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## *Propagation of Adenoviral Vectors: Use of PER.C6 Cells*

**W. W. Nichols,\* R. Lardenoije,† B. J. Ledwith,\*  
K. Brouwer,† S. Manam,\* R. Vogels,† D. Kaslow,\*  
D. Zuidgeest,† A. J. Bett,\* L. Chen,\* M. van der Kaaden,†  
S. M. Galloway,\* R. B. Hill,\* S. V. Machotka,\*  
C. A. Anderson,\* J. Lewis,\* D. Martinez,\* J. Lebron,\*  
C. Russo,\* D. Valerio,† and A. Bout†**

\*Merck Research Laboratories  
Merck & Company, Inc.  
West Point, Pennsylvania

†Cruell NV  
Leiden, The Netherlands

### **I. Introduction**

#### **A. Scope of the Chapter**

The goal of gene therapy is the introduction of genes into human somatic cells for therapeutic purposes. The success of gene therapy is therefore dependent on the efficiency by which a therapeutic gene can be transferred to the patient's target tissues. In many cases, viruses are exploited for gene transfer purposes and in particular gene transfer vectors derived from adenoviruses (adenoviral vectors) are often used to achieve this (for review see [1]).

The reason for this is that adenoviral vectors:

- efficiently transfer genes to many different cell types;
- can be propagated on well-defined production systems to high yields; and
- are very stable, which makes purification and long-term storage possible, thereby making pharmaceutical production feasible.

This contribution will focus on the production systems for clinical lots of adenoviral vectors. Particular attention will be paid to the generation and use of complementation cell lines that carry the E1 genes. Particular emphasis will be on the PER.C6 cell line, which was developed to prevent generation of replication-competent adenovirus (RCA) during propagation of E1-deleted adenoviral vectors. In addition, safety issues with respect to the use of the cell line for making clinical grade material will be addressed.

## B. Adenoviruses

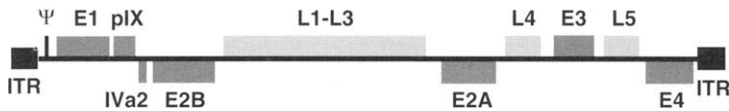
Human adenovirus was isolated for the first time in 1953 from cultured adenoidal tissue [2, 3]. Since then, 51 different serotypes have been isolated from various tissues and excretions of humans, of which serotypes 42–51 were obtained from immunocompromised individuals [4–6]. A serotype is defined on the basis of its immunological distinctiveness as judged by quantitative neutralization with animal antisera (horse, rabbit). If neutralization shows a certain degree of cross-reaction between two viruses, distinctiveness of serotype is assumed if (i) the hemagglutinins are unrelated, as shown by lack of cross-reaction on hemagglutination-inhibition, or (ii) substantial biophysical/biochemical differences in DNA exist [7].

Human adenoviruses are subdivided into six different groups (A–F), which are based mainly on differences in hemagglutination, restriction enzyme analysis, and DNA homology [8]. The adenoviruses were found to be associated with different disease patterns (see, e.g., [9, 10]). In addition to the human adenoviruses, some 40 different serotypes have been isolated from various animal species [11].

All adenoviruses possess a DNA molecule that is surrounded by a capsid consisting essentially of hexon, penton-base, and fiber proteins. The virion has an icosahedral symmetry and, depending on the serotype, a diameter of 60–90 nm.

The well characterized adenovirus serotypes 2 and 5 have a linear double-stranded DNA genome of approximately 36,000 base pairs (Fig. 1). Other adenoviruses have genome sizes ranging from 30 to 38 kb. The genome contains, at both its ends, identical inverted terminal repeats (ITRs) of approximately 90–140 base pairs with the exact length depending on the serotype. The viral origins of replication are within the ITRs exactly at the genome ends. Sequences required for encapsidation ( $\Psi$ ) of the viral genome are located in a region of approximately 400 bp downstream of the left ITR.

The structure of the adenoviral genome is described on the basis of the adenovirus genes expressed following infection of human cells, which are called early (E) and late (L), according to whether transcription of these regions takes place prior to or after onset of DNA replication.



**Figure 1** Map of the adenovirus genome. The 36-kb (for adenovirus type 5) double-stranded DNA molecule is usually divided into 100 map units (mu). The early (E) and late (L) regions are indicated on the map. The ITR sequences (inverted terminal repeats) are identical, inverted, terminal repeats of approximately 100 bp, depending on the serotype, which are required for replication.  $\Psi$  is a stretch of sequences involved in packaging of the viral DNA into particles. E1 comprises the E1A and E1B region, both encoding two proteins, which are described in detail in section I.C. E2A encodes the DNA binding protein, E2B the precursor terminal protein and DNA polymerase. E3 encodes a number of proteins that are predominantly involved in modulating the host's immune response against adenoviral infected cells. E4 proteins (six in total) are involved in modulation of gene expression and viral replication, mainly through interactions with the host cell. IVa2 (transcriptional activator of major late promoter) and pIX (essential for assembly of the virion) are intermediate proteins. L1–L5 encode the late proteins, which are mainly capsid proteins, including penton (L2), hexon (L3), hexon-assembly (L4), and fiber (L5) protein.

Infection of a target cell starts by interaction of the fiber with a receptor on the surface of the cell. Many, but not all [12], adenoviruses use the Coxsackie-adenovirus receptor (CAR) for this [13, 14], which is present on the cell surface. Integrins act as secondary receptors by binding to the viral penton-base protein. Subsequently, the virus is internalized by receptor-mediated endocytosis. The adenoviruses escape from the endocytic vesicles (or receptosomes) by virtue of a change in the configuration of the virion surface due to the low pH in these vesicles. As a consequence, the virus particles are released in the cytoplasm of the cell, where they are further degraded [15], with the DNA ending up in the nucleus, where a complex with histone proteins is formed, which may attach to the nuclear matrix for replication [16].

The adenovirus DNA is usually not integrated into the host cell chromosomal DNA but remains episomal (extrachromosomal) unless transformation or tumorigenesis has occurred.

### C. Adenovirus Replication

As indicated before, a productive adenovirus infection is divided into two distinct phases: the early and the late phases. In the early phase, the so-called early genes (E1, E2, E3, and E4) of adenovirus are expressed to prepare the host cell for virus replication. During the late phase, actual viral DNA replication and production of viral structural proteins takes place, leading to the formation of new viral particles. Adenovirus replication requires both host-cell and viral proteins (see [8, 16] for reviews). The cellular proteins needed for replication are nuclear factors I, II, and III [16], which are involved in initiation of viral DNA replication and elongation, as well as in increasing the efficiency of replication.

Adenovirus DNA replication starts with expression of the “immediate-early” E1 genes. The E1 region comprises two different transcription units, E1A and E1B. The main functions of the E1A gene products are (i) to induce quiescent cells to enter the cell cycle and resume cellular DNA synthesis and (ii) to transcriptionally activate the E1B gene and the other early regions (E2, E3, E4). The E1A region encodes two major RNA products, 12S and 13S, which are generated by one transcription unit and which differ in size due to alternative splicing. The RNAs encode acidic proteins of 243 and 289 amino acids, respectively (for adenovirus 5). These are phosphorylated proteins, present in the nucleus of the cells. In addition, during lytic infection mRNAs of 9S, 10S, and 11S are produced, but these proteins were found to be not essential for adenoviral replication [17, 18]. The function of these proteins has not yet been resolved.

The E1B region codes for one 22S mRNA, which is translated into two proteins, with molecular weights (for adenovirus 5) of 21 and 55 kDa. E1B proteins assist E1A in redirecting the cellular functions to allow viral replication. The E1B 55-kDa protein forms a complex with the E4 open reading frame 6 (ORF6) 34-kDa protein, which is localized in the nucleus [19, 20]. Its main function is to inhibit the synthesis of host proteins and to facilitate the expression of viral genes. In addition, it also blocks the p53 tumor-suppressor protein, thereby inhibiting apoptosis [21]. The E1B 21-kDa protein is important for quenching the cytotoxic effects to the target cells induced by E1A proteins. It has anti-apoptotic functions similar to the human Bcl-2 protein, which is important for preventing premature death of the host cell before the virus life cycle has been completed [22]. Mutant viruses incapable of expressing the E1B 21-kDa gene-product exhibit a shortened infection cycle that is accompanied by excessive degradation of host cell chromosomal DNA (*deg*-phenotype) and an enhanced cytopathic effect (*cyt*-phenotype) [23].

The E2 region encodes three different proteins that function in viral DNA replication: an Ad-specific DNA polymerase, the precursor terminal protein (pTP), and the DNA-binding protein [16]. The DNA-binding protein, which is encoded by the E2A gene, binds to single-stranded DNA and is involved in unwinding duplex DNA. It might also be involved in the regulation of transcription. The precursor of the terminal protein (pTP) and the DNA polymerase, which are present as a heterodimer, are encoded by the E2B region. The pTP is attached to the adenoviral DNA and is cleaved by the viral protease late in infection. It has a function in protection of the DNA from nucleolytic breakdown and in attaching the adenoviral DNA to the nuclear matrix, which may localize the viral genome to areas of the nucleus in which high concentrations of replication and transcription factors are present. The polymerase is involved in the synthesis of new DNA strands.

None of the E3 products are required for virus replication. They do, however, play an important role in virus multiplication *in vivo*, since they protect virus-infected cells from being eradicated by the host's immune response (reviewed in [9]). Several differentially spliced mRNAs are synthesized from the E4 region during infection and six different polypeptides have been identified in infected cells [24]. These proteins are involved in modulation of gene expression and viral replication, mainly through interactions with the host cell.

The E4 ORF3 and E4 ORF6-encoded proteins are involved in post-transcriptional processes that increase viral late protein synthesis. They do so by facilitating the cytoplasmic accumulation of the mRNAs encoding these proteins and by expansion of the pool of late RNAs in the nucleus, most likely by influencing splicing. In addition, the E4 ORF6-encoded protein forms a complex with the E1B 55-kDa protein that selectively increases the rate of export of viral late mRNAs from the nucleus. The complex is located in so-called viral inclusion bodies, the region where viral DNA replication, viral late gene transcription, and RNA processing occur [25]. The E4 ORF6 protein, either alone or in a complex with the E1B 55-kDa protein, binds the cellular protein p53, thereby blocking its potential to activate the transcription of tumor-suppressing genes [26, 27].

E4 ORF1 sequences are related to dUTPase enzymes. It has been hypothesized that this gene has a role in stimulating quiescent cells [24].

The E4 ORF4 protein binds to protein phosphatase 2A, which results in hypophosphorylation of some proteins, including the adenovirus E1 proteins. This perhaps limits cytotoxic effects of E1A and may lead to a more productive infection. It is also in line with the observation that E4 ORF4 mutants are more effective than wild-type viruses in killing nonpermissive rodent cells [28]. E4 ORF4 also induces apoptosis in transformed cells like 293 cells [29].

The E4 ORF6/7 modulates the activity of the cellular transcription factor E2F, which may subsequently activate cellular genes which are important for the S phase [30]. The functions of E4 ORF1, ORF2, and ORF3/4 during lytic infection are less clear and are dispensable for growth of the virus in laboratory cell lines.

After onset of DNA replication, expression of the late genes L2–L5, which are all under the control of one promoter, is switched on. These genes encode the structural components of the virus particles, including L2 the penton, L3 the hexon, L4 the hexon assembly, and L5 the fiber protein. These proteins form the new virus particles into which the adenoviral DNA becomes entrapped. Depending on the serotype, 10,000–100,000 progeny adenovirus particles can be generated in a single cell. The adenoviral replication causes lysis of the cells.

## II. Cells Expressing E1 of Adenovirus

### A. Transformation of Cells by E1 of Adenovirus

In the previous section of this chapter, the function of adenoviral gene products in the replication of adenovirus was described. There is extensive influence of adenoviral proteins on a large number of cellular functions. In the absence of lytic viral replication, adenoviral genes may have a profound effect on cellular functions, the most striking being transformation by the adenoviral E1A and E1B proteins. Clearly, these proteins interfere with the regulatory mechanism of cellular proliferation.

Human adenoviruses have a narrow host range for productive infections, and can only be propagated in cells of human, chimpanzee [31], pig [32], and cotton rats [33]. In rodent cells, e.g., from rat (with the exception of the cotton rat), hamster, or mouse, they bring about an abortive infection, which occasionally leads to transformation [34]. In the transformed cells the adenoviral DNA is integrated into the genome and at least the genes of the viral E1 region are expressed (reviewed in [35]).

The viruses that were used for such studies were mainly adenovirus serotypes 2, 5, and 12. The various Ad serotypes differ in their ability to induce tumors upon inoculation into newborn hamsters; for example, Ad type 5 (Ad5) is nononcogenic [36], whereas Ad12 is highly oncogenic [34]. However, all Ad serotypes or their DNA can transform rodent cells [37, 38]. Ad5E1-transformed cells can form tumors only in immunodeficient mice and rats, whereas Ad12E1-transformed cells are oncogenic both in immunodeficient and in immunocompetent animals [39], which correlates with the ability of Ad12E1 to repress expression of MHC class I genes [40].

In culture, both rodent cells, e.g., from rat, mouse, or hamster, and human cells can be transformed by Ad DNA, although human cells, including fibroblasts and epithelial cells, are relatively refractory to transformation. Adenovirus DNA transformed human cell lines have been made from cultures of human embryonic kidney [41, 42], human embryonic retina [43–46], human embryonic lung [44], and recently, human amniocytes [47].

As described before, the E1 region consists of two transcriptional units, E1A and E1B. For complete morphological transformation, both regions are needed, but the E1A region by itself can immortalize rodent cells [48] and occasionally human cells [43], albeit with very low efficiency. Expression of E1A usually results in induction of programmed cell death (apoptosis), which can be prevented by coexpression of E1B [49]. The E1A associates with a number of cellular proteins, including the tumor suppressor gene product pRb, as well as p107, p130, cyclins A and E, cyclin-dependent kinase 2 (cdk2), and p300 (reviewed in [50–52]). Most of these proteins are involved in cell-cycle control, and, with the exception of p300, regulate the activity of the

transcription factor E2F [51]. The E1A proteins do not exert their activity in initiation of transcription by direct, sequence-specific binding to DNA, but rather do so by binding to cellular transcription factors.

The E1B 55-kDa [19] and 21-kDa [53] proteins cooperate independently with E1A in transformation, and are required to inhibit the apoptotic response initiated by E1A. The 55-kDa E1B protein inhibits apoptosis by blocking the function of the p53 tumor-suppressor protein, which mediates E1A-induced apoptosis [21]. The 21-kDa E1B protein inhibits apoptosis in a way similar to the cellular Bcl-2 protein [22].

## B. E1-Expressing Cell Lines for Adenoviral Vector Production

Most adenoviral vectors currently used in gene therapy have a deletion in the E1 region, where novel genetic information can be introduced [54]. The E1 deletion renders the recombinant virus replication-defective, which is a prerequisite for most of the clinical applications. In order to be able to produce E1-deleted recombinant adenoviral vectors, complementing cell lines have to be used that express the E1 proteins of adenovirus. One of the main challenges here is to express sufficient levels of the E1 protein to achieve this. However, adenovirus E1 proteins, and in particular E1A proteins, are very toxic to cells. E1A has a profound effect on the transcription of many cellular genes, which leads to alteration of the morphology and growth of the cells and may lead to apoptosis.

A few examples have been reported in literature, where cells have been immortalized (but not transformed) with E1A only. This has been described both for rodent [48] and for human cells [43]. It is not known whether cells that express E1A only are able to complement adenoviral vectors that are deleted for both E1A and E1B. Attempts have also been made to express E1 proteins in established cell lines such as A549. Growth of established cells is not dependent on E1 expression and the toxicity of E1 proteins made it difficult to isolate clones that show stable expression of the E1 proteins, although a few papers report encouraging results [46, 55, 56]. To the best of our knowledge, there is limited use of such cells and therefore this chapter will deal mainly with the group of E1-expressing cells that use the transforming capacity of the adenoviral E1 genes.

Typical examples are the cell lines derived from human embryonic kidney (HEK) [41, 42], human embryonic retina (HER) [43–46], and human amniocytes [47]. The advantage of using E1 for immortalization is that such cells are dependent on E1 expression for growth, and therefore the levels of E1 expression are remarkably constant over time.

The vast majority of cell lines that were made by immortalization and transformation of primary cells, were made to study immortalization and transformation and were not made for propagation of E1-deleted adenoviral



vectors. The only documented cell lines based on the E1 immortalization principle, which were made specifically for use in gene therapy are the PER.C6 cell line [46] and the amniocyte-derived cell line [47].

These cell lines have been tailor-made for the manufacture of clinical lots of adenoviral vectors, with special attention to avoiding generation of RCA (see below). In addition, proper documentation and adequate safety testing are pivotal to ensure manufacture of safe batches of adenoviral vectors. As PER.C6 is the only cell line currently used for making clinical lots of adenoviral vectors, a description of the generation of PER.C6 is given below. Also, the performance of the cell line in production of recombinant adenovirus as well as results of safety and genetic testing are provided.

### **III. PER.C6 Prevents RCA during Vector Production**

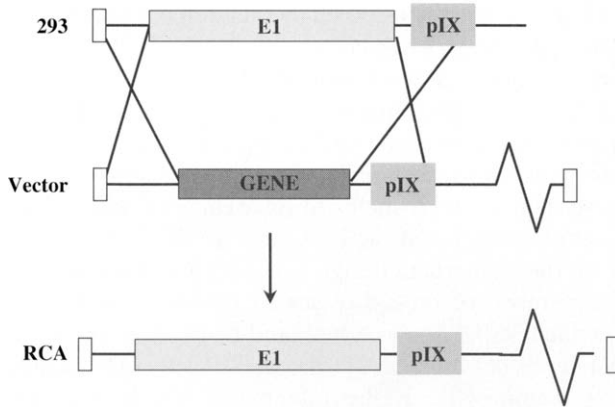
#### **A. RCA**

The majority of preparations of E1-deleted adenoviral vectors have been produced on 293 cells. This cell line was generated in Leiden in the group of Prof. Van der Eb, by transfection of E1 sequences of adenovirus type 5 into primary human embryonic kidney cells [41]. The aim of this experiment was to study the transforming potential of adenoviral E1 sequences, and the DNA used for it was sheared adenoviral DNA [41]. Precise mapping of the adenoviral sequences present in this cell line indicated that the cell line had integrated bases 1–4137 of the adenoviral DNA [57]. Adenoviral vectors carry a deletion in the E1 region that runs from approximately nt 400 to nt 3500 of the adenoviral genome. This means that there is a substantial sequence overlap between the E1 sequences present in the cell line and the adenoviral vector DNA (see Fig. 2). This sequence overlap may result in homologous recombination between the sequences. Due to a double crossover, the E1 region present in the cellular chromosome may end up into the E1-deleted adenoviral vector [58] (Fig. 2). The resulting virus is E1-positive and therefore capable of replicating independently in cells that do not contain E1 sequences in the chromosome. Several reports have described the occurrence of RCA in adenoviral vector batches produced on 293 cells [46, 55, 58–60].

RCA in clinical preps is unwanted, both from the manufacturing and the safety points of view.

Its appearance in batches is a chance process and is therefore unpredictable and difficult to control. This is a significant problem for GMP manufacturing, in particular if large-scale batches have to be prepared.

It is also unwanted from a safety point of view, as upon coinfection of a cell RCA causes the E1-deleted adenoviral vector to replicate in an



**Figure 2** Mechanism of generation of RCA in 293 cells. Adenoviral vectors contain sequences that overlap with sequences present in the genome of 293 cells, indicated by the crossing lines. Due to the sequence homology, crossover events can occur, which lead to exchange of DNA. E1 sequences replace the transgene in the adenoviral vectors, resulting in E1-containing adenoviruses that are replication-competent.

uncontrollable way. It causes shedding of the vector [61]. In addition, RCA has been shown to cause inflammatory responses [59, 62]. Therefore, RCA generation during production of E1-deleted adenoviral vectors has to be circumvented.

#### B. PER.C6: Absence of Sequence Overlap Eliminates RCA Generation

The strategy to prevent RCA occurrence was to eliminate sequence overlap between the E1 sequences present in the cellular genome and the adenoviral vector [46]. A potential hurdle to do this is the way the E1B and pIX gene are regulated. Both E1B and pIX use the same poly(A) sequences [63]. Furthermore, the pIX gene is not expressed upon transfection in cultured cells [64], but can be expressed only if present in an adenoviral genome. Therefore, an RCA-free packaging system should consist of two components: (i) an adenoviral vector that is deleted for E1A and E1B, but contains the pIX expression cassette and (ii) a cell line that expresses E1A and E1B and is devoid of pIX sequences.

##### 1. E1 Construct Used for Making PER.C6

To create the novel cell line, the aim was to use only a minimal number of human adenovirus-type-5-derived sequences, i.e., the E1 protein coding sequences only, to prevent sequence overlap with E1-deleted Ad. The E1 promoter and poly(A) sequences were therefore obtained from nonadenovirus

sources. The E1 promoter was replaced by the human phosphoglycerate kinase (PGK) promoter (see below), which is a known housekeeping promoter [65] and the poly(A) sequences were isolated from hepatitis B virus [66, 67]. The construct pIG.E1A.E1B contains, in addition to the E1A and E1B coding regions, sequences upstream of the E1A gene, including E1A enhancer elements and the cap sequence. Untranslated E1A sequences were also retained in the construct. These elements were included since earlier studies indicated that this results in efficient expression of the E1A gene [68].

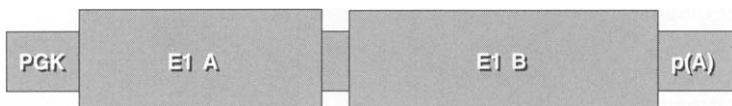
A map of the construct, designated as pIG.E1A.E1B, is presented in Fig. 3. Despite removal of the splice site at position 3509 of the adenoviral genome [63], which is highly conserved, and truncation of the E1B transcript, high expression levels of both E1B 21 kDa and E1B 55 kDa were obtained [46]. In fact, the expression of the E1B proteins was even higher than in 293 and 911 cells, whereas equal expression levels of E1A were observed [46].

To prevent sequence overlap with E1 present in PER.C6 cells, adenoviral vectors were constructed that carry a deletion of the complete E1 region. These vectors were shown to propagate efficiently in PER.C6 cells (see below) and were found to express the pIX gene [46].

## 2. Generation of PER.C6

The primary cells selected for making a new E1-complementing cell line were human embryonic retinoblasts (HER). The choice for retinoblasts [43] was based on the observation that Ad12 could transform hamster retinal cells *in vitro* [69] and induce retinoblastomas following intraocular injection into newborn baboons [70]. It has been described that these cells can be immortalized relatively easily by E1 of human Ad5 [43, 44, 71] and Ad12 [72]. In addition, the 911 cells, which are derived from HER cells, are very efficient in production of recombinant adenoviral vectors and easy to use [71], thus providing a second argument for the use of primary HER cells as the source of primary cells to make a novel cell line.

Primary HER cells have a limited life span. Such cells can be cultured for only a few passages, after which the cells senesce. Transfection of HER cells with E1 constructs results in immortalization and transformation of the cells, reflected by focus formation in the cultures. This is easily recognized by both macroscopic and microscopic examination of the cultures. Such foci



**Figure 3** The E1 construct used to generate PER.C6. The pIG.E1A.E1B construct contains adenovirus type 5 sequences 459–3510. E1A expression is driven by the human PGK promoter. E1B transcription is terminated by hepatitis B virus-derived poly(A) sequences.

can be isolated and cultured further. Therefore, the pIG.E1A.E1B construct was transfected into primary HER cells, and PER.C6 cells were isolated as described in detail before [46].

After propagation of the cells to passage number 29, a research master cell bank was laid down, which was extensively characterized and tested for safety, including sterility testings (see below).

Immortalization of primary cells with E1 sequences of adenovirus guarantees (i) a stable expression of E1 proteins, as the cells need E1 expression for growth, and (ii) that no external selection marker is needed to distinguish E1 expressing from nonexpressing cells. Human adenovirus serotype 5 was taken as the donor for E1 sequences.

### C. Frequency of RCA Occurrence

In order to test whether PER.C6 cells are able to propagate adenoviral vectors without concomitant generation of RCA, E1-deleted adenoviral vectors were propagated on 293 cells and on PER.C6 cells. The adenoviral vectors used did not have any sequence overlap with E1 sequences in PER.C6. The batches of vector were analyzed for the presence of RCA, using cell culture based assays, as described before [46, 58]. The results (summarized in Table I) clearly indicate that adenoviral vectors when propagated on 293 cells, get contaminated with RCA. On the other hand, the data provided in Table I clearly demonstrate that PER.C6 cells support RCA-free propagation of E1 deleted adenoviral vectors, even if large-scale batches (produced on 1–3E10

**Table I**  
**Frequency of RCA Occurrence in 293 Cells and in PER.C6 Cells**

Helper cell	No. of productions	No. of cells per production	No. of RCA positive batches	
			2.5E9 IU	2.5E10 IU
293	22	1E8–3E9	13/22	ND
PER.C6	8	1E8–3E9	0/8	0/2
PER.C6	3	1E10–3E10	ND	0/3

*Note.* Batches of E1 deleted adenoviral vectors, propagated on either 293 and PER.C6 cells, were tested for the presence of RCA at a level of sensitivity of either 1 RCA in 2.5E9 infectious units (IU) or 1 RCA in 2.5E10 IU of E1 deleted adenoviral vector. The number of batches that were produced on either cell line, as well as the number of cells used for the production, are indicated as well.

PER.C6 cells) were tested for RCA in a very sensitive assay (1 RCA/2.5E10 infectious units).

In a separate experiment, an E1- and E3-deleted Ad5 vector was derived and propagated in PER.C6 cells. A master virus seed (MVS), prepared from passage 12, was used to generate 8 virus-production lots (passage 13). The unprocessed virus harvest (vector-infected suspension culture) of the MVS and the virus-production lots were tested for RCA. In brief, test articles were frozen and thawed and then assayed by inoculation onto the human-lung-carcinoma (A549, ATCC CCL 185) cell line for approximately 1–2 h at 37°C, after which the inoculum was removed and the culture was refed with medium. Cultures were passaged three times to amplify any putative RCA present, with incubation times ranging from 4 to 7 days for the early passages and 2 to 5 days for the final passage. The cultures were examined for cytopathic effects at each passage. The virus-production scale was approximately 20 L and a 60 mL volume (diluted to 600 mL to avoid toxicity and interference with detection of RCA) was tested for RCA for each lot. The testing volume was selected on the basis of a worst-case calculation to ensure the testing of at least three dose equivalents of virus. Earlier virus-production studies suggested that the freeze–thaw extract would contain at least  $5 \times 10^9$  particles/mL (or  $10^{11}$  particles/20 mL). Thus, at least  $3 \times 10^{11}$  Ad5 particles (three dose equivalents) would be tested. Assuming a random (Poisson) distribution of RCA, if there were an average of one RCA per  $1 \times 10^{11}$  particles (20 mL), one would predict the probability of not detecting it by testing only  $1 \times 10^{11}$  particles to be  $= e^{-1}$  or 0.3679 (36.79% chance). By testing  $3 \times 10^{11}$  particles (60 mL), the (binomial) probability of not detecting 1 RCA/ $1 \times 10^{11}$  particles is reduced to  $= e^{-3}$  or 0.04979 (4.98% chance). Mathematically, this is equivalent to three independent tests of 20 mL each (60 mL total).

No RCA was detected in the MVS or in any of eight virus-production lots assayed. Using the ratio of particle/TCID<sub>50</sub> determined for purified virus (15.6 particles/infectious units), the virus-production lots were estimated to have an average of  $1.9 \times 10^{10}$  particles/mL. It was estimated that the mean probability of not detecting at least one RCA in a dose of  $10^{11}$  particles of virus-production lots was 0.000887%. Besides having directly tested the infected cell suspension of the MVS for RCA, the repeated inability to detect RCA in the various clinical batches bodes well for the RCA-free nature of the MVS. For the clinical production runs, 1 mL of MVS is used to inoculate each of 100 roller bottles (RBs). This means a total of 800 mL of MVS have been used for these “clinical lots.” Following the same calculation scheme as above, if there were one RCA per 20 mL of the MVS, there would be  $e^{-1 \times 5}$  or 0.00674 probability (0.674% chance) of not transmitting an RCA when preparing a single clinical batch. Moreover, cumulatively across the eight clinical production runs, there would be only  $(e^{-1 \times 5})^8$  or a  $4.25 \times 10^{-18}$  probability ( $4.25 \times 10^{-16}$ %) chance of not

transmitting RCA in the preparation of eight lots. In conclusion, the 60-mL freeze–thaw sample used for RCA testing provided adequate assurance for the detection of RCA in virus-production lots, at a level of one RCA for a  $10^{11}$  dose. However, for testing of future Ad5 vector lots, we plan to use a clarified lysate. In this case, the probability estimated for detection of RCA will be based on more direct measurement of virus concentration.

In summary, eliminating overlap between E1 sequences in the cell and the E1-deleted adenoviral vector eliminates RCA.

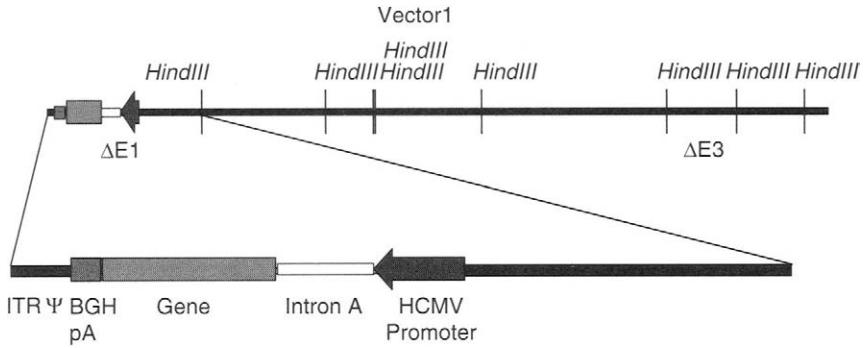
## IV. Production of Adenoviral Vectors

### A. Vector Stability

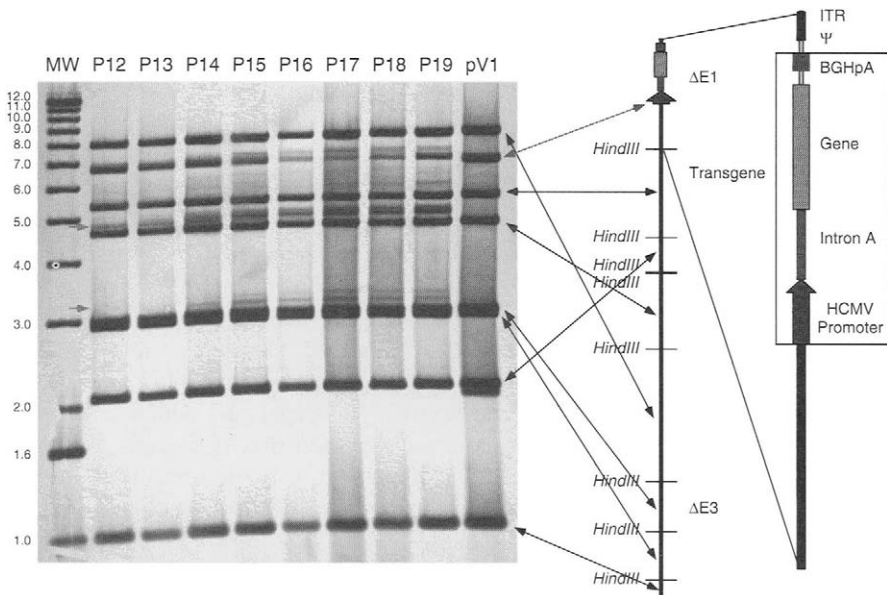
When constructing E1-deleted adenoviral vectors, a number of choices must be made regarding the structure of the vector backbone and the composition of the transgene. One must determine if the size of the E1 deletion will be adequate to accommodate the size of the transgene or if additional deletions, such as in the E3 region, will be needed. One must also decide on the placement of the transgene within the genome (E1 vs E3) and the orientation of the transgene (E1 parallel vs E1 antiparallel). Finally one must decide on the composition of the transgene in terms of the transcriptional regulation elements that are utilized (promoter and polyadenylation signals).

All of these parameters make constructing adenoviral vectors that express the transgene to the desired level, are genetically stable and propagate well enough to allow high-level production, a somewhat empirical process. The net genome size of the vector, the deletions used, transgene orientation, the composition of the transgene and the transgene product itself can all affect the growth and productivity of the vector. The degree to which vector and transgene structure can effect genomic stability and productivity is illustrated by our experience with Ad5 vector 1 (Fig. 4). Vector 1 contains an E1 deletion into which the transgene was introduced in the E1 antiparallel orientation. The transgene is composed of our gene of interest flanked by the immediate-early gene promoter and intron A from the human cytomegalovirus, and the bovine growth hormone polyadenylation signal sequence. In addition to the deletion of the E1 region, the vector has an E3 deletion [73].

When the genetic stability of vector 1 was assessed after serial passage in PER.C6 it was found to be unstable. Restriction analysis of purified viral DNA recovered from passages 12 to 19 indicated that the virus population contained genetic variants (Fig. 5). Over this passage series, the proportion and number of variants appeared to increase. An analysis of the novel restriction fragments and close to 1000 individually recovered, circularized viral genomes, indicated that two genetic mechanisms could account for all of the observed



**Figure 4** Genetic structure of Ad5 vector 1.



**Figure 5** Genetic structure of serially passed vector 1. Viral DNA was purified from passages 12 to 19 of vector 1 digested with *HindIII* and end-labeled with [<sup>32</sup>P]-dATP. The end-labeled restriction fragments were then size-fractionated by gel electrophoresis and detected by autoradiography. pV1, the plasmid used to derive Vector 1 is shown for comparison. The position in the vector 1 genome to which the restriction fragments correspond is indicated on the right. The reduction and upward shift in the 6.6-kb transgene-containing restriction fragment (uppermost double arrow) is due to amplification of the 107-bp sequence in the packaging region. Novel bands seen at approximately 4.8 and 3.2 kb (arrowheads) are due to deletions in the transgene in association with amplification in the packaging region.

RFLPs: (i) deletions of the transgene expression cassette, particularly in the region of the hCMV promoter and intron A, and, in two instances, deletion of only adenovirus sequence; and (ii) amplification (two to four repeats) of a 107-bp sequence in the region containing the viral packaging elements. No rearrangements or insertions in the E3 region were detected.

The genetic analysis of vector 1 has led to the development of highly stable vectors that can be easily propagated in PER.C6 cells, suggesting that the genetic instability can be overcome by vector design and is not necessarily related to the use of PER.C6 cells.

## B. The Production Process

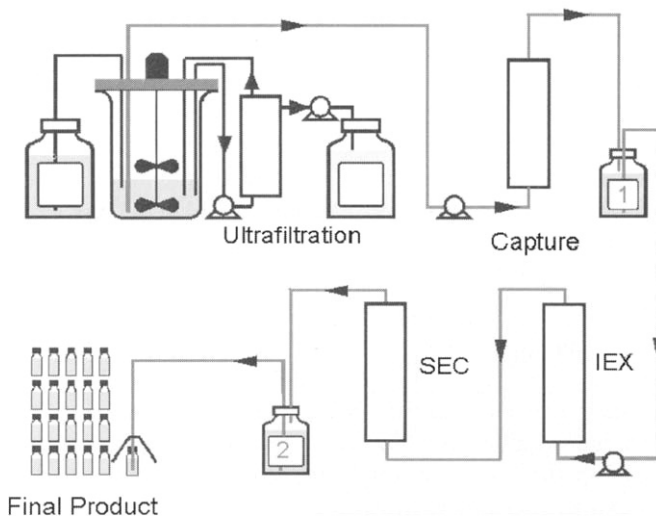
To make E1-deleted adenoviral vectors for human gene therapy, a scalable process suitable for commercial manufacturing under GMP conditions was developed. One of the key factors in the development of cell-culture-based production processes is the culture system. In particular, if scaling of the process is needed, culture of the cells in a bioreactor is highly desired. For robust and scalable systems, suspension growth of the required cell line is extremely advantageous. PER.C6 cells can be cultured both as adherent cells and in suspension culture. For suspension growth, specific well-defined serum-free media have been developed (e.g., ExCell 525; JRH Biosciences). These media do not contain any protein that is derived from human or animal tissues or specimens. This results not only in many fewer contaminants to be removed during downstream processing but also a favorable safety profile with respect to pathogens which might be introduced by animal/human-derived components.

The serum-free culture medium (SF-medium) supports the growth of PER.C6 cells to densities of  $1.5\text{--}2.5 \times 10^6$  cells/mL in routine T-flask and roller bottle cultures. In perfused bioreactor systems, cell densities up to  $10^7$  cell/mL are easily obtained.

An overview of the process of production of E1-deleted recombinant adenoviruses is presented in Fig. 6 and is summarized below.

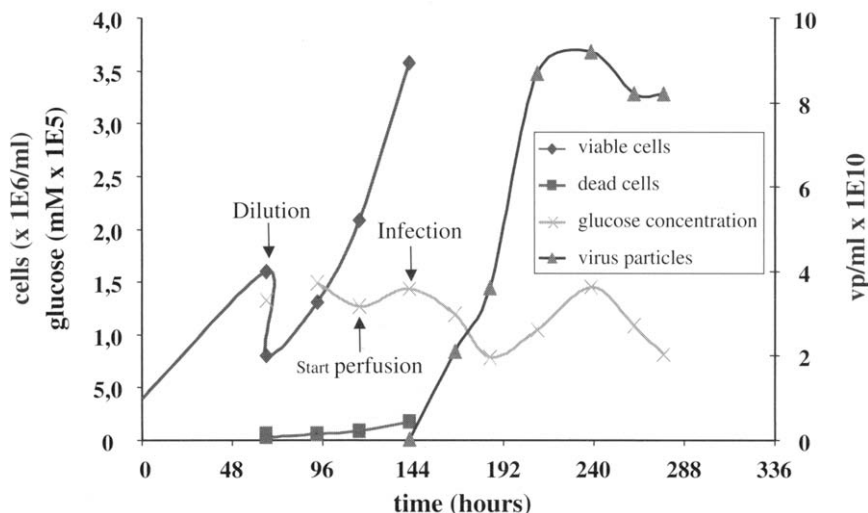
After thawing a vial of PER.C6, expansion in a T-flask containing SF-medium is done, followed by transfer of the suspension culture to roller bottles. Then these roller bottles are cultured until sufficient cells are generated to inoculate a bioreactor. In the standard batch-wise production process (e.g., in 2- or 20-L bioreactor) half of the bioreactor working volume is inoculated at  $0.5 \times 10^6$  cells/mL. Then PER.C6 is grown in 2 days to  $2 \times 10^6$  cells/mL and diluted once to  $1 \times 10^6$  cells/mL by adding the same volume of fresh medium. Then the seed virus is added and temperature is lowered from  $37^\circ$  to  $35^\circ\text{C}$ , followed by harvest after 3 days by pelleting. The latter is necessary if the purification process consists of ultracentrifugation with CsCl density gradients. After these 3 days, the virus particles become suspended utilizing





**Figure 6** Overview of the process of production of E1-deleted recombinant adenovirus. The process is described in section 4B of this chapter.

cell lysis. The batch process is very robust but not economical since only low cell densities can be obtained due to the rapid consumption of nutrients from the medium. When high cell densities are required a perfusion system can be used. Nutrients are replaced and metabolites removed by perfusion of fresh medium. A suitable perfusion system can be obtained with hollow fiber modules. These modules are operated externally on the bioreactor and can therefore easily be replaced when malfunction occurs. Hollow-fiber technology also has the opportunity for virus retention, easy scale-up, and its potential application as a first step in the virus isolation. To take full advantage of high-density cultures the virus replication should last longer than 3 days to enable the utilization of all cells present because a repeated infection can occur with newly released particles from lysed cells. A typical example of a 20-L bioreactor run is presented in Fig. 7. Because a large part of the total produced virus will be in suspension, the volume of such a culture is too large to enable purification by ultracentrifugation. Hollow-fiber ultrafiltration and chromatography are methods of choice for virus isolation and purification. With these systems directly connected to the bioreactor, thereby ensuring a closed system, all virus can be isolated from the culture medium. After capture of the virus, the bulk product can be further purified utilizing ion exchange chromatography and/or size exclusion chromatography systems. The obtained product is of high purity and infectivity. Final formulation can be done by ultrafiltration, bringing the product to the final concentration in the required buffer.



**Figure 7** Example of production of E1-deleted adenoviral vectors in PER.C6 in a 20-L bioreactor. PER.C6 cells are seeded at a density of  $0.5E6$  cells/mL, in ExCell525 culture medium. Perfusion is started 48 h later, at a rate of 1 bioreactor volume/24 h. The glucose concentration remains constant during perfusion. Under these conditions, cell densities of  $1 \times 10^7$  cells/ml are obtained.

### C. Yields of Adenoviral Vectors

The yields of virus obtained after propagation in PER.C6 cells in 20-L suspension cultures ranges from  $0.6 \times 10^{11}$  to  $1.1 \times 10^{11}$  vp/mL culture medium with an average yield of  $0.8 \times 10^{11}$  vp/mL ( $n = 5$ ). The cell density during infection was approximately  $3 \times 10^6$  cells/mL. The calculated virus yield per cell is therefore  $0.2 \times 10^5$ – $0.4 \times 10^5$  vp/cell. As the cultures are inoculated at a multiplicity of infection of 40 vp/cell, an amplification factor of 500 was achieved. The loss during isolation and purification can be held to 70–80%. This figure was consistently obtained in multiple runs for three different adenoviral vectors.

Similar yields of E1-deleted adenoviral vectors obtained on PER.C6 have been obtained by others [74].

### D. Scale of Adenoviral Vector Production

The estimated scale of the required bioreactor and cell-line stability is calculated as follows. The cell density used for virus production in perfusion mode is  $3$ – $6 \times 10^6$  cell/mL. Therefore, assuming at least 20,000 virus particles per cell yield, the overall expected yield in the crude bioreactor harvest is  $2 \times 10^4$  vp/cell  $\times 5 \times 10^6$  cell/mL =  $1 \times 10^{11}$  vp/mL. Further, after optimization,

maximum expected loss of virus particles after downstream processing (DSP) by column chromatography is 75%. Therefore, from a 20-L perfusion bioreactor  $1 \times 10^{11}$  vp/mL  $\times$  0.25 (recovery)  $\times$   $10^4$  mL =  $5 \times 10^{14}$  vp can be obtained. This gives  $5 \times 10^{14}$  vp/ $1 \times 10^{10}$  vp/dose = 50,000 doses (assuming  $1 \times 10^{10}$  vp/dose). When during product development 40% of the batch is retained for QC and archiving purposes 3000 patients can receive  $50,000 \times 0.6/3000 = 10$  doses each. Therefore, using the currently developed technology, this 20-L bioreactor is sufficient for the generation of material for the first clinical studies. However, to be able to do process development on a larger scale, needed for full commercial production, a larger vessel is required. Full production scale is expected to be about five times larger, and therefore a 100-L bioreactor is expected to be the maximum volume required for application with single doses up to  $10^{10}$  vp. To propagate the cells from a working cell bank ampoule, containing  $5^6$  cells, to a  $5E6$  cell/mL culture in a 100-L bioreactor would take 17 cell doublings. So a reliable production process would require a cell line which is at least stable over 20 cell doublings. PER.C6 was shown to be stable with respect to E1 expression for at least 98 cell doublings.

## V. Safety Considerations of PER.C6

### A. QC Testing of PER.C6 Cells for Use in the Manufacture of Biologicals and Vaccines

The safety of vaccines and biologicals manufactured in continuous cell lines of animal or human origin is of *paramount importance* and *must be ensured* by the manufacturer through a program of quality control (QC) testing applied to the product before release for human administration. This QC testing is intended to (i) ensure the *identity* of the product, (ii) ensure the *safety and sterility* of the product by demonstrating the absence of adventitious microbial agents, and (iii) ensure the *safety and sterility* of the product by demonstrating the absence of adventitious viral agents. The program for QC testing applied to a biological product, formalized as a *release protocol*, is developed as a responsibility of a Department of BioAnalytical Development. The release protocol is developed through an evaluation and integration of (i) relevant compendial literature and precedents, (ii) the origin of the cell line used for production and its development as a master cell bank, (iii) the sourcing and quality control testing of raw materials of animal origin used in manufacture, and (iv) the method of good manufacturing practice (cGMP) manufacture of the bulk and intermediate and final product considering, among other things, the quality of environment in which bioprocessing is conducted, the method of manufacture, in particular the isolation of the culture system from operators, and the consistency of preparation.





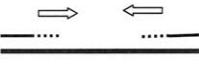
The release protocol prescribes the QC testing to be applied not only to final product but, importantly, master cell banks, master virus seeds, and other bioprocess inputs, raw materials of animal origin, and intermediate bulk products developed during downstream processing, purification and formulation. The release protocol specifies testing methods and volumes to be tested relying upon bacterial broth and agar cultures, embryonated eggs, small animals, and *in vitro* cell culture in a variety of primary and continuous cell lines of mammalian or human origin. These methods are well known to be sensitive to the detection of a variety of bacterial and viral agents and applied in concert provide a comprehensive and sensitive analytical approach upon which to ensure product safety. More recently, with the development of exquisitely sensitive polymerase chain reaction (PCR) methods for the detection of agents which are refractory to animal or cell culture, these classical propagation methods are commonly supplemented with agent-specific testing, using PCR and polymerase-enhanced reverse transcriptase (PERT) assays. The general methods of testing to ensure product safety are presented in illustrated form in Fig. 8.

### 1. QC Testing for the Release of PER.C6 Master Cell Bank

The development of PER.C6 research master cell bank (rMCB) A068-016 to support manufacture of biologics has been previously described. The release protocol to ensure the (i) identity, (ii) sterility, and (iii) viral safety of the rMCB is presented in Table II. The QC testing was conducted by contract at Inveresk Research (Tranent, Scotland) and at MicroSafe (Leiden, The Netherlands).

**Table II**  
**Release Protocol for Crucell rMCB A068-016**

Test	Method
Identity	Isoenzyme analysis
Sterility	Broth and agar for cultivation of bacteria, fungi, mycoplasma <i>In vitro</i> indicator cells for detection of mycoplasma using Hoechst stain
Viral safety	
<i>In vivo</i> eggs	Eggs (allantoic and yolk sac)
<i>In vitro</i> cell culture	MRC-5, HeLa, Vero, bovine cells
Agent-specific testing using PCR	HBV, HCV, EBV, HHV6, HIV-1, HIV-2, HTLV-1, HTLV-2, AAV, B19, SV40
Agent-specific testing for retroviruses	PERT, S <sup>+</sup> L <sup>-</sup> , XC testing

<i>Method</i>	<i>Criteria for Evaluation</i>
 <p><b>Sterility</b> Inoculation of Broth and Agar Culture and Cell Cultures with Observation of 14-21 days</p>	<p><b>Turbidity, Colony Formation Cytoplasmic Fluorescence</b></p>
 <p><b>In Vivo Testing in Eggs</b> Injection of Eggs by Amniotic, Allantoic or Yolk Sac Routes with Observation for 7-14 days</p>	<p><b>Viability Gross morphology Hemagglutination</b></p>
 <p><b>In Vivo Testing in Animals</b> Injection of Adult or Suckling mice, Guinea pigs or Rabbits by IM, IP, or SC Routes with Observation for 7-60 days</p>	<p><b>Viability Fitness Evidence of Disease</b></p>
 <p><b>In Vitro Testing in Cell Culture</b> Inoculation of Primary or Continuous Cell Lines of Human, Primate or Animal Origin with Observation for 14-28 days</p>	<p><b>Evidence of Cytopathology Hemadsorption Hemagglutination</b></p>
 <p><b>Testing for Specific Virus Agents</b> Use of Sequence Specific Primers for PCR Amplification or PERT, or TEM</p>	<p><b>Evidence of Gene Specific Product</b></p> <p><b>Evidence of Enzymatic Activity of RT</b></p>

**Figure 8** Testing methods for the demonstration of product safety.

## 2. QC Testing for the Release of a PER.C6 Working Cell Bank

The release protocol of research working cell bank (rWCB) A068-043W, according to the panel of testing, is presented in Table III. The QC testing was conducted by contract at Inveresk Research and at MicroSafe. This testing included tests for (i) identity, (ii) sterility, and (iii) viral safety in cells of human and simian origin.

## 3. Development of a Master Cell Bank at the Merck Research Laboratories

Cryopreserved vials of the rWCB were obtained from Crucell by the Merck Research Laboratories and expanded under conditions of cGMP manufacture to create a master cell bank (MCB) for future manufacturing use. This MCB has been released for use in the propagation of recombinant adenovirus according to a release protocol presented in Table IV. The preponderance of this QC testing was conducted by Q-One BioTech (Glasgow, Scotland).

This release protocol for the rWCB provides persuasive demonstration of the (i) identity, (ii) sterility, and (iii) viral safety of the PER.C6 MCB. This release protocol specifies animal testing in small animals to supplement the egg safety testing applied to the rWCB, expands the variety of primary and continuous cell lines used for viral safety using *in vitro* cell culture, and greatly broadens the variety of agent-specific testing using PCR-based testing and biochemical testing for retroviruses. The human cell line 293 was included in the panel of tissue culture cell lines in an attempt to detect the presence of any defective adventitious virus that requires the presence of E1 in the host cell. The direct assay for reverse transcriptase, as well as the detection of RT in cocultivation supernatant fluids, was done with the highly sensitive PCR-based reverse transcriptase (PBRT) assay. The supplemental PCR tests were included

**Table III**  
**Release Protocol for Crucell rWCB A068-043W**

Test	Method
Identity	Isozyme
Sterility	Broth and agar for cultivation of bacteria, fungi, mycoplasma <i>In vitro</i> cell culture testing for mycoplasma
Viral Safety	
<i>In vitro</i> cell culture	Vero, MRC-5, PER.C6
Agent-specific testing using PCR	Adeno-associated virus

**Table IV**  
**Protocol for Release for the  $\alpha$  PER.C6 Master Cell Bank**

Test	Method
Identity	Isozyme analysis DNA Fingerprinting PCR-Based Test for E1
Sterility	Broth and agar for cultivation of bacteria, fungi, mycoplasma  <i>In vitro</i> cell culture testing for mycoplasma
Viral safety	
<i>In vivo</i> eggs	Eggs (allantoic and yolk sac)
<i>In vivo</i> animals	Guinea pig, adult and suckling mouse
<i>In vitro</i> cell culture	VERO, MRC-5, 293, Rabbit Kidney 13, Vero, bovine turbinate, porcine kidney
Agent-specific testing	Transmission electron microscopy PERT for RT Raji, RD, H9 cell-cocultivation for retroviruses  PCR for HBV, HCV CMV, EBV, HHV6, HHV7, HHV8, HIV-1, HIV-2, HTLV-1 & HTLV-2, SiFV, SFV, AAV, B19, bovine polyoma, SV40

with due consideration for the human origin of the cell line and the use of bovine serum for the derivation of the cell line. The tumorigenic potential of the cell line was tested beyond the anticipated manufacturing cell-passage level.

Satisfactory results were obtained from all QC testing. The results of the testings are presented in Table V.

## B. Tumorigenicity

### 1. Tumorigenicity Studies of PER.C6 Cells

Three tumorigenicity studies were carried out on the PER.C6 cell line. The results of these studies are summarized in Table VI. In the first study, nude (nu/nu) mice were injected subcutaneously with  $10^7$  PER.C6 cells. Positive control animals were injected subcutaneously with  $10^7$  KB cells. KB is a known tumor-producing cell line derived from an epidermoid carcinoma (American Type Culture Collection; CCL-121). The study was conducted over 28 days, at which point all animals were necropsied and examined grossly and histologically. All of the positive control animals had growing nodules, and 8 of 10 male mice and 7 of 10 female mice receiving PER.C6 cells had growing nodules, thus producing a positive test (Table VIA).

At the time of the first study, 21 or 28 days was the duration that was usually used. Subsequently the Center for Biologics Evaluation and

**Table V**  
**Summary of Testing of PER.C6 Research Master Cell Bank (Passage No. 29)**

Test	Specification	Result
Sterility (EP)	Negative	Negative
Mycoplasma (broth, agar and DNA staining)	Negative	Negative
<i>In vitro</i> virology for adventitious viruses (28 days, with cytopathic effect and haemadsorption) on Vero, MRC-5, HeLa and PER.C6 cells (PTC)	Negative	Negative
Specific viruses		
Human immunodeficiency virus types 1 and 2	Negative	Negative
Human T-lymphotropic virus types 1 and 2	Negative	Negative
Human hepatitis B + C	Negative	Negative
Human cytomegalovirus	Negative	Negative
Human parvovirus B 19	Negative	Negative
Human herpes virus 6	Negative	Negative
Simian virus 40	Negative	Negative
Adeno-associated virus	Negative	Negative
Epstein–Barr virus	Negative	Negative
Bovine viruses (BVD, IBR and PI3)	Negative	Negative
<i>In vivo</i> virology in suckling mice (i.c. and i.p.), and embryonated eggs, allantoic and yolk sac injections (PTC)	Negative	Negative
Isoenzyme test for human origin	Confirmed	Confirmed
<i>In vivo</i> virology (adult mice, guinea pigs and suckling mice) and transmission electron microscopy (TEM)	Absence of adventitious microbial contamination	Free from infectious adventitious microbial contamination
Reverse transcriptase assay	Negative	Negative
S <sup>+</sup> L <sup>-</sup> focus forming assay and XC plaque assay	Negative	Negative
Tumorigenicity in nude mice	Report result	Tumorigenic
Restriction analysis	No evidence of mutation or rearrangements	No evidence of mutation or rearrangements
Sequencing	Report sequence	Sequence reported

(continued)



**Table V**  
(continued)

Test	Specification	Result
DNA profiling rMCB (passage 29) and late passage cells (passage 98)	Late passage banding pattern resembles rMCB	Late passage banding pattern resembles rMCB
Karyotyping/chromosomal analysis	Report chromosome numbers	Modal No. 86. Range 68–106
Fluorescent product enhanced reverse transcriptase (PERT) assay	Negative	Negative
S <sup>+</sup> L <sup>-</sup> focus forming assay and XC plaque assay	Negative	Negative
Multicolor fluorescent <i>in situ</i> hybridization (M-FISH)	Report integration site	Chromosome 14
Copy no. determination (fiber FISH analysis)	Report results	13.6 ± 6.1
Prions Determination of prions Sequence analysis	No evidence for infectious PrP <sup>Sc</sup>	Confirmed

Research (CBER) of the Food and Drug Administration had suggested the observation period be extended to 84 days. This was to give more time for slow growing tumors to appear and for nontumorigenic nodules to regress or disappear. Therefore, the tumorigenicity study on the PER.C6 cells was repeated.

The second study was performed in nude (nu/nu) mice over an 84-day period. Thirty nude mice were injected subcutaneously with 10<sup>7</sup> PER.C6 cells in 0.2 mL of serum-free medium. As a positive control, 10 mice were injected subcutaneously with 10<sup>6</sup> HeLa cells in 0.2 mL of serum-free medium. As a negative control, 30 mice were injected with 0.2 mL of medium. The mice were palpated at the injection site every 3 to 7 days and any nodules found were measured in two dimensions. The PER.C6 cell test arm and the negative control arm had 10 mice necropsied 21, 42, and 84 days postinjections. The positive control arm was necropsied at 42 days postinjection. Gross and histological examinations were performed on all injection sites and nodules if they appeared. During the initial days after injection, palpable nodules were present at the subcutaneous injection sites in all animals inoculated with PER.C6 cells. Between postinjection days 5 and 14, the detectable masses disappeared from the injection sites. However, in several of these mice, the masses subsequently reappeared by around day 21 and continued to enlarge until the animals were necropsied. Of the mice injected with PER.C6 cells, 5 of 10 sacrificed on day 21, 5 of 10 sacrificed on day 42, and 1 of 10

**Table VI**  
**Tumorigenicity of PER.C6 Cells**

A. Day 28 tumorigenicity of PER.C6 and KB cells in nude mice				
Cell type	No. of cells	Male	Female	
KB	$1 \times 10^7$	10/10	10/10	
PER.C6	$1 \times 10^7$	9/10	7/10	

B. 84-Day tumorigenicity study of PER.C6 and HeLa cells				
Cell type	No. of cells	Day 21	Day 42	Day 84
HeLa	$1 \times 10^6$	NA	10/10	NA
PER.C6	$1 \times 10^7$	5/10	5/10	1/10
Medium control	—	1/10 <sup>a</sup>	0/10	0/10

C. Titration tumorigenicity study of PER.C6 cells in nude mice				
Cell type	No. of cells	Day 21	Day 42	Day 84
PER.C6	$1 \times 10^3$	0/10	0/10	0/10
PER.C6	$1 \times 10^5$	0/10	0/10	0/10
PER.C6	$1 \times 10^7$	5/10	9/10	7/10 <sup>b</sup>
Medium	—	0/10	0/10	0/10

*Note.* Details of the experiment are presented in Section 5B.

<sup>a</sup> Benign lung adenoma.

<sup>b</sup> Seven animals sacrificed, with tumors on day 56 and leaving 0/3 at day 84.

sacrificed on day 84 (actually sacrificed on day 49 due to tumor size) had gross or microscopic evidence of a tumor (Table VI B). Histologically, these recurrent nodules were composed of sheets of large pleomorphic cells with numerous, sometimes abnormal, mitotic figures. These masses compressed the surrounding tissues but were not invasive. No tumors were observed outside the injection sites. The interpretation of the test is that PER.C6 cells are positive for tumorigenicity.

In view of the positive tumorigenicity results obtained following injection of  $10^7$  PER.C6 cells, a titration study was performed in which nude mice were injected with PER.C6 cells at doses of  $10^7$ ,  $10^5$ , or  $10^3$  cells per animal. Mice were necropsied 21, 42, or 84 days postinjection. No animals receiving  $10^3$  PER.C6 cells had palpable masses at the injection site from the first palpation day until necropsy. None of these animals had gross or microscopic evidence of nodules or tumor cell collections at any necropsy time point. Two of the 30 mice receiving  $10^5$  PER.C6 cells had palpable nodules on postinjection day 3.

These masses disappeared by day 7 and did not recur. Gross and histological examination of the injection sites were negative at all necropsy time points. In the mice that received  $10^7$  PER.C6 cells, 29 of 30 animals had palpable nodules on Day 3—some of which disappeared or became smaller but most of these recurred and grew progressively until necropsy. At necropsy, 5 of 10 mice on day 21 had tumors, 9 of 10 mice sacrificed on day 42 had tumors, and 7 of 10 in the group scheduled for day 84 had tumors but were sacrificed on day 56 because of tumor size (Table VI C). The histological and gross features of the PER.C6 cell tumors were similar to those described for the previous study (above). No metastatic nodules were found. Thus, the tumorigenicity studies of PER.C6 cells were positive at  $10^7$  cells per animal and negative at  $10^5$  and  $10^3$  cells per animal. This would indicate that not all of the PER.C6 cells are tumorigenic and/or a critical mass of tumorigenic cells are necessary for tumor formation.

## 2. Tumorigenicity Studies of Residual DNA from PER.C6 Cells

In view of the positive tumorigenicity studies with  $10^7$  PER.C6 cells, the oncogenic potential of residual DNA from these cells was tested in both nude mice and newborn hamsters. For these studies, DNA was isolated from passage 61 PER.C6 cells using standard procedures. The DNA preparation was shown to be of high molecular weight (average size  $\sim 100$  kb) and devoid of significant protein or RNA impurities. In the nude mouse study, 20 female nude (nu/nu) mice were injected subcutaneously with 225  $\mu$ g of PER.C6 DNA (in a volume of 0.25 mL). For negative controls, two groups of 20 female mice each were injected subcutaneously with 0.25 mL of vehicle. Approximately 5 months after injection, the mice were necropsied and examined histologically for tumor growth. None of the mice in this study exhibited gross or microscopic evidence of tumors at the injection site. One treated mouse had a lymphoma at a distant site. However, nude mice—particularly females—are known to have a high incidence of spontaneous lymphoma [75–78], and the occurrence of a single lymphoma in 20 treated mice is consistent with the spontaneous incidence. Although the lymphoma was almost certainly a spontaneous event, a polymerase chain reaction (PCR) study was performed on the lymphoma DNA to determine if there was any evidence for the presence of the adenovirus E1 region—the transforming agent of PER.C6 cells. The study was negative, with a sensitivity of approximately one copy of E1 per 750 tumor cells. Previously, E1 expression has been shown to be necessary to maintain the transformed state of 293 cells, which, like PER.C6 cells, were transformed by E1 [79]. The results of the PCR analysis support the conclusion that the lymphoma was a spontaneous event, not induced by PER.C6 DNA.

A second tumorigenicity study using DNA from PER.C6 cells was carried out in newborn hamsters. Between 18 and 36 h after birth, female and male

hamsters (28 total) were injected subcutaneously with approximately 100  $\mu\text{g}$  of PER.C6 DNA (in a volume of 110  $\mu\text{L}$ ). Two groups of control hamsters (50, mixed sex, per group) were injected with 100  $\mu\text{L}$  of vehicle. Several pups in each group were lost due to maternal cannibalism, reducing the group sizes to 20 (11 female, 9 male) in the PER.C6 DNA group, 40 (19 female, 21 male) in control group 1, and 45 (27 female, 18 male) in control group 2. After weaning, the hamsters were palpated on a weekly basis. The hamsters were necropsied approximately 5 months after injection and examined grossly and histologically for tumor growth. One female hamster in control group 2 died approximately 21 weeks after injection of a malignant ovarian teratoma. No evidence of tumors was found in the 20 hamsters that were injected with PER.C6 DNA.

### 3. Concerns about Using a Tumorigenic Cell Substrate

The basis for concern about using a tumorigenic cell substrate to produce a vaccine includes three theoretical possibilities. First, DNA from the cells carrying a putative activated oncogene or cancer-causing mutation could be integrated into the recipient's genome and produce a tumor. Second, a transforming protein in the cells could be transmitted and result in a tumor. Third, an adventitious tumor virus may be present and could be transmitted to the recipient and produce a tumor.

Concerning residual DNA from a tumorigenic cell substrate, there have now been several reports demonstrating that DNA extracted from tumorigenic cell lines or tumors growing *in vivo*—and even purified activated oncogenes—do not produce tumors when injected into animals at levels up to 1000  $\mu\text{g}$  of DNA [80–87]. The negative results obtained with PER.C6 DNA in nude mice and newborn hamsters are consistent with these findings. In the case of the PER.C6 studies, the amount of DNA injected ( $\sim 100$  or 225  $\mu\text{g}$ ) represents a  $>10^6$ -fold excess compared to the amount of residual DNA present in a dose of vaccine produced on this cell substrate. Others have calculated that 100 pg of residual DNA from tumorigenic cells would be equal to less than a billionth of a tumor-producing dose [80–87].

The second concern, transforming proteins or growth factors, has been considered by a WHO study group to be significant only if they are continually produced by cells or have continued administration [80, 81]. The study group did not consider the presence of contaminating known growth factors, in the concentrations that they would be found, to constitute a serious risk in biological products prepared from continuous cell lines.

The third category of concern, viruses or other adventitious agents, does present a potential risk. This risk is greatest when primary cells are used because of the frequent need for newly acquired cells that require repeats of the extensive testing for adventitious agents. Human diploid cell lines and continuous tumorigenic cell lines are thoroughly and routinely tested for a

wide variety of known and unknown adventitial agents in a series of *in vitro* and *in vivo* assays, thus providing adequate assurance that adventitial agents will not be transmitted.

### C. Prion-Related Issues

It is now generally accepted that an abnormal form of the cell surface glycoprotein PrP, or prion protein, is the main infectious agent in transmissible spongiform encephalopathies like scrapie, bovine spongiform encephalopathy (BSE), and Creutzfeldt–Jakob disease (CJD) ([88] and reviewed in [89]). The abnormal form of PrP, called PrP<sup>sc</sup> or PrP-res, is characterized by a remarkable resistance to denaturing agents and to degradation by Proteinase K (Prot K). Diagnostic tests take advantage of this unusual stability that allows a distinction between PrP<sup>c</sup> and PrP<sup>sc</sup> using antibodies that recognize both forms of PrP (e.g., [90]).

Human prion diseases occur in sporadic, acquired or inherited forms with different clinical and pathological phenotypes (reviewed in [91]). In 1996 a new variant of CJD (vCJD) was reported in the United Kingdom in relatively young patients with clinical features different from the known CJD forms [92]. It was also found by strain typing that the prion protein of these patients was indistinguishable from the one that causes BSE, thus raising the question whether vCJD could be acquired by consumption of meat from cattle suffering from BSE [93, 94]. The possibility of transmission of PrP<sup>sc</sup> from bovine to human raises safety issues for cultured cell lines used for the production of human drugs.

Therefore, PER.C6 cells were carefully examined for the PrP phenotype (see below) as well as genotype. It has been found that specific mutations in the PrP gene are associated with hereditary forms of human prion disease (reviewed in [89] and [91]). Furthermore, a common methionine/valine polymorphism at codon 129 of the PrP gene appears to be associated with phenotypic variability and susceptibility to sporadic and iatrogenic CJD. The vast majority of patients suffering from sCJD and also from vCJD were found to be homozygous for 129 M, whereas patients heterozygous at codon 129 were strikingly underrepresented [95–97]. To examine whether the PER.C6 PrP gene contains any of the known mutations associated with susceptibility to prion diseases, the PER.C6 PrP gene was sequenced. For these sequencing studies, genomic DNA from PER.C6 cells was isolated, and used to amplify the PrP gene sequences by PCR. The resulting PCR product was cloned into a vector, and the PrP gene in each of 13 PrP-containing clones was sequenced by BaseClear (Leiden, The Netherlands). Five of these clones contained sequences coding for the 129 Methionine PrP<sup>c</sup> protein, while the other eight contained the 129 Valine PrP<sup>c</sup> sequence, demonstrating the heterozygosity at this position. To confirm this observation, the resulting PCR product was also sequenced. As expected, a double peak (g/a) was observed in the 129 codon at a position

defining it as a valine (if the nucleotide is a guanine) or as methionine (if the nucleotide is an adenine). The PER.C6 PrP gene sequence was then compared to the wild-type sequence published in GenBank (Accession No. M12899) and was found to be identical to the wild type gene; thus, ruling out the possibility that these cells possessed a hereditary mutation that would be predisposing for prion diseases. The sequence also revealed that PER.C6 cells are heterozygous for methionine/valine at codon 129.

PrP<sup>c</sup> is constitutively expressed in adult brain [90, 98, 99] and at lower levels in other tissues like liver and spleen [100]. PrP expression has also been found in a variety of rodent and human cell lines. Our studies on PER.C6 and 293 cells have shown that these cells also express the cellular form of PrP. A validated Western blot analysis of Prot K-treated protein extracts of PER.C6 cells and their parental HER cells has failed to detect any Prot K-resistant forms of PrP at passages 33 and 36 of PER.C6 cells and passage 6 of their parental HER cells.

In addition to the sequencing of the prion gene and testing for the presence of abnormal prion protein in the PER.C6 cells at an early and late passage level of the culture, serum and trypsin batches that were used were traced to see if any were derived in the United Kingdom.

Finally, it has been possible to adopt the PER.C6 cells to serum-free suspension so that bovine sera can be completely avoided in the future if desired.

The above-mentioned characteristics of PER.C6 make it a safe manufacturing cell line in this respect.

## D. Genetic Characterization of PER.C6 Cells

### 1. Sequence Analysis of E1

The integrity of the E1A and E1B coding regions present in PER.C6 was tested by sequence analysis. This was done by bidirectional sequencing of PCR fragments generated from these regions, and the sequence of these fragments was compared to the original pIG.E1A.E1B sequence, the construct that was initially used in transfection.

No mutations, deletions, or insertions were detected between the sequence of the PCR fragments and pIG.E1A.E1B, indicating that no genetic alterations were introduced in the E1A and E1B regions during transfection and subsequent culture of the cells.

### 2. Site of Integration of E1

The chromosomal integration site of the plasmid pIG.E1A.E1B in PER.C6 was determined by using the multicolor fluorescent *in situ* hybridization (MFISH) technique in combination with the principle of combined binary ratio labeling (COBRA) [101]. This technique combines 24-color COBRA-MFISH

using chromosome-specific painting probes for all human chromosomes with plasmid probe (pIG.E1A.E1B) visualization (25th color).

The pIG.E1A.E1B integration site was determined using PER.C6 cells that are derived from the research master cell bank (passage number 29). Cells were analyzed at passage numbers 31, 41, 55, and 99. Two hundred and fifty metaphases and interphases were studied.

pIG.E1A.E1B integration was detected only on chromosome 14 (Fig. 9, see color insert) and in both sister chromatids of the chromosome in all PER.C6 passage numbers screened. Of the 47 metaphases and 203 interphases, 75–80% consisted of integration of pIG.E1A.E1B in one chromosome 14, whereas 20–25% consisted of integration in two chromosomes 14 [102].

### 3. Copy Number of the E1 Construct

The number of copies of pIG.E1A.E1B present in the PER.C6 chromosome was studied by Southern blot analysis, dot blot analysis and fiber FISH analysis [102]. Southern hybridization revealed the presence of several integrated copies of pIG.E1A.E1B in the genome of PER.C6 [46].

In addition, dot blot analysis showed a pIG.E1A.E1B plasmid copy number of  $19 \pm 3$  (research master cell bank) and  $24 \pm 16$  (extended cell bank, passage number 99) per genome.

From the results it was concluded that PER.C6 consists of five to six copies of pIG.E1A.E1B per haploid genome.

Fiber FISH enables physical length measurements of *in situ*-hybridized DNA probes on linearized DNA fibers with a resolution equal to the theoretical length of a linearized DNA molecule according the model of Watson and Crick (1 kb is 0.34  $\mu\text{m}$ ). Therefore, fiber FISH was conducted to measure the length of the integrated construct in the PER.C6 cell line at passage numbers (pns) 31, 41, and 99. Twenty fibers were measured. It was determined that pIG.E1A.E1B was integrated in tandem copies in chromosome 14 of PER.C6. The copy number of these in-tandem integrations was determined to be as follows: pn31,  $13.6 \pm 6.1$ ; pn41,  $18 \pm 4.5$ ; and pn99,  $20.1 \pm 7.9$ .

### 4. Chromosome Analysis

PER.C6 cells from cellular passages 44 and 66 were harvested for chromosome analysis to determine the modal chromosome number and the karyotype in a sample of metaphase plates. Cells were harvested, and slides were prepared and stained using a standard giemsa banding (GTG) technique. At each passage level, the chromosomes in 50 metaphase plates were counted. Also, full karyotypes were prepared from each passage level.

At passage level 44, the chromosome number ranged from 43 to 160. The mean number of chromosomes was 72 and the modal number was

61. All metaphase plates examined had structural chromosomal changes and rearrangements. A marker chromosome 19 with additional material in the long arm ( $19q^+$ ) was the most common alteration and was found in 14 of the 20 metaphase plates that were karyotyped.

At passage 66, the chromosome number ranged from 42 to 112. The mean number of chromosomes was 63 and the modal number was 64. All metaphase plates karyotyped again were found to have structural changes. The  $19q^+$  was again the most common change, observed in 15 of 20 karyotypes. There was also a marker chromosome 11 with extra material in the short arm ( $11p^+$ ) in 14 of the 20 karyotypes and a marker chromosome 9 with additional material in the short arm ( $9p^+$ ) in 8 of the 20 karyotypes.

Several of the markers differed at the two passage levels, but the most common marker,  $19q^+$ , was the same. The continuing changes seen as passage level increases is typical of heteroploid continuous cell lines.

### 5. DNA Fingerprinting

PER.C6 cells were also analyzed on two occasions by DNA fingerprinting. DNA profile analysis of PER.C6 indicated no changes in the banding pattern obtained between the research master cell bank (pn 29) and an extended cell bank that was laid down at passage number 99. On a second occasion, a consistent DNA fingerprint was obtained between pn 45 and pn 67. There was no evidence of cross contamination with other cell lines.

## VI. Conclusions

At the present time, the PER.C6 cell line is the best substrate for the production of adenoviral vectors for gene therapy or vaccines. This conclusion is based on the ability to obtain good yields and safety considerations.

The major safety considerations are the possibility of:

- i. the production of replication-competent adeno virus (RCA);
- ii. a tumorigenic risk from the transformed cell line;
- iii. the presence of abnormal prions;
- iv. contamination by adventitial agents.

As described in this chapter, the lack of any overlap between the genome of the adenoviral vectors that carry the E1 deletion and the adenoviral E1 sequences carried in the PER.C6 cells makes homologous recombination impossible, thereby preventing the formation of RCA.

It is well known that many transformed cell lines can produce tumors when injected into immunodeficient animals. As described, PER.C6 cells produce tumors in nude mice when  $10^7$  cells are injected. They do not produce



tumors, however, when  $10^5$  or  $10^3$  cells are injected. Since it is not anticipated that there will be any PER.C6 cells in a final product, this leaves the question of possible tumorigenicity of residual PER.C6 cellular DNA. Studies in nude mice and newborn hamsters in which DNA from PER.C6 cells was injected were negative for tumor production.

The possibility of the presence of abnormal prions that could produce a neurodegenerative disease was also considered. This could occur if the PER.C6 cells had a mutation in a prion gene or if the cells were contaminated with abnormal prions such as in bovine spongiform encephalopathy. As far as possible, all serum and trypsin batches used from the time of origin of the culture were traced and no contact of serum from British sources was identified. The PER.C6 cell line was also adapted to serum-free suspension cultures.

The prion protein gene of PER.C6 cells was sequenced and no mutations were found and the cell line was shown to be heterozygous for the 129 M/V polymorphism. The cell line was also analyzed for the presence of abnormal prions at an early and late passage and an early passage of the HER parental line and none were found. In total, these studies indicate that the risk of a prion disease from the use of PER.C6 cells is vanishingly small.

Finally, extensive studies for known and unknown adventitial agents have been documented and are negative.

While there can be no absolute elimination of risk, this body of studies indicates a minimal, if any, risk from the use of this cell substrate for the production of adenoviral vectors. As new studies are developed they will also be applied to ensure that no hazards are present. It has often been pointed out that a continuous cell line such as PER.C6 permits extensive analysis for adventitial agents and other safety concerns and thus is less hazardous than short-lived primary cell cultures for which testing must be repeated for each newly established culture.

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