HUMAN GENE THERAPY 25:318–327 (April 2014)

DOI: 10.1089/hum.2014.007

Challenges in Manufacturing Adenoviral Vectors for Global Vaccine Product Deployment

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Abstract

Once adenovirus vector-based vaccines are licensed for the prevention of important infectious diseases, manufacturing processes capable of reliably delivering large numbers of vaccine doses will be required. The highest burden of disease for many infectious pathogens under investigation occurs in resource-poor settings. Therefore, the price per dose will be an important determinant of success. This review describes common practices for manufacturing replication-incompetent adenovirus vectors at clinical scale. Recent innovations and strategies aimed at improving the cost-effectiveness of manufacturing and ensuring high-volume vaccine production and purification are described. Hereto, technologies to increase bioreactor yields are reviewed. In addition, the use of single-use perfusion bioreactors, modification of some purification steps to avoid the use of expensive endonucleases, and use of charged filters during anion exchange all have the potential to bring down the cost of goods and are thus described. Finally, processes for ensuring quality throughout the manufacturing process, methods for testing viral identity, and safety of master seeds through to the end vaccine product are described.

Introduction

ADENOVIRAL VECTOR-BASED VACCINES are intensively studied because of their potential to prevent infectious diseases for which current vaccines are suboptimal (such as influenza and anthrax vaccines) or for which licensed vaccines are not available (such as malaria and human immunodeficiency virus [HIV]). Once adenovirus vector-based vaccines will be licensed for the prevention of infectious diseases, manufacturing methods capable of reliably delivering large numbers of vaccine doses will be required. The use of current, clinical-scale, traditional manufacturing processes for production of adenovirus vector vaccines is not deemed to be commercially viable. Hence, technical innovations are studied to increase production yield and reduce the cost of purification. Such innovations are deemed pivotal since, in order to combat diseases such as malaria, tuberculosis, and HIV, which are most prevalent in low-resource settings, the price per dose will be an important determining factor of success. This review describes common best practices for manufacturing replication-incompetent adenovirus vectors at clinical scale, as well as discusses promising strategies aimed at improving the cost-effectiveness of manufacturing replication-deficient adenoviral vectors.

Adenoviruses and Replication-Deficient Adenoviral Vectors

Within the mastadenovirus genus, many human adenovirus serotypes have been identified (Ishiko and Aoki, 2009) and are classified into seven subgroups (A to G) based on neutralization with specific antibodies present in antisera generated against the known serotypes (Madisch *et al*., 2005), or by computational analysis (Robinson *et al*., 2011; Walsh *et al*., 2011). Adenoviruses are able to infect a wide range of tissues and cells and can replicate efficiently in both dividing and nondividing cells. To generate a replication-deficient adenoviral vector, DNA encoding for proteins that *trans*-activate transcription of proteins pivotal for viral DNA replication and viral gene expression is deleted from the viral genome (the *E1* region). Additionally, a large portion of the *E3* gene, which is nonessential for *in vitro* replication, is deleted. This is because products of the *E3* gene have immune-modulating characteristics that might negatively influence the vaccine potency (Ginsberg *et al*., 1989). The space created in the viral genome by deletion of *E1* and *E3* is used to insert an expression cassette (promoter–polylinker–polyadenylation signal) that drives transcription of selected vaccine antigens (for instance, DNA encoding for HIV envelope or hemagglutinin antigen from

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influenza virus). In order to produce E1-deficient viruses, the E1 proteins have to be provided *in trans* using a cell line that stably expresses the E1 proteins. A commonly used E1-complementing cell line is HEK293 (Graham *et al*., 1977), which contains approximately 4300 bp of the left end of the adenovirus type 5 (Ad5) genome, including the left inverted terminal repeat, the packaging domain, and *E1* and *pIX* (Hehir *et al*., 1996). The HEK293 cell line allows efficient growth of E1-deleted Ad5 vectors, but is also known to induce replication-competent adenoviruses (RCA) because of homologous recombination between the E1-deleted Ad5 vector and the Ad5 sequences within the cell line (Lochmuller *et al*., 1994). The presence of RCAs is considered a safety risk because of their potentially transmissibility in humans. In order to avoid RCAs, a cell line was developed (PER.C6) that contained the Ad5-derived *E1A* and *E1B* genes only, and thus lacks sequences from outside the *E1* region (Fallaux *et al*., 1998). As a consequence, homologous recombination between the E1-deleted adenoviral vector and sequences within the cell line cannot occur and no RCA can be formed when matching vectors devoid of sequence overlap with the cell line are used. Rare events of nonhomologous recombination, however, have been observed in the past. Formation of the so-called helper-dependent E1-positive particles has been described at low frequency, but these particles cannot replicate independent from adenovirus and as such can disseminate in the host and consequently do not pose a direct safety risk (Murakami *et al.*, 2004; Farson *et al.*, 2006). When using serotypes other than Ad5, the risk of RCA formation in HEK293 cells is virtually nonexistent because of the lack of sequence homology between the non-Ad5 viral genome and the Ad5 sequences present in HEK293. The lack of sequence homology may, however, require further modification of the vector backbone. For some serotypes, the native *E4-orf6* sequence (*E4-orf6* interacts with E1 protein) was replaced with a sequence from Ad5 to obtain efficient growth of non-Ad5 replication-deficient vectors on HEK 293 or PER.C6 cells (Havenga, *et al.*, 2006; Abbink, *et al.*, 2007) or by providing the Ad5 E4-orf6 protein *in trans* by the HEK 293-orf6 cell line (Brough *et al*., 1996). Figure 1 provides a schematic overview of the vector backbone modifications and *trans*-complementing cell lines that in some cases is required to be able to produce human replication-deficient adenoviral vectors.

Current Common Practices for Small-Scale (10–50-liter) Adenovirus Vector Manufacturing

Clinical-grade production of replication-deficient adenoviral vectors is typically performed at 10–50-liter scale for

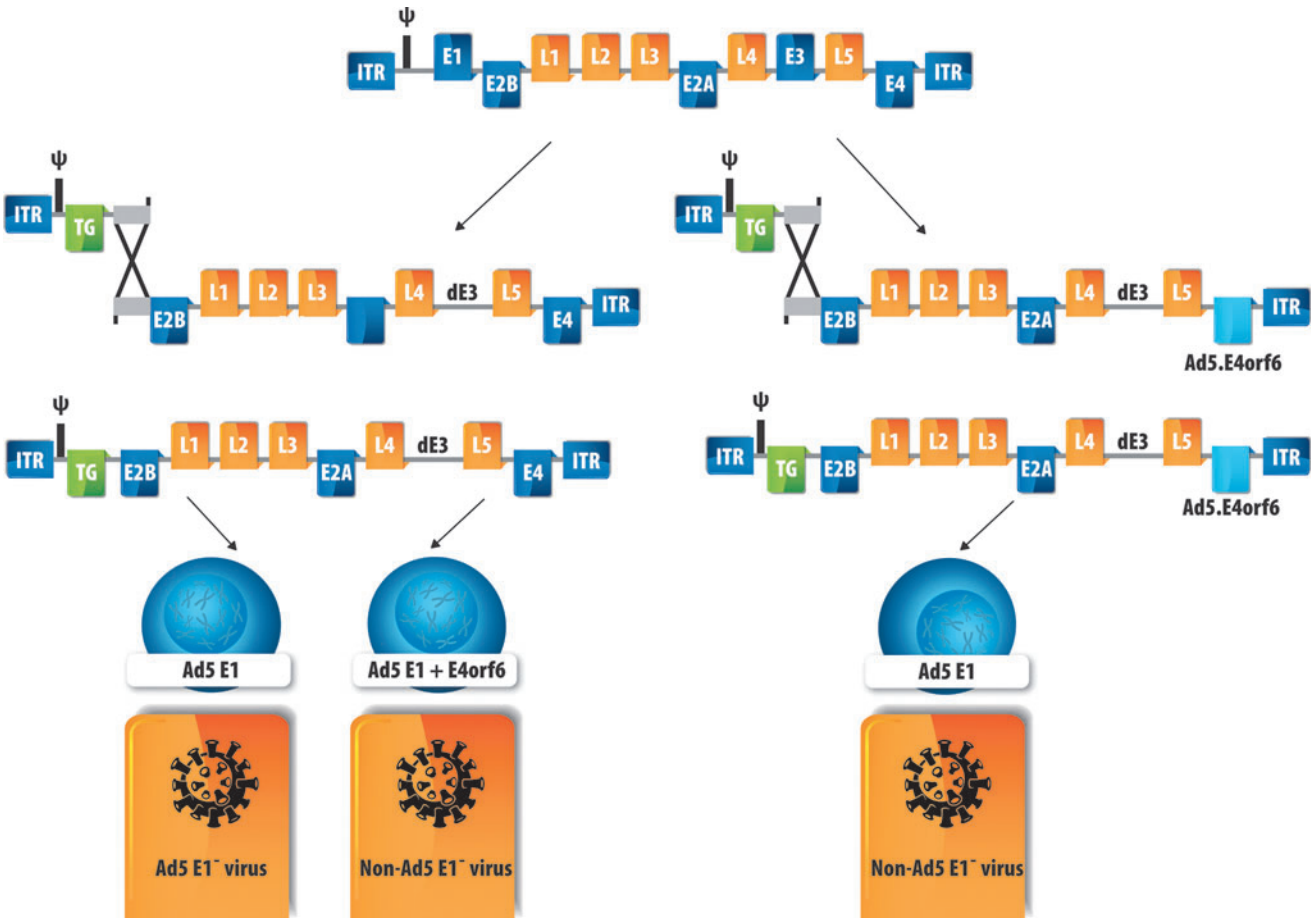

FIG. 1. Ad5-based adenoviral vectors (Ad5 E1− virus) are grown on Ad5 E1-complementing cell lines such as HEK293 and PER.C6. To grow non-Ad5 E1− vectors, the Ad5 E4-orf6 has to be provided *in trans* as well, either by the cell line (Ad5 E1+E4orf6 cell line) or by replacing the native E4-orf6 sequence with the Ad5 E4-orf6 sequence in the viral backbone.

phase I and II studies, often using a six-step purification process after production of the virus on the E1-complementing cell line (see Fig. 2 for a schematic representation). Production has historically been performed in a host of different systems ranging from multilayer culture flasks, cell factories, to roller bottles, and traditional bioreactors. One common denominator in all production systems is a cell deck confluence of around 70% when infection occurs under adherent conditions, or a cell density at infection between 0.5×10^6 and 1.0×10^6 cells/ml when using suspension cell growth (Kamen and Henry, 2004; Altaras *et al*., 2005). Typical yields have been reported for Ad5 of 10^{13} virus particles (VPs) per liter for standard batch bioreactor processes (Kamen and Henry, 2004; Altaras *et al*., 2005).

The steps involved in purification of adenovirus are (1) cell lysis and genomic DNA breakdown, (2) clarification with dead end filters, (3) concentration with ultrafiltration/diafiltration (UF/DF), (4) anion-exchange (AEX) purification, (5) gel filtration, and (6) dead-end filtration. Common practices for these processes are outlined briefly below.

Step 1: cell lysis and genomic DNA breakdown

Different methods have been explored to achieve effective cell lysis, the most common being freeze–thaw disruption (Huyghe *et al*., 1995; Altaras *et al*., 2005) or detergent lysis (Zhang *et al.*, 2001; Altaras *et al.*, 2005; Goerke *et al*., 2005a,b). Freeze–thaw is laborious and scalability is limited, whereas detergent lysis, in particular Triton X-100 at up to 1% (v/v), overcomes these difficulties (Zhang *et al.*, 2001; Altaras *et al*., 2005; van Corven and Weggeman, 2005).

The use of detergent has been reported to have additional benefits in that it enhances the efficiency of endonucleases, specifically Benzonase (Merck, 2003), and facilitates viral clearance for enveloped viruses (WHO, 2004). While the cell lysis step significantly increases the yield of adenoviral vector, it also results in release of contaminants such as intracellular proteins and cellular genomic nucleic acids. The presence of nucleic acid is a particular concern for adenovirus as DNA is known to mediate virus particle aggregation (Konz *et al*., 2005) and therefore the majority of current practices rely on postlysis incubations with nuclease (specifically Benzonase; up to 100 U/ml), to rapidly reduce the DNA concentration (Altaras *et al*., 2005; van Corven and Weggeman, 2005; Eglon *et al*., 2009; Puig *et al*., 2014). Benzonase acts to reduce nucleic acid chain length, which facilitates its removal in later steps, reduces viscosity, and helps to reduce nucleic acid mediated aggregation (Konz *et al*., 2005).

Step 2: clarification

After cell lysis and Benzonase treatment, clarification is usually carried out using depth filters at industrially relevant scales (Altaras *et al*., 2005; Puig *et al*., 2014). Clarification often consists of a filter train containing 1–2 depth filter(s) and a series of membrane filters with the last filter often employing a 0.2 μm pore size to protect subsequent (tangential flow filtration and column steps) and to reduce the bioburden load. This allows the postclarification material to be an in-process

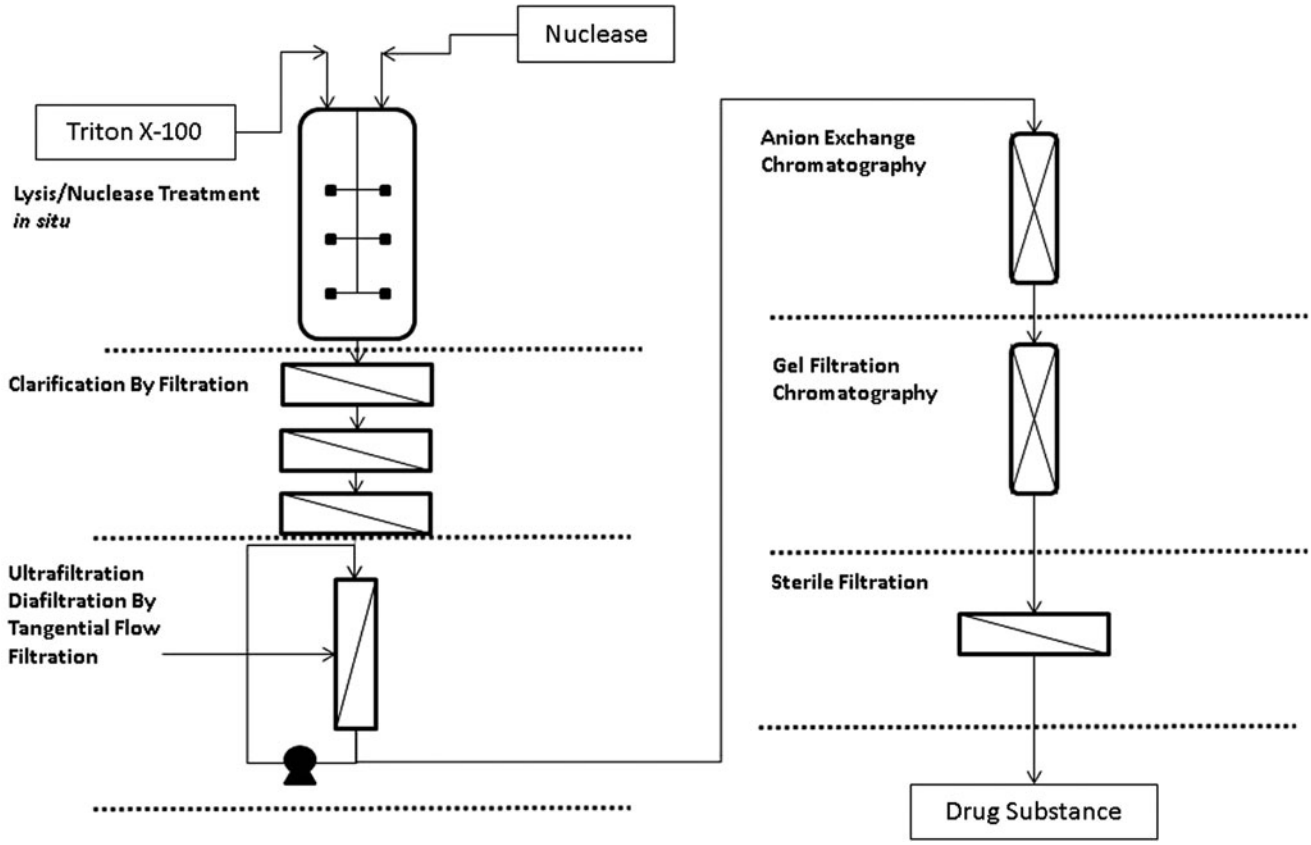

FIG. 2. Process flow diagram for the current best practice for small-scale (10–50-liter) adenovirus vector purification.

hold point. The clarification process allows the adenovirus to pass all the membranes, but particulate contaminants are removed. Depth filters may use a variety of materials, such as glass, polyether sulfone, and polypropylene (Lee *et al*., 2009).

Step 3: concentration

The concentration step reduces the bulk volume with (UF/DF) via tangential flow (Altaras *et al*., 2005; Segura *et al*., 2011; Puig *et al*., 2014). This step is scalable to large volumes and does not involve any phase change that could conceivably be detrimental to the virus (Altaras *et al*., 2005; Segura *et al*., 2011). While concentration is mainly used to reduce process volume for subsequent steps (Altaras *et al*., 2005; Segura *et al*., 2011), this step can also provide a high degree of purification via the reduction of small molecules, such as small proteins that interact with the virus (van Corven and Weggeman, 2005). The size of Ad5 is approximately 170 MDa (Shabram *et al*., 1997; Klyushnichenko *et al*., 2001), and therefore 100–500 kDa pore sizes are frequently used (Segura *et al*., 2011; Puig *et al*., 2014). Historically, the majority of UF/DF steps have used a hollow fiber configuration (Puig *et al*., 2014) as shear rates can be calculated.

Steps 4 and 5: anion exchange and gel filtration

The next step is usually a capture step using column chromatography—typically bead AEX chromatography (Altaras *et al*., 2005; Segura *et al*., 2011), this is because adenovirus is negatively charged at physiological pH (Altaras *et al*., 2005; Burova and Ioffe, 2005; Goerke *et al*., 2005b). A number of different resins have been used: Source Q (Shabram *et al*., 1997; Goerke *et al*., 2005b), Q Sepharose XL (Blanche *et al*., 2000; Eriksson *et al*., 2000; van Corven and Weggeman, 2005; Eglon *et al*., 2009), and Fractogel DEAE 650M (Huyghe *et al*., 1995; Green *et al*., 2002; Kamen and Henry, 2004). The use of anion exchange can result in coelution of moieties with similar charges (Segura *et al*., 2011). AEX chromatography in general gives high step recoveries: 60–90% in the majority of cases (Burova and Ioffe, 2005; Eglon *et al*., 2009; Segura *et al*., 2011). The eluate from this step contains adenovirus in high levels of salt, which needs to be removed in the polishing step. Polishing of the AEX eluate is normally achieved by gel filtration (Altaras *et al*., 2005; van Corven and Weggeman, 2005; Eglon *et al*., 2009; Segura *et al*., 2011). The aim of this step is further purification of the adenovirus by removal of small moieties, removal of salt, and to buffer exchange to the final formulation buffer. Resins used in this step typically include Toyopearl HW75F (Huyghe *et al*., 1995), Sepharose-4FF (van Corven and Weggeman, 2005; Eglon *et al*., 2009), Sephadex-G25 (Altaras *et al*., 2005), and Sephacryl-400HR (Kamen and Henry, 2004). Variable yields of 15–90% have been reported with gel filtration for adenovirus (Segura *et al*., 2011). This variability of yield is likely because of initial resin choice, with pore entrapment occurring when resins are used with pore sizes of a similar size to adenovirus (Huyghe *et al*., 1995).

Step 6: membrane filtration

The final step for the production of a GMP-grade adenovirus product is bioburden reduction through dead end filtration using a 0.2 μm sterile membrane. Because of commonality of this step with other biological products, manufacturers have a range of specific filters for this purpose.

Innovations to Improve Commercial Attractiveness of Adenoviral Vector-Based Vaccines

The development of adenovirus-based vaccines faces challenges (like any new vaccine) that need to be overcome in order to bring a vaccine to the market. Given the complexity of the pathogens being investigated as targets for adenovirus vectored vaccines, it is highly likely that such vaccines will require multiple antigens to augment the breath of the immunological responses in order to induce protective efficacy. The level of vaccine complexity can have a tremendous impact on the number and type of clinical studies required to demonstrate immunogenicity, efficacy, and safety and, moreover, can significantly impact the cost of manufacturing (Wilson, 2010). Therefore, it can be a disadvantage when a vaccine requires two or more vectors in order to be effective. In this respect, it is noteworthy that mapping the molecular biology of the adenoviral genome has progressed significantly, allowing for multiple antigens to be inserted in one expression cassette, or the use of multiple expression cassettes in one viral genome (Vogels *et al*., 2007). In addition, to the requirement for sophisticated vaccine designs and clinical evaluation, there are challenges to overcome with respect to vaccine production, purification, fill, finish, and characterization. Promising innovations that indicate the feasibility of manufacturing global adenoviral vector-based vaccines are described below.

Production (upstream processing)

Current common practice for manufacturing of adenoviral vectors results in a yield of approximately 10^{13} VP/liter of batch bioreactor process. If one was to pursue a birth cohort HIV vaccination strategy in countries with HIV incidence rates equal to or greater than 5 per 10,000, then the number of doses required could amount to 160 million (80 million newborns annually receiving two doses) (Sachs *et al*., 1999). This would translate to an annual demand for at least 8×10^{18} VP, at an estimated vaccine dose of 5×10^{10} VP per vial (Baden *et al*., 2013). If we consider a 10% retention and a 50% recovery from purification, then the fermentation process would need to deliver approximately 1.76×10^{19} VPs. Taking the current common fermentation yield of 10^{13} VP/liter, this would translate into an annual fermentation process of 1,760,000 liters. Such large-scale manufacturing is not deemed commercially viable using current processes, and further innovations to increase yield in production compared with such processes are essential for commercially successful product deployment of adenoviral vectors.

Attributes such as production temperature, pH, and medium components may play a role in obtaining higher adenoviral vector yields, and as such, establishing optimal parameters has been intensively researched. It has, however, become clear that optimization of these parameters alone will likely be insufficient to make a stirred tank batch process attractive for commercial production of a vaccine that is to be deployed at a global scale, especially in resource-poor settings. Innovations in the last decades have led to production systems that are capable of growing cell cultures

to densities beyond 10^7 cells/ml (cell density achievable using batch bioreactors) without significantly decreasing the VP productivity per cell (10^5 VP/cell). Notably, hollow fiber and fed batch processes in perfusion fermentation processes have been investigated.

Hollow fibers. Hollow fibers are flexible and relatively easy to implement technologies. Cells are cultured in the extracapillary space surrounding the hollow fibers, while serum-free medium is pumped through the semipermeable hollow fibers. Only medium components, gasses, and waste products are able to pass through the membrane. The adenoviral vectors are too large to pass through and are retained in the extracapillary space. Cell densities of up to 10^8 HEK293 cells/ml have been reported using hollow fiber technology (Gardner *et al.*, 2001; Isayeva *et al.*, 2003), thus increasing cell densities approximately 10-fold as compared with current common practices. Another advantage of this technology is the flexibility of using single-use cartridges that avoid long decontamination and cleaning times. Monitoring cell cultures inside hollow fibers, however, is difficult, and therefore such cultures are hard to control (Isayeva *et al.*, 2003). Also, infection of the cells needs to be done by injection of the adenoviral vector seed stock into the extracapillary space of the hollow fiber (Gardner *et al.*, 2001). Distribution of the viral seed can thus be difficult at high cell densities, and therefore not all cells may become infected. It thus remains questionable at this point in time whether this technology is robust and sufficiently scalable to meet the needs for global vaccine production.

Fed batch and perfusion. Fed batch and perfusion bioreactor processes are based on the principle of extending the culture of a standard batch cell culture by supplying media and culture components over time. Toxic waste products that limit cell cultures in standard batches can be efficiently targeted by additive components. This process is widely used in pharmaceutical industries for production of biological products. For instance, successful scale-up of the E1-complementing cell line PER.C6 to 20,000 liters using an optimized fed-batch process has already been successfully achieved (Rolli, 2012). In perfusion bioreactors, retention devices retain cells and desired product(s) within the bioreactor while fresh medium is added, and waste products are withdrawn from the culture. The use of retention devises such as tangential flow filtration system, alternating tangential flow system, spin-filters, centrifuges, and acoustic separators (Kamen and Henry, 2004) enables cell densities of at least 100 times higher than can be achieved with batch or fed batch processes. For PER.C6, cell densities of $>1\times10^8$ cells/ml can be achieved in a perfusion system (Rolli, 2012). As such, a 1000-liter perfusion bioreactor could theoretically yield up to 1×10^{19} VP (10^5 VP/cell$\times1\times10^{11}$ cells/liter$\times$1000 liter). Thus, two perfusion bioreactors could suffice for a global vaccine against HIV as described above. One decade ago the optimal cell density at infection for adenovirus production was approximately 1×10^6 cells/ml. Today adenoviral vectors based on adenovirus serotype 26 and 35 are produced at a cell density up to 16×10^6 cells/ml (more than 1 log increase per volume), without losing the characteristic yield per cell (Herk and Luitjens, 2011). Besides the technical innovation, the optimization of medium has contributed to the success of perfusion bioreactor production of adenoviral vectors. The potential of the perfusion technology has been proven by growing E1-complemeting cells 2 logs higher than with traditional bioreactor processes. An additional advantage of using perfusion systems is that because of their relatively small volume, perfusion systems can make use of single-use bioreactors. The use of disposable fermenters reduces both the time required for decontamination and cleaning of the manufacturing facility between production campaigns as well as reducing the footprint of the manufacturing facility. Thus, the cost of manufacturing using perfusion fermentation can be significantly reduced not only because of increased yield but also because of more efficient use of a modestly sized manufacturing facility.

Purification (downstream processing)

In order to further reduce the cost of manufacturing, several innovations are being investigated, of which the most promising currently include omitting the use of endonuclease and using new chromatographic technology for purification processes.

Benzonase. Current common practices for manufacturing a replication-deficient adenoviral vector requires the use of circa 100 Units/ml of Benzonase, which is, as described earlier, an enzyme used to break down cellular genomic DNA. As such, the cost for Benzonase stands at approximately 5,000 USD per 10-liter batch fermentation. If one assumes that the high cell density process will require approximately 10-fold more Benzonase to break down DNA within the same time frame, a 100-liter fermentation would cost an estimated 0.5 million USD in terms of the cost of Benzonase alone. It is for this reason that alternatives to nuclease treatment have been sought.

Implementation of a DNA precipitation step within adenovirus purification has proved successful (Goerke *et al*., 2005a,b), demonstrating removal of DNA in both traditional fermentation processes (Goerke *et al*., 2005a) as well as with increased cell densities (de Vocht and Veenstra, 2010) without significantly affecting the infectivity of adenovirus (Goerke *et al.*, 2005a,b). Cationic detergents, specifically domiphen bromide, are relatively inexpensive as they are produced for use as antimicrobials in cosmetics and antiseptics and as antigingivitis agents in toothpaste and mouthwash (Council of Europe, 2008). The cost of domiphen bromide is approximately 1000 USD/kg, and can efficiently be used at low molarities of around 1.52 m*M* even for high-cell-density cultures (de Vocht and Veenstra, 2010). Less than 25 kg would be needed per year, which at a cost of less than 36,000 USD, thus representing a significant cost saving compared with processes that rely on Benzonase.

Chromatography. For AEX chromatography resin, the direct material cost of the resin itself is low, but because of low binding capacities (Altaras *et al*., 2005), large-volume columns are required along with large volumes of process buffers. Using the lowest estimated binding capacities as a worst-case scenario (0.5×10^{12} VP/ml) (Altaras *et al*., 2005) and for an arbitrary 20,000 doses at 5×10^{10} VP/ml (Baden

et al., 2013), a total of 2 liters of resin would be needed. During the AEX chromatography process there are usually eight major stages: preuse sanitization, flush of sanitization buffer, equilibration, wash, elution, postuse sanitization, flush of sanitization buffer, and column storage, which need up to three-column volumes per stage; therefore, 48 liters of buffer and solutions would be required. Charged filters for AEX are a logical replacement, as these have higher binding capacities than resins and monoliths; 1×10^{13}–1.9×10^{14} VP/ml (Peixoto *et al.*, 2008; BIA Separations, 2010; de Vocht and Veenstra, 2010; Pall, 2011). Therefore, applying a similar arbitrary calculation, with the lowest binding capacity as a worst-case scenario for 20,000 doses at 5×10^{10} VP/ml, approximately only 0.1 liter of filter would be required. The estimated buffer usage therefore would be 2.7 liters to process the same number of doses. The approximate 20-fold reduction in buffer usage would further decrease costs and facility size.

Gel filtration. Gel filtration is the fifth purification step and has inherent limited scalability because of the relative low loading capacity: around 20% of column volume (GE Healthcare, 2007). Therefore, large amounts of resin (five-fold larger than the volume of material requiring purification) are required. Hence, large buffer/solution volumes are also required (around 100-fold larger volume than the material for purification). If the purity from previous purification steps is sufficient, which is likely the case if selective precipitation is adopted, gel filtration may be replaced by tangential flow DF (Goerke *et al*., 2005a,b). DF offers the added advantage that the concentration can be increased if required. It is estimated that the volume of buffer/solution required would be reduced from 100-fold to around 6-fold larger than the volume of material for purification. This would significantly reduce buffer/solution usage and scale of the production facility.

Therefore, significant cost savings during the purification can be made by replacing the (expensive) Benzonase step with a cationic detergent-mediated precipitation of nucleic acid as well as using advances in chromatography as described in Fig. 3. These cost savings would increase the commercial viability of manufacturing facilities for adenovirus vector vaccines and would flow on to the final cost of goods.

Characterization, Testing, and Release of Replication-Deficient Adenoviral Vectors

Development of innovative vaccines and vaccine manufacturing processes strongly depends on availability of accurate and reproducible characterization assays and release assays.

Regulatory guidance is available for release testing of viral vectors in general and adenoviral vectors in particular (Ph. Eur 5.14.; Ph. Eur 5.2.3.; Ph. Eur 2.6.16.; FDA 1998; FDA 2010; EMA 2010). Release tests for adenoviral vectored products include tests to measure quantity, potency, vector genetic stability, identity, residuals (i.e., Triton and Benzonase) aggregation, protein content, and safety. Although every vaccine has

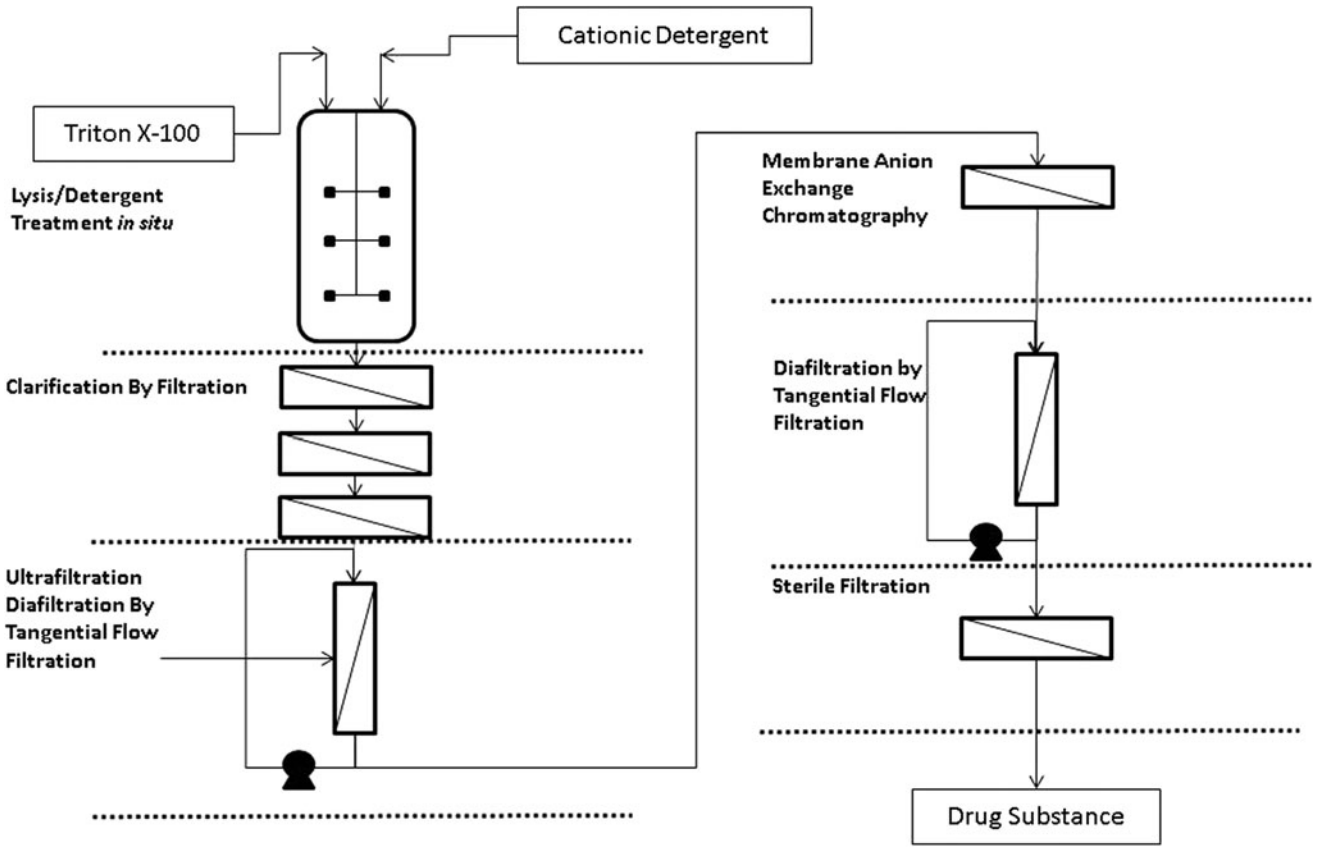

FIG. 3. Process flow diagram for the large-scale purification of adenoviral vector-based vaccines for product deployment.

his own characteristics that need to be taken into account during development and qualification of an assay, this review touches on testing of extraneous agents, RCAs, and testing of vector genetic stability in more detail because these assays are specific for adenoviral vectors. Adenoviral vector quantity and potency assays will be touched upon as well, as these are of importance for both process development and product release and specifically need to be developed for adenoviral vector vaccine products, where assays such as sodium dodecyl sulfate polyacrylamide gel electrophoresis and silver staining are commonly used for determination of product purity of biotechnology products.

Extraneous agents

Manufacturing processes for recombinant adenovirus-based vaccines and therapeutics do not involve wide-ranging viral inactivation and clearance steps that are generally performed when manufacturing inactivated vaccines. Therefore, a comprehensive viral safety strategy needs to be in place to ensure the lowest risk with regard to introduction and carryover of these types of contaminants. Extraneous agents, adventitious viruses, and transmissible spongiforme encephalopathy causing agents can be introduced at several steps in the manufacturing process. Proper control must be gained over raw materials used in construction and manufacturing of adenoviral vectors. Use of animal-derived components should be avoided as much as possible, and when unavoidable, should have excellent traceability and testing in place to mitigate any risk of introduction and transfer of extraneous agents in the process. Extensive testing (by PCR, *in vitro*, *in vivo*) to confirm the absence of adventitious viruses in master cell banks and master viral seeds (MVSs) is performed according to ICH Q5A and ICH Q5D guidelines. Recently, massive parallel sequencing has been employed to detect and identify adventitious agents in cell supernatants and virus stocks. Massive parallel sequencing has been shown to potentially provide a powerful and sensitive tool for adventitious agent testing (Onions and Kolman, 2010).

Replication-competent adenoviruses

Batches of recombinant replication-deficient adenoviruses for clinical use need to be devoid of RCAs that may arise from sequence overlap with complementing sequences in the production cell line. While cell lines such as PER.C6 do not share any sequence overlap with the vector, regulatory agencies still require testing for emergence of RCAs. Testing for the absence of RCAs in batches of replication-deficient recombinant adenovirus is routinely done, but can potentially be omitted when there is no risk to obtain RCAs during manufacturing, as is the case for E1-deleted rare serotype adenovirus vectors that do not have sequence overlap with the complementing cell line. The maximum RCA level of 1 RCA/3×10^{10} VP is based on current FDA guidelines for Ad5 vectors (FDA Gene Therapy Letter, 2000). Assays to detect RCAs in batches of replication-deficient adenovirus are based on the detection of virus replication in noncomplementing cell lines by a combination of cytopathic effect and PCR detection with the inclusion of spiked controls that validate the sensitivity of the test system (Marzio *et al.*, 2007). These cell-based RCA assays are laborious and complex and require handling of large amounts of permissive cells to allow testing of acceptable amounts of test article to achieve the sensitivity required.

Vector genetic stability

Genetic stability testing is performed to confirm stability throughout the manufacturing process, whereby the vector is preferably propagated for a number of passages until or beyond the envisioned stage of commercial manufacturing (vaccines for human use-general considerations; www.usp.org/). Although mutation frequency in replication-deficient adenovirus is relatively low, analysis of the transgene region to assure that proper antigen expression is maintained is crucial. Extended propagation of the test material provides the sensitivity required to detect any recombinants or mutants that have gained a growth advantage over the target vector. PCR detection, combined with sequence analysis of PCR fragments, provides sufficient specificity and sensitivity to detect any variants in the transgene region that might arise. Although next-generation sequencing technologies can be employed at MVS stage for characterization of vector variants that might be present in vaccine preparations or viral seeds, the relevance of detected variants can only be shown by using the approach of extended propagation.

Quantity and potency

Replication-incompetent adenoviral vectors are live viruses that exert their clinical effect through *in vivo* expression of the transgene or antigen in transduced target cells. Infectious titer measurements are important for process development, for product release, and stability assessments of adenoviral vector preparations. In general, it is accepted that adenoviral vector preparations should consist of a high proportion of active virus, that is, virus capable of transducing host cells. Infectious titer assays are considered to be representative of quantifying active, infectious adenoviral particles. Standard plaque assays or tissue culture infectious dose ($TCID_{50}$) assays using complementing cell lines can be employed to assess infectious titers. Although laborious and lengthy, these infectious titer assays have been shown to be suitable for this purpose. To improve the accuracy and precision of the plaque assay and $TCID_{50}$ assay, higher numbers of replicates can be employed either in parallel and/or on different days. Use of quantitative PCR technologies to increase ease and throughput improves accuracy and precision of infectious titer determinations, as a higher number of replicates can be achieved fairly easily (Wang *et al.*, 2005).

The number of physical adenoviral particles (quantity) is an important parameter to ensure accurate control of the amount of viral proteins injected within the acceptable safety window. In general, absorbance measurements are used for quantitation of VP. Maizel *et al.* (1968) established a correlation between protein content of adenovirus preparations and absorbance at 260 nm. Alternative methods have been developed especially to allow accurate in-process adenovirus particle quantitation and, at the same time, improve the lower limit of quantitation. Analytical anion exchange-based methods allow in-test separation of VPs from significant impurities, such as nucleic acids, host cell proteins and ''free'' viral proteins, and quantitation of peak fractions

using absorbance measurements (Shabram *et al*., 1997; Transfiguracion *et al*., 2001; Kuhn *et al*., 2007). For quantitation of low concentrations of adenoviral vector preparations, methods have been developed based on quantitative PCR to determine the number of viral genome DNA copies (Wang *et al*., 2006). Quantitative PCR-based assays allow automation of some key steps in the assay process, permitting high-throughput sample analysis for processing the large numbers of samples that are generated during process development activities (Wang *et al*., 2006).

A resultant parameter considered to be representative of the quality of a replication-deficient adenoviral vector preparation is the ratio between the amount of physical particles and infectious particles. The VP/IU ratio was initially set to prevent administration of quantities of VP and proteins that would pose a safety risk in human trials. For recombinant Ad5 gene therapy vectors, an FDA Advisory Committee recommended a targeted VP/IU ratio of < 30 (FDA Gene Therapy Letter, 2000). Achieving this value for alternative serotype vectors could possibly depend on design of the vector, infectious titer assay design, and the manufacturing process employed.

Evidence for the proper expression of the transgene present in the vector after transduction of permissive cells can be provided as an additional potency test. For early stage clinical trials, semi-quantitative assays such as Western blotting are considered sufficient. The expectation is that for late-stage clinical development more quantitative assessments will be required to accurately assess potency, linked to a clinical relevant response. It remains challenging to design assays that are predictive for *in vivo* potency, quantitative, and sufficiently straightforward and robust for use in quality-control laboratory settings.

Adenoviral vectors are manufactured in human complementing cell lines that contain several copies of the adenovirus *E1* gene. This requires control over the levels of host cell DNA and protein removal in the downstream process. In the final product drug substance, host cell DNA and protein levels should be well below the specification. For host cell DNA levels in injectable vaccine preparations, the specification is set by the European Pharmacopoeia (Ph. Eur 5.2.3.) and the World Health Organization (WHO Expert Committee on Biological Standardization, WHO Technical Report Series 878, 47th report, 1998) at less than 10 ng/dose. Host cell DNA levels can be determined using quantitative PCR-based assays and by immune enzymatic threshold methods (Gijsbers *et al*., 2005; Mehta and Keer, 2007). As well as requiring evidence that host cell DNA levels are below the specified level, regulatory bodies might request evidence that the DNA removal process fragments the host cell DNA sufficiently, to a median DNA size of 200 bp or less (Meeting Report, WHO study group on cell substrates for Production of Biologicals, 2007; Knezevic *et al.*, 2008).

Cold Chain and Vaccine Presentation

The success of an adenoviral vector-based vaccine may rely not only on the clinical properties of the vaccine and the ability to manufacture at low cost, but also on other factors such as cold chain and vaccine presentation. Both issues are only briefly touched upon below.

Cold chain

Development of vaccines that are stable at ambient temperatures of target countries would be ideal, but is difficult to achieve with current knowledge. At research scale, it is a common practice to store adenoviral vectors at $-80°C$. It is clear that a cold chain at this temperature is not feasible, and for that reason, development of formulation buffers has been carried out. Today a cold chain of 2–8°C is achievable for adenoviral vector-based vaccine formulations with stable infectivity profile of up to 2 years (Evans and Volkin, 2001). Advanced formulation development in the coming years will be required to be able to use the vaccine in a broader temperature chain, ideally ranging from 2°C to 37°C (Wilson, 2010).

Vaccine presentation

Vaccine presentation can impact costs of a vaccine in various ways. Prefilled syringes are easy to use but are more expensive than vials. Multiple-dose vials can be economically attractive for commonly used vaccines, whereas more infrequently used vaccines do not benefit because of wastage. The choice of vaccine presentation has severe impact on storage and transport capacity and therefore also influences the cold chain. Naturally, all these factors heavily contribute to the cost of deployment of a vaccine.

Conclusions and Future Challenges

The availability of effective adenovirus-vectored vaccines that prevent morbidity and mortality caused by important global infectious pathogens is an exciting public health prospect. Achieving a cost-effective, high-volume manufacturing process will be critical for success, particularly because many of the diseases currently targeted by adenovirus vector-based vaccines have their highest prevalence in resource-poor settings. Our review of currently available technologies suggests that the use of single-use perfusion bioreactors, replacement of endonuclease with cationic detergent-mediated nucleic acid precipitation (specifically domiphen bromide) during purification, and the use of charged filters during anion exchange are key process modifications that can contribute significantly in lowering the cost of goods. In spite of these recent advances, the technology for production of adenoviruses at high cell densities remains under development, and the final manufacturing processes and costs will heavily depend on the success of this innovation. Characterization, testing, and release play an important role in the adenoviral vector vaccine development and manufacturing but are not deemed key factors in vaccine cost. Although guidelines are available, rigorous scientific reasoning is required for assay development to meet the requirements for release of the adenovirus vector vaccine.

Ultimately, it is the development and manufacturing costs that will form the foundation for pricing of the final vaccine product. Attributes such as presentation, packaging, wastage, cold chain transport, and storage will also play an important role in determining the final price per dose per country.

Acknowledgments

The authors acknowledge Richard Verhage and Harold Backus for their valuable scientific input. Furthermore, the

authors acknowledge the FP7-PEOPLE-Marie-Curie Actions Agreements ADVANCE (290002) and AD-VEC (324325).

Author Disclosure Statement

J.V., D.M., M.v.O., P.I., and J.C. are employees of Crucell Holland BV. J.P.S., Ag.L., An.L., C.Y., and M.H. are employees of Batavia Bioservices, The Netherlands. This does not alter the authors' adherence to all the *Human Gene Therapy* policies on sharing data and materials.

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Received for publication January 14, 2014;
accepted after revision March 1, 2014.

Published online: March 4, 2014.