molecular mechanisms for improved vehicle design. *Biotechnol Bioeng* 70(6):593-605.

Vasconcelos D, Norrby E, Oglesbee M. 1998. The cellular stress response increases measles virus-induced cytopathic effect. *J Gen Virol* 79 (Pt 7):1769-73.

Voisard D, Meuwly F, Ruffieux PA, Baer G, Kadouri A. 2003. Potential of cell retention techniques for large-scale high-density perfusion culture of suspended mammalian cells. *Biotechnol Bioeng* 82(7):751-65.

von Groll A, Levin Y, Barbosa MC, Ravazzolo AP. 2006. Linear DNA low efficiency transfection by liposome can be improved by the use of cationic lipid as charge neutralizer. *Biotechnol Prog* 22(4):1220-4.

Wang F, Puddy AC, Mathis BC, Montalvo AG, Louis AA, McMackin JL, Xu J, Zhang Y, Tan CY, Schofield TL and others. 2005. Using QPCR to assign infectious potencies to adenovirus based vaccines and vectors for gene therapy: toward a universal method for the facile quantitation of virus and vector potency. *Vaccine* 23(36):4500-8.

Wang Q, Greenburg G, Bunch D, Farson D, Finer MH. 1997. Persistent transgene expression in mouse liver following in vivo gene transfer with a delta E1/delta E4 adenovirus vector. *Gene Ther* 4(5):393-400.

Wang Q, Jia XC, Finer MH. 1995. A packaging cell line for propagation of recombinant adenovirus vectors containing two lethal gene-region deletions. *Gene Ther* 2(10):775-83.

Weaver LS, Kadan MJ. 2000. Evaluation of adenoviral vectors by flow cytometry. *Methods* 21(3):297-312.

Wu SC, Huang GY, Liu JH. 2002. Production of retrovirus and adenovirus vectors for gene therapy: a comparative study using microcarrier and stationary cell culture. *Biotechnol Prog* 18(3):617-22.

Xie L, Metallo C, Warren J, Pilbrough W, Peltier J, Zhong T, Pikus L, Yancy A, Leung J, Aunins JG and others. 2003. Large-scale propagation of a replication-defective adenovirus vector in stirred-tank bioreactor PER.C6 cell culture under sparging conditions. *Biotechnol Bioeng* 83(1):45-52.

Xie L, Pilbrough W, Metallo C, Zhong T, Pikus L, Leung J, Aunins JG, Zhou W. 2002. Serum-free suspension cultivation of PER.C6(R) cells and recombinant adenovirus production under different pH conditions. *Biotechnol Bioeng* 80(5):569-79.

Xu Q, Arevalo MT, Pichichero ME, Zeng M. 2006. A new complementing cell line for replication-incompetent E1–deleted adenovirus propagation *Cytotechnology* 51(3):x-x.

Yeh P, Dedieu JF, Orsini C, Vigne E, Denefle P, Perricaudet M. 1996. Efficient dual transcomplementation of adenovirus E1 and E4 regions from a 293-derived cell line expressing a minimal E4 functional unit. *J Virol* 70(1):559-65.

Yoshimura K, Rosenfeld MA, Seth P, Crystal RG. 1993. Adenovirus-mediated augmentation of cell transfection with unmodified plasmid vectors. *J Biol Chem* 268(4):2300-3.

Youil R, Toner TJ, Su Q, Casimiro D, Shiver JW, Chen L, Bett AJ, Rogers BM, Burden EC, Tang A and others. 2003. Comparative analysis of the effects of packaging signal, transgene orientation, promoters, polyadenylation signals, and E3 region on growth properties of first-generation adenoviruses. *Hum Gene Ther* 14(10):1017-34.

Yuk IH, Olsen MM, Geyer S, Forestell SP. 2004. Perfusion cultures of human tumor cells: a scalable production platform for oncolytic adenoviral vectors. *Biotechnol Bioeng* 86(6):637-42.

Zhang C, Ferreira TB, Cruz PE, Alves PM, Haury M, Carrondo MJ. 2006. The importance of 293 cell cycle phase on adenovirus vector production. *Enzyme and Microbial Technology* 39:1328-32.

Zhang WW, Koch PE, Roth JA. 1995. Detection of wild-type contamination in a recombinant adenoviral preparation by PCR. *Biotechniques* 18(3):444-7.

Zhou H, Beaudet AL. 2000. A new vector system with inducible E2a cell line for production of higher titer and safer adenoviral vectors. *Virology* 275(2):348-57.

Zhou H, O'Neal W, Morral N, Beaudet AL. 1996. Development of a complementing cell line and a system for construction of adenovirus vectors with E1 and E2a deleted. *J Virol* 70(10):7030-8.

Zhou H, Zhao T, Pastore L, Nageh M, Zheng W, Rao XM, Beaudet AL. 2001. A Cre-expressing cell line and an E1/E2a double-deleted virus for preparation of helper-dependent adenovirus vector. *Mol Ther* 3(4):613-22.

Zhou HS, Zhao T, Rao XM, Beaudet AL. 2002. Production of helper-dependent adenovirus vector relies on helper virus structure and complementing. *J Gene Med* 4(5):498-509.

Zhu J, Grace M, Casale J, Chang AT, Musco ML, Bordens R, Greenberg R, Schaefer E, Indelicato SR. 1999. Characterization of replication-competent adenovirus isolates from large-scale production of a recombinant adenoviral vector. *Hum Gene Ther* 10(1):113-21.

Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, Chesnut K, Summerford C, Samulski RJ, Muzyczka N. 1999. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther* 6(6):973-85.