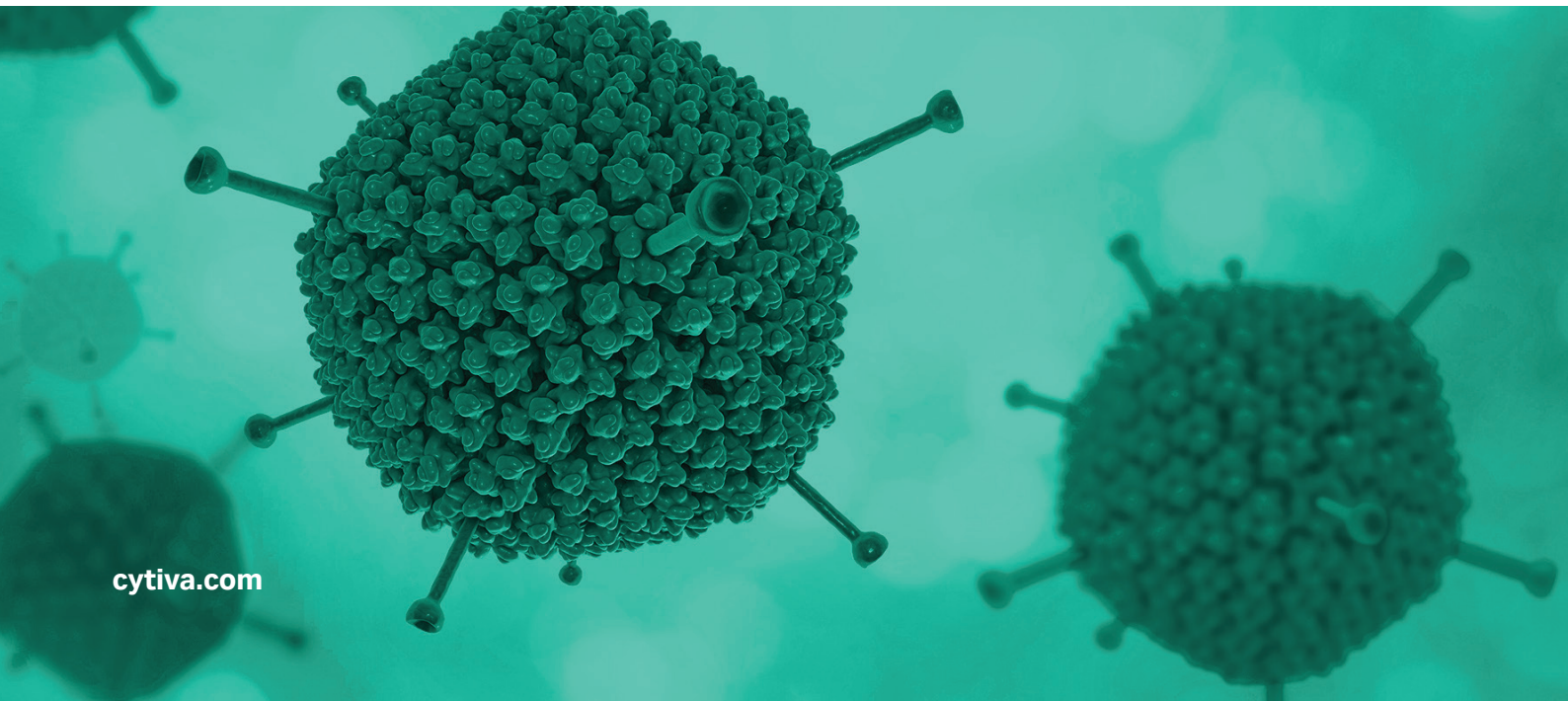
Minimizing RCA in large-scale adenoviral vector production using CAP™ Ad cells

By Silke Wissing*, Helmut Kewes* and Nico Scheer*#
*Cytiva, Cologne, Germany; #FH Aachen, University of Applied Sciences, Germany

Adenoviral vectors are frequently used as gene delivery tools for various medical applications. In most cases, replication-incompetent adenoviruses are employed, which, once they have infected a target cell, cannot spread further in the patient's body. In such applications, regulatory guidelines require that the formation of replication-competent adenoviruses (RCA) in the production process must be minimized. Since the risk of RCA occurrence depends on the adenoviral vector and the cell line used for generating the virus stock and for manufacturing the vector at large scale, the choice of an appropriate cell line and production platform is a critical decision at the beginning of each project. Ideally, it should allow, among other things, easy, scalable and GMP-compliant production of adenoviral vectors at high titers while minimizing the risk of RCA formation. Here we present the CAP™ Ad cell line for Ad vectors, specifically designed to minimize RCA during production. CAP Ad cells can be cultivated in common bioreactors using serum-free, chemically defined media. They grow to high densities as single cells in suspension and support high titer adenoviral vector production while minimizing RCA formation. This white paper provides further background information on the CAP Ad cell line and typical process flows used for adenoviral vector manufacturing at industrial scale.



Adenovirus background

Adenoviruses (Ad) are non-enveloped viruses with an icosahedral nucleocapsid containing a double stranded DNA genome of 26-46 kb. Ad viruses infect a broad range of vertebrate species. In humans more than 50 distinct Ad serotypes have been reported[1]. Wildtype Ad can cause different illnesses in humans, usually mild respiratory infections, but sometimes also life-threatening multi-organ diseases, in particular in immunocompromised individuals[2]. Ad vectors have been used as delivery tools since the 1980s in various medical applications. These include the use in gene therapy, as oncolytic viruses to target and destroy cancer cells, and as transfer vehicles to introduce antigens in vaccine development. In particular the latter has recently received increased attention with the approval of several Ad-based vaccines for the prophylaxis of Covid-19 (e.g., Johnson & Johnson's Janssen COVID-19 Vaccine, and AstraZeneca's Vaxzevria) and Ebola (e.g., Janssen's Ad26.ZEBOV)[3–5]. There are many different reasons for the frequent use of Ad vectors[6].One of them is that the Ad biology has been intensively studied for a long time and is very well understood which has contributed largely to the development of safe Ad-based vectors. Furthermore, vector construction is comparatively simple and straightforward, and Ad vectors enable an efficient transduction of proliferating and quiescent cells. Because these vectors are very stable, their handling is relatively easy. There are established purification methods and long-term storage is possible without any problems. With regard to the use of Ad vectors as vaccines, their ability to stimulate a robust cellular as well as humoral immune response is also important. In this context, the fact that an Ad vector may use the same entry route as the virus for which immune protection is to be established can be an additional advantage. In addition, if a strong promoter is used, this can result in higher and longer expression of the antigen compared to vaccines based on recombinant protein or inactivated viruses. Lastly, the vaccine products which have been approved for Covid-19 and their world-wide safe use represent strong precedents of the high suitability of Ad-based vectors.

The importance of minimizing RCA in Ad vector production

Ad vectors used as vaccines and in gene therapy are usually replication incompetent[6]. This means that the administered recombinant virus can introduce its genome, and thus also the gene to be expressed, into the human target cells, but cannot replicate in these cells. This prevents the uncontrolled spread of virus in the human body. Adenoviruses are not completely apathogenic and therefore it is of great importance that their reproduction in the human body is prevented. Otherwise, adverse reactions and an undesired strong immune response may occur[7,8]. Replication incompetence is usually achieved by deleting the E1 region of the viral genome. These so-called AdΔE1 vectors carry a deletion in the E1 region of the Ad genome from nucleotide 400–3500 (9). The viral E1A and E1B functions encoded by the E1 region are absolutely essential for virus replication, so that loss of this area efficiently prevents replication of the virus. In most Ad vectors used, the gene of interest (GOI) is placed at the site of the E1 region (Fig 1). In addition, these vectors usually have the Ad E3 gene deleted to create additional space in the viral genome for inserting a larger GOI.

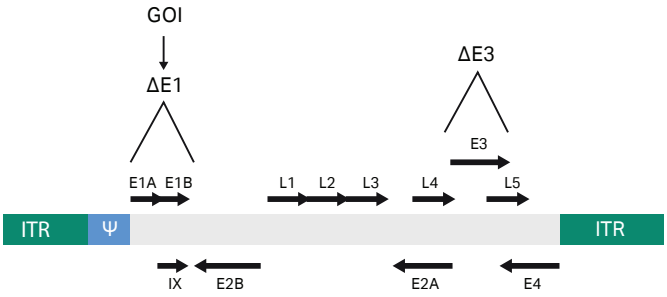


Fig 1. Genome structure of a typical AdΔE1 vector. The location and orientation of the adenoviral early (E) and late (L) transcription units are shown as black arrows. The inverted terminal repeats (ITRs), the packaging signal Ψ, and the pIX transcription unit are also shown. The E1 region of AdΔE1 vectors is usually replaced with the gene of interest (GOI), and the E3 gene is additionally removed.

Due to the use of AdΔE1 vectors and the associated inability to replicate, their production requires special cell lines that complement the E1 function in trans. Thus, these production cell lines carry a stable insertion of the adenoviral E1 region in their genome. After transfection with an AdΔE1 vector, recombinant vector is produced which is replication incompetent as the E1 region provided in trans cannot be packaged into the virus particle. Consequently, these viruses can infect a target cell when administered to a patient and introduce their genome together with the GOI, but cannot replicate and form new virus particles. However, depending on the production cell line used, there is a certain probability of homologous recombination between the E1 region integrated into the genome of the production cell line and the AdΔE1 vector genome due to existing homology regions, which may result in replication competent adenoviruses (RCA). The occurrence of RCA has been observed, for example, in the commonly used HEK-293 cell line, because the E1 region inserted in these cells has a relatively long region which is homologous to most of the Ad vectors used[10]. For the reasons described above, the occurrence of RCA is highly undesirable, and FDA guidelines restrict their appearance to less than 1 RCA in 3×10^{10} virus particles[9].

Minimizing RCA using the CAP Ad cell line

Since the risk of RCA occurrence depends on the homology between the trans-complementing E1 region in the production cell line and the Ad vector, the choice of an appropriate production platform is a critical decision at the beginning of each project. The CAP Ad cell line was developed from the onset with the intention of establishing a cell line that minimizes RCA formation for the industrial scale production of Ad vectors for clinical use. CAP Ad cells are human amniocyte cells (Fig 2), acquired during a routine amniocentesis and immortalized with AdE1A/E1B and pIX gene functions. pIX has been reported to have an important function in stabilizing the Ad capsid and providing temperature resistance, both being important for commercial scale manufacturing[9].

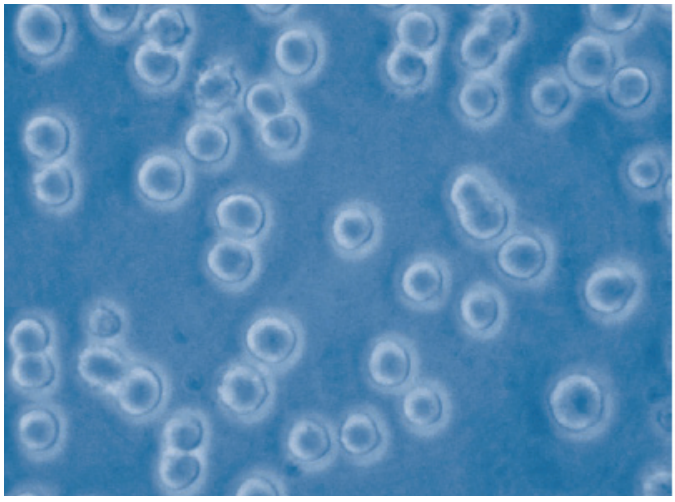


Fig 2. CAP Ad suspension cells. CAP Ad cells grow well as a single cell suspension with high viabilities and high number of viable cells in serum-free, chemically defined media.

Importantly, the plasmid used to immortalize CAP Ad cells contained a special arrangement of elements, providing a very low degree of homology to Ad vectors and also carrying a reverse orientation in parts of the sequence, so that the risk of RCA occurrence is dramatically reduced (Fig 3). Indeed, RCA contaminants were found undetectable in 5×10^{10} VPs in a batch of CAP Ad produced Ad vector compared to 5-500 detected RCA particles in 293-F productions (at high-sensitivity propagation on ΔE1 cells; data not shown).

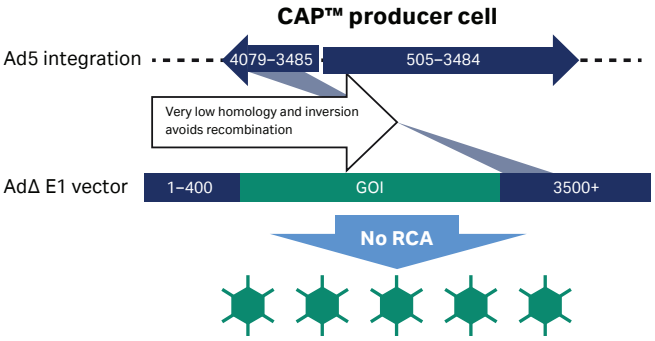


Fig 3. Ad production in CAP Ad cells. The Ad integration in CAP Ad cells contains only a short, inverted region of homology to the AdΔE1 vector minimizing the risk of RCA formation.

Additional benefits of the CAP Ad cell line

Minimizing RCA is an important characteristic when manufacturing Ad vectors. But a suitable cell line must also meet numerous other requirements, such as allowing easy, scalable and GMP-compliant production of Ad vectors at high titers. The CAP Ad cell line meets all these needs. CAP Ad cells grow to high cell densities of up to 15–20 × 10^6 cells/mL in single cell suspension with good viabilities over the process (Fig 4). They can be easily cultured in shake flasks and common bioreactor types, with a straightforward scale-up to high volumes. A range of commercially available serum-free, animal component-free and chemically defined media are suitable for CAP Ad cell culturing and Ad production. Furthermore, the CAP Ad cell line is of non-tumor origin and, due to its derivation from standard amniocentesis, ethically accepted.

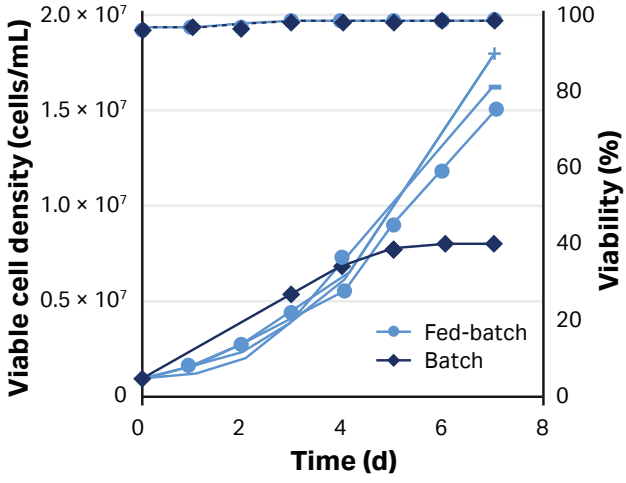


Fig 4. CAP Ad cells grow to high cell densities with good viabilities. Cell densities of up to 15–20 × 10^6 cells/mL in fed-batches with various feed strategies are typically achived in various formats, including shake flasks and common bioreactor types (Data shown here present CAP Ad cells cultured in the ambr15 stirred tank bioreactors).

Regulatory aspects

Ad vector production for clinical use requires a large-scale production process under GMP conditions using GMP qualified materials, such as a GMP Master Cell Bank (MCB) and a Working Cell Bank (WCB) as well as Master Virus Seed Stock (MVSS) and Working Virus Seed Stock (WVSS). Importantly, a CAP Ad GMP master cell bank has been established and a comprehensive quality control was performed according to ICH and FDA guidelines. In addition, a biological master file has been submitted to the FDA and the cell line has been made available for licensing.

The typical workflow of Ad vector production in CAP Ad cells

The recombinant Ad production process using the CAP Ad cell line is usually carried out in several successive steps (Fig 5). Initially, an Ad plasmid is generated using a shuttle vector containing the GOI and a pAd vector carrying the Ad genes required for virus production. The pAd vector including the GOI is then linearized and transfected into the GMP-grade CAP Ad cells, resulting in a primary Ad stock which is subsequently amplified, usually in three rounds. The final amplification steps can be done in CAP Ad suspension cell culture in serum-free, animal component-free media in shake flasks or small bioreactors. Optionally, clonal Ad can be obtained from GMP-grade CAP Ad cells using a plaque assay. After an additional amplification step, the Ad preparation is purified and the titer is calculated using standard methods. Ad preparations are then used for generating the MVSS and the WVSS.

After extensive quality control testing, the viral seed is used for the large-scale production utilizing GMP-grade CAP Ad cells. For this purpose, the CAP Ad cells from the GMP bank are expanded and infected at a fixed multiplicity of infection (MOI). The infected cells are harvested at a defined time post infection, followed by cell lysis resulting in the crude virus harvest. Extensive purification, concentration and quality control steps are carried out before the recombinant virus preparation is finally filled with the desired titer. In summary, the process is carried out under highly controlled conditions, and due to the suspension growth in bioreactors it is readily scalable to large volumes.

Conclusions

Scalable production with minimized RCA is an important prerequisite for many different applications of recombinant Ad vectors, e. g. in vaccine production and in gene therapy. However, depending on the production platform chosen, there is a risk of emergence of replication-competent adenoviruses during the manufacturing process. The CAP Ad cell line is designed to minimize RCA during adenovirus production. With its excellent safety profile, scalability and productivity under serum-free culture conditions, the CAP Ad technology is well-suited for the industrial production of Ad vectors.

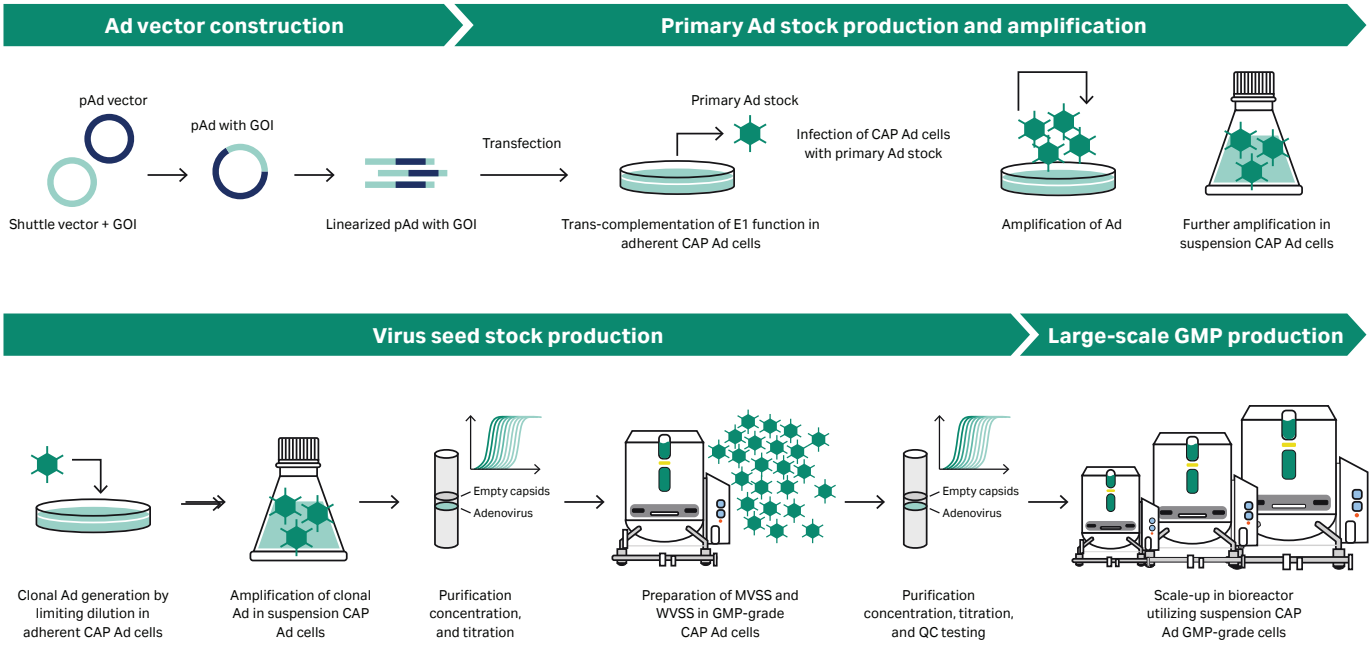


Fig 5. Workflow of an industrial scale Ad production in CAP Ad cells. From the top left to the bottom right. Construction of the Ad plasmid by homologous recombination of a shuttle vector carrying the gene of interest (GOI) and a pAd vector containing the adenoviral genes required for virus production. The final vector is linearized and transfected into adherent CAP Ad cells to generate and amplify the primary Ad stock. Subsequent amplification steps are done in serum-free, animal component free suspension cell culture in shake flasks or small bioreactors. A clonal Ad virus is then produced in GMP-grade CAP Ad cells using a standard plaque assay. After an additional amplification step, the Ad vector is purified and the titer is determined. The Ad preparation is then used for the generation of the Master Viral Seed Stock (MVSS) and the Working Viral Seed Stock (WVSS) in GMP-grade suspension CAP Ad cells. Following another round of purification, concentration, titer determination, and a rigorous QC testing program, expanded GMP-grade CAP Ad cells are infected at a pre-determined multiplicity of infection (MOI) to produce the final Ad preparation and harvested at a defined timepoint post infection. The final large scale GMP production comprises several steps, such as an engineering run, the actual GMP run, purification, concentration, GMP fill and finish, and GMP release tests, which are omitted here for simplicity.

Literature

1. Lynch JP, Fishbein M, Echavarria M. Adenovirus. *Semin Respir Crit Care Med.* 2011;32(4):494-511. doi: 10.1055/s-0031-1283287
2. Ghebremedhin, B. Human adenovirus: Viral pathogen with increasing importance. *Eur J Microbiol Immunol (Bp).* 2014;4(1):26-33. doi: 10.1556/EuJMI.4.2014.1.2.
3. U.S. Food and Drug Administration. fda.gov/emergency-preparedness-and-response/coronavirus-disease-2019-covid-19/covid-19-vaccines. Accessed December 15, 2023.
4. ema.europa.eu/en/human-regulatory/overview/public-health-threats/coronavirus-disease-covid-19/treatments-vaccines/vaccines-covid-19/covid-19-vaccines-authorised
5. ema.europa.eu/en/news/new-vaccine-prevention-ebola-virus-disease-recommended-approval-european-union
6. Wold WSM, Toth K. Adenovirus vectors for gene therapy, vaccination and cancer gene therapy. *Curr Gene Ther.* 2013 Dec;13(6):421-33. doi: 10.2174/1566523213666131125095046.
7. Lochmüller H, Jani A, Huard J. et al. Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants (ΔE1 + ΔE3) during multiple passages in 293 cells. *Hum Gene Ther.* 5(12) (1994).
8. Hermens WT, Verhaagen J. Viral vectors, tools for gene transfer in the nervous system. *Prog Neurobiol.* 1998 Jul;55(4):399-432. doi: 10.1016/s0301-0082(98)00007-0.
9. Kovesdi I, Hedley SJ. Adenoviral producer cells. *Viruses.* 2010 Aug;2(8):1681-1703. doi: 10.3390/v2081681.
10. Murakami P, Pungor E, Files J. et al. A single short stretch of homology between adenoviral vector and packaging cell line can give rise to cytopathic effect-inducing, helper-dependent E1-positive particles. *Hum Gene Ther.* 2002;13(8).
11. Schiedner G, Hertel S, Kochanek S. Efficient transformation of primary human amniocytes by E1 functions of Ad5: Generation of new cell lines for adenoviral vector production. *Hum Gene Ther.* 2000;11(15): 2105–2116.

cytiva

cytiva.com

For local office contact information, visit cytiva.com/contact

CY41089-15Mar24-WP