Rapid titration of adenoviral infectivity by flow cytometry in batch culture of infected HEK293 cells

Vincent Gueret, Juan A. Negrete-Virgen, Andrew Lyddiatt & Mohamed Al-Rubeai*

Department of Chemical Engineering, University of Birmingham, Birmingham, B15 2TT, UK

(* Author for correspondence; E-mail: m.al-rubeai@bham.ac.uk; Fax: +44(0) 121 414 3888)

Accepted 31 March 2002

Key words: adenovirus titration, adenoviral vectors, flow cytometry, GFP, infectivity

Abstract

There is a constant and growing interest in exploiting adenoviruses as vectors for gene therapy when transient expression of a therapeutic protein is necessary. The requirement for an increased viral titre has prompted a search for techniques by which this virus may be assayed with greater speed and simplicity. Conventional plaque assay for quantification of adenoviral vectors titre in current use is laborious and time-consuming (up to 14 days). We report herein a method for the monitoring of adenovirus expressing green fluorescent protein that incorporates rapid and easy sample handling by means of flow cytometric analysis. Cells (HEK293) were infected with adenovirus at various multiplicity of infection (MOI), harvested 17 to 20 h post infection and analysed by flow cytometry. Assumptions were made that one fluorescent cell was infected by a single infectious particle at a relatively low MOI. The adenoviral titre was subsequently estimated from cell analysis in a relatively short time. The results obtained with an E1-complementing cell line (HEK293) were compared with that obtained using a non-complementing cell line (A549). A Poisson distribution successfully modelled the profile of infection as a function of MOI. This provided a better understanding of adenoviral infection at the earliest stage possible. Monitoring of GFP fluorescence and virus propagation in a batch culture of infected cells was subsequently used as a practical application of the validated method.

Introduction

Adenoviruses are now widely used in gene therapy as vectors to deliver therapeutic genes when transient expression of a protein is required. The advantages of these vectors include their ability to infect a large range of cell types, their high transduction efficiency and the high concentrations of samples that can be achieved (10^{12} particles/ml) due to their stability (Kay et al., 1997; Wang and Huang, 2000). Recently potential applications of adenoviral vectors have been reported for the treatment of various diseases including cystic fibrosis, muscular dystrophy, Parkinson and autoimmune diabetes and the treatment of tumours in prostate, colon, cervix and ovary (Russell, 2000). Key data for administration of clinical trials is the P:I ratio representing the total viral particle number relative to infectious units whereas the bioactivity is defined

as the inverse of the P:I ratio. The total number of particles includes both functional (infectious) and non-functional vectors. In order to set an acceptable quality for adenovirus preparations used in clinical trials, the US Food and Drug Administration has recommended a maximum P:I ratio of 100:1, corresponding to a minimum bioactivity of 1% (Nyberg-Hoffman et al., 1997). Accurate and rapid adenovirus titration is thus essential to monitor and optimise the manufacture and preparation of clinical doses.

The most commonly used physically based methods to assess the total number of adenoviral particles are electron microscopy (Pinteric and Taylor, 1962), optical absorbance (Maizel et al., 1968), Pico Green fluorescent staining (Murakami and McCaman, 1999) and anion-exchange HPLC (Shabram et al., 1997; Blanche et al., 2000). Each method has advantages and

limitations when compared in terms of speed, sensitivity, and compatibility with sample components.

In contrast, biologically based methods are required for the determination of the titre of infectious particles. Such methods rely on the ability of infectious adenoviral vectors to produce quantifiable biological events. The plaque assay has long been considered as a standard method because of the inherent accuracy and reproducibility. Here, a monolayer of permissive cells are infected with serial dilutions of virus stock and incubated until visible and quantifiable plaques are formed due to viral propagation and multiple rounds of cell lysis (Cooper, 1961). Plaque assays are tedious, time-consuming (up to 14 days) and operator dependent in terms of absolute determinations.

Further available methods include fluorescent focus assays (Philipson, 1961), the end point assay (Nielsen et al., 1992) and gene transfer assays (Mittereder et al., 1996). These demand pre-treatment of samples such as cell fixation and staining that often require the use of labelled antibodies (Weaver and Kadan, 2000; Eykholt et al., 2000). Most of these methods are relatively rapid but accuracy and reproducibility commonly vary depending on experimental conditions and the operator.

Flow cytometry has been extensively used for the detection and quantitation of green fluorescent protein (GFP) in plant, mammalian and bacteria cells (Galbraith et al., 1999). Such applications include the screening of transduction efficiency (Subramanian and Srienc, 1996), the on-line monitoring of cell growth in bioreactors (Zhao et al., 1999), and the detection of adenovirus (Côté et al., 1997) and vaccinia virus expression (Domáñquez et al., 1998).

In this study a method based on flow cytometric analysis of infected GFP-expressing cells was used to titre adenoviral vectors carrying the GFP reporter gene. This method was validated by the standard method plaque assay. Furthermore the Poisson distribution modelled the infection efficiency of HEK293 and A549 cells as a function of the MOI and the state of infection was qualitatively described by flow cytometric data. The method was successfully used to monitor GFP expression and virus propagation in batch cultures of attached and HEK293 cells in suspension infected at different MOIs.

Materials and methods

Cell culture

The A549 lung epithelial carcinoma cell line was kindly donated by Dr Peter Searle, Institute of Cancer Studies, University of Birmingham. The HEK293 human embryonic kidney and the human retinal 911 cell lines were obtained from Life Technologies®. Both HEK293 and 911 cell lines are E1-complementing cell lines that can support the replication of E1-deleted replication-defective adenovirus. The A549 cell line is non-complementing. The three cell lines were cultured in Dulbecco's Modified Eagle's medium (Life Technologies®) supplemented with 10% heat-inactivated (56 °C, 30 min) foetal calf serum (hiFCS). Cell lines were subcultured every 3/4 days following digestion at room temperature with 0.5 ml trypsin/EDTA (Sigma). HEK293 cell line was adapted to grow in suspension in serum free medium SFM II (Life Technologies®) and was subcultured every 48 h by centrifuging and resuspending in fresh medium to maintain the cell density of 2×10^5 cells ml⁻¹ in 75 cm² vented T-flasks. Cell densities were determined using the standard trypan blue method and a hemocytometer. All cultures were maintained at 37 °C in an incubator with 5% CO₂ atmosphere.

Adenoviral vector

The adenoviral vector Ad5GFP used was obtained from Dr Peter Serale, CRC Institute for Cancer Studies, University of Birmingham. It comprised an E1⁻E3⁻ replication-defective, serotype 5 vector containing, in the E1 position, an enhanced green fluorescent protein (EGFP) reporter gene driven by a cytomegalovirus promoter. The EGFP has an excitation peak and an emission peak at 488 nm and 515 nm, respectively.

Flow cytometric assay

HEK293 cells were grown in T-25 flasks to at least 80% of confluence. The cells were counted by hemocytometer and infected with 200 µl of serial dilutions of the virus stock prepared in infection medium (DMEM + 2% hiFCS). After 90 min of incubation at 37 °C (gentle rocking with a periodicity of 20 min), 3 ml of infection medium were added to each flask (virus containing medium was not removed). After 17 to 20 h of incubation the supernatant was collected in a Universal sample tube and the cells were

sedimented by centrifugation and washed with 2 ml sterile Dulbecco's Phosphate Buffered Saline (PBS, Life Technologies®). Cells were trypsinised and 2 ml of infection medium were added before the cells were collected in the same container.

Aliquots of 500 μ l of each suspension were immediately analysed in the flow cytometer. Flow cytometric data were acquired using a Coulter EPICS® Elite Analyser equipped with an argon ion laser set at 488 nm. The green fluorescence signal was collected by a photomultiplier tube after passing through a 525 ($\pm$ 20) nm band pass filter (PMT2). The PMT2 amplifier was calibrated every day with standard fluorospheres Flow-Check™ (Beckman Coulter). Single viable cells were gated on the basis of the side-angle light scatter (PMT1 LOG) and the forward-angle light scatter (FS). GFP fluorescence of 3×10^4 single viable cells per sample selected on FS versus SS scatter basis, were analysed and high fluorescent cells were gated. The cytometric parameters were set to provide accurate discrimination between non-fluorescent (or self-fluorescent) negative cells and positive GFP-fluorescent cells on a PMT2 LOG (gain 1, voltage ranging from 400 to 600 V depending on the signal) versus FS density plot in order to estimate the proportion of infected cells. Listmode data analyses were performed using data analysis software (FCS Express version 1.0 for windows 95/98NT) for accurate exploitation.

Calculation of the titre in flow cytometric assay

At low multiplicity of infection (MOI < 0.3 infectious viral particles ml^{-1}) a linear relationship between the proportion of infected cells, assessed by flow cytometry, and the dilution factor can be drawn. This defines the linear zone of infection where one infected cell corresponds to a single infectious event as described previously (Nadeau et al., 2000). In these conditions, the titre can be calculated by

$$\text{TITRE (gfu ml}^{-1}\text{)} = \frac{\% \text{INF} \times \text{TOTCELLNUM} \times \text{DIL}}{100 \times \text{VOL}} \quad (1)$$

where gfu refers to green fluorescent units, %INF is the proportion of infected cells, TOTCELLNUM is the total cell number at the time of infection, DIL is the dilution factor and VOL is the volume of diluted virus stock added (here 0.2 ml). As the MOI is defined as:

$$\text{MOI (gfu/cell)} = \frac{\text{TITRE (gfu ml}^{-1}\text{)} \times \text{VOL}}{\text{DIL} \times \text{TOTCELLNUM}} \quad (2)$$

at low MOI, the linear relationship holds where:

$$\% \text{INF} = 100 \times \text{MOI} \quad (3)$$

Plaque assay

911 cells were grown in 6-well plates until even monolayers were formed. The medium was replaced with 500 μ l of serial dilutions of the virus stock. After 90 min of incubation at 37 °C the virus containing medium was replaced with 2ml overlay consisting of 0.7% noble agar (Sigma), 1.8% FCS and 0.24% MgCl_2 in DMEM. The overlay was maintained at 42 °C prior to application to prevent solidification. Titre plates were cultured in a humidified, 5% CO_2 atmosphere at 37 °C. After 7 days the cell monolayers were fed with 3 ml of overlay medium. One day before counting, the plaques were stained with 0.2% (v/v) neutral red to enhance visualisation. Plaques were counted on day 14 by visual inspection. The mean number of plaques from duplicates was determined and the titre was calculated by multiplying this value by the dilution factor and dividing by the volume of the sample.

Virus harvest

Suspension cells were spun down and resuspended in PBS+10% glycerol. Attached cells were trypsinised and then treated similarly to the suspension cells. Cells were processed through 3 cycles of freezing-thawing in liquid nitrogen and 37 °C water bath respectively. After centrifugation the supernatant was collected in a universal container for titration.

Growth of cells and virus infection in batch culture

HEK293 attached cells were grown in 75 cm^2 T-flasks using DMEM supplemented with 10% foetal calf serum. Once confluence was reached (48 h in this case) one of six flasks was used for cell counting and to calculate the volume of virus stock necessary to infect at particular MOI. The medium from the remaining five flasks was replaced with the virus stock. Cultures were incubated at 37 °C in 5% CO_2 for 90 min with gentle rocking every 20 min. After infection 30 ml of fresh DMEM was added. One flask was harvested every day for five days according to the virus harvest protocol described before.

Suspension cells HEK 293 were grown in 250 ml spinner flasks in SFM II. Cultures were incubated at

37 °C in 5% CO₂. For infection cells were centrifuged and infected with the virus stock for 90 min with gentle rocking every 20 min. After infection 100 ml of fresh medium were added and virus harvest followed as described before.

Poisson distribution

The Poisson distribution best describes the distribution of virus infection in the monolayer (Luria et al., 1978; March et al., 1995; Mittereder et al., 1996; Nyberg-Hoffman et al., 1997). At a given MOI, the probability for a cell to be infected by k viruses is given by

$$\Pr(X = k) = \frac{\text{MOI}^k \times e^{-\text{MOI}}}{k!} \quad (4)$$

The probability p for a cell to be infected is the probability for this cell to receive at least one virus, where:

$$\begin{aligned} \Pr(\text{cell infected}) &= p = \Pr(X \leq 1) \\ &= 1 - \Pr(X = 0) = 1 - e^{-\text{MOI}} \end{aligned} \quad (5)$$

This individual probability p can be used in a binomial law to predict the proportion of cells that are infected at a given MOI. As the total number of events is relatively high ($n = 3 \times 10^4$ cells analysed by flow cytometry), a Gaussian distribution $\text{LG}(np, \sqrt{np(1-p)})$ is more suitable. In these conditions, p gives a very good estimation of the proportion of infected cells

$$\% \text{INF} = 100 \times (1 - e^{-\text{MOI}}) \quad (6)$$

If we develop Equation 6 for low values of MOI, Equation 3 is obtained.

Results

Linear relationship between proportion of infected cells and number of viruses introduced

In previous reports the number of infected cells was proportional to the number of viruses introduced when less than 30% of cells were infected (Nadeau et al., 2000). In this study a correlation of $r^2 = 0.83$ ($n = 88$) was found with HEK293 cells under the same conditions. In contrast, a correlation of $r^2 = 0.98$ ($n = 81$) was obtained when less than 23% of the cells were infected, and this latter value was considered as the upper limit of the range of application of the method.

Effect of the cell line upon the method

In order to assess the effect of the complementing nature of the cell line upon the method, the results obtained using HEK293 and A549 cell lines were compared. A549 cells exhibited a lower level of GFP expression than that exhibited by HEK293. The signal obtained with A549 cells 24 h post infection was not high enough to provide rapid and accurate discrimination between non infected (non fluorescent) and infected (fluorescent) cells and required a further 24 h incubation before effective flow cytometric analysis was possible.

At low MOIs, a linear relationship between the proportion of infected cells and the MOI was obtained with HEK293 and A549 cell lines (Figure 1A). Similar titres with both cell lines were obtained $2.6 \times 10^{10} \pm 9.8 \times 10^8$ gfu ml⁻¹ and $2.4 \times 10^{10} \pm 1.2 \times 10^9$ gfu ml⁻¹, respectively (Figure 1B).

Validation of the flow cytometric assay with plaque assay

Virus stocks characterised by different titres were assayed by flow cytometry (using HEK293) and plaque assay. A linear regression can be drawn between the titres obtained by flow cytometry and that obtained by plaque assay with a correlation of $r^2 = 0.996$ (Figure 2).

Modelling of infection

To better understand the dose effect of adenovirus on target cell monolayers of A549 and HEK293, cells were infected with MOI ranging from 0 to 15 gfu/cell. The proportion of infected cells was determined by flow cytometry (Figure 3). The data obtained appeared to follow the pattern of an exponential rise to a maximum proportion of infected cells with increasing MOI. The data were subsequently fitted to the general form of an equation for the type of relationship given by

$$\% \text{INF} = 100 \times A \times (1 - e^{-B \times \text{MOI}}) \quad (7)$$

where A and B are constants. The coefficients A and B were optimised separately for HEK293 and A549 cells, and found equal to $A = 0.90$ and $B = 0.96$ ($r^2 = 0.986$) and $A = 0.95$ and $B = 1.01$ ($r^2 = 0.991$) respectively.

Figure 1. Impact of choice of cell lines HEK293 (○) and A549 (Δ) upon the analytical method. Identical virus stocks were titrated using HEK293 and A549. (A) Percentage of green fluorescent cells analysed by flow cytometry versus number of viruses introduced. This is proportional to the inverse of the dilution factor or the MOI. (B) Adenovirus titre obtained by flow cytometric assay using HEK293 and A549 cell lines at MOI = 0.23.

State of infection qualitatively described by flow cytometry data

The mean GFP intensities of GFP-expressing HEK293 cells were comparable when less than 23% of cells were infected (Figure 4). Below this limit, the relative number of GFP expressing cells was linearly virus dose-dependant. The GFP signal was shifted to higher intensities as the percentage of infected cells increased. A sub-population of higher fluorescence intensity was detected within GFP positive cells at 68% and 94% of infected cells (pointed in Figure 4). This sub-population was not detected when A549 cells were analysed (data not shown).

Monitoring of GFP fluorescence and virus propagation in batch cultures of infected HEK293 cells

Batch cultures of attached and suspension HEK293 cells were infected with AdGFP at MOIs of 10 and 50. The viral production was assessed daily during 5 days post infection by plaque assay and the validated flow cytometric assay as described previously. Cell densities, GFP fluorescence intensities and titres obtained by both plaque assay and flow cytometry from suspension and attached HEK293 were compared at the different MOIs tested (Figures 5 and 6). The results showed that the flow cytometric method is suitable for

both attached cells and cells in suspension. It is notable that there is a good agreement between the two monitoring methods in the later infection phase when the heterogeneity of the culture increases.

Discussion

Figure 2 demonstrates that the cytometric titre is proportional to the plaque titre with a coefficient of 3.3 ($r^2 = 0.996$). Previous studies have reported that estimated titres strongly depend upon the method of determination (Mittereder et al., 1996; Nyberg-Hoffman et al., 1997). In addition, discrepancies are commonly observed when different conditions are used (depth of vector containing fluid and time of infection) and different biological events are detected (transgene expression or formation of plaques). Physical and biological mechanisms have to be addressed for both flow cytometric assay and plaque assay in order to have a better understanding of the coefficient of proportionality.

Cell infection is a diffusion-limited process, in which the viral particles are constantly removed from the supernatant by the cell monolayer. Only a fraction (F_{arriving}) of viruses present in the suspending fluid diffuses to the cellular monolayer within the time of infection. Amongst F_{arriving} , only a fraction ($F_{\text{successful}}$) eventually results in a detectable infection. These

Figure 2. Comparison between the titres obtained by flow cytometry and by plaque assay. Linear regression was performed through the data points. The correlation coefficient ($r^2=0.996$) indicated a good correlation between the two methods.

mechanisms were formally described (Mittereder et al., 1996) by the expression

$$F_{\text{detected}} = F_{\text{arriving}} \cdot F_{\text{successful}} \quad (8)$$

where F_{detected} is the fraction of the total virions in the fluid that are detected in the assay and subsequently referred to as infectious particles.

In this study, the depths of adsorption fluid were maintained constant at 0.008 cm and 0.052 cm for the flow cytometric and plaque assays respectively. In previous reports little change occurred in the estimated titre at infection medium heights less than 0.052 cm (Mittereder et al., 1996). Therefore this parameter was not considered limiting in the present study. In the plaque assay, the infection medium was removed prior to agar overlay in contrast to the flow cytometric assay where infection could still occur during the 17 h incubation. In agreement with previous works (Mittereder et al., 1996), the higher titre obtained by flow cytometry can be jointly attributed to this extended time of infection and the complex biological mechanisms involved in plaque assays. The similarity of the titres obtained using HEK293 and A549 cell lines (Figure 1B) confirmed that the method was not affected by virus replication, as reported previously (Côté et al., 1997), and that the method has the potential to be used with different cell lines.

As MOI increased up to 15 gfu/cell, saturation in the proportion of infected cells was observed (Figure

3). At MOI higher than 0.23 gfu/cell, some infected cells received more than one virus and a linear approximation was no longer possible. In such cases, the behaviour was best described by Equation 7 wherein the parameter A corresponds to the maximum proportion of cells that can be infected. This represents the efficiency of infection with the given protocol. The main limiting factor upon efficiency is the small volume to surface area ratio of the infection medium, resulting in an inhomogeneous dispersal of the viruses on the cellular monolayer. Equations applied in Figure 3 have the form of Equation 6. Thus the Poisson distribution is an adequate statistical law to describe the infection. However the linear relationship at MOI below 0.23 gfu/cell was best modelled by Equation 3.

Figure 3 shows that a minimum MOI of 5 gfu/cell is required to infect the highest number of cells with the given protocol. The variability observed at MOI higher than 5 gfu/cell for HEK293 (Figure 3A) may be due to inconsistency of the mixing induced by rocking during infection at the low working volumes. This effect was not noticeable at lower MOI. The model can be exploited to predict the number of viruses introduced even when some cells receive more than one virus. This raises the upper application limit of the titration method from 23% to 80% of infected cells.

The similarity between the mean GFP intensities obtained when less than 23% of cells were infected

Figure 3. Percentage of infected cells versus MOI. Models for HEK293 (A) and A549 (B) cell lines were established by fitting the equation of an exponential rise towards the maximum with the data points. Good correlations ($r^2 = 0.986$ and $r^2 = 0.991$ respectively) between the models and experimental points were obtained.

Figure 4. Histograms depicting the GFP intensity distribution of cells infected with different serial dilutions of adenovirus stocks and analysed by flow cytometry (3×10^4 cells analysed). Depending on the number of viruses, 7.5% (MOI 0.08 gfu/cell), 17.5% (MOI 0.17 gfu/cell), 68% (MOI 1.7 gfu/cell) and 94% (MOI 8.3 gfu/cell) of the cells were infected. The percentages were determined on a 2D FS vs PMT2 density plot for accurate discrimination. As the MOI increased, the GFP intensity distribution shifted to higher intensities and a sub-population of more intense green fluorescing cells appeared (pointers). Uninfected cells show a low GFP intensity signal (Control).

Figure 5. Virus propagation in attached HEK293 cell line infected at a) 50 MOI and b) 10 MOI. Cells were grown in DMEM supplemented with 10% foetal calf serum. Cell density was measured by standard trypan blue method. Titres were obtained by plaque assay and flow cytometry. GFP fluorescence intensity was obtained by flow cytometric method. Virus propagation was carried out for 5 days.

provides additional support for the assumption of one infectious particle per infected cell at low MOI (Figure 4). Since the cells were analysed at the same time, the GFP fluorescence intensity was indicative of the number of viral genomes per cell. The amplification in the mean GFP intensity that was observed when 68% and 94% of cells were infected (Figure 4) suggested that multiple infection occurred. The apparent sub-population at 68% and 94% of infected cells corresponded to the cells in which early viral replication took place (Weaver and Kadan, 2000). These results

showed that flow cytometry analysis provides qualitative data on the GFP intensity distribution of the cell population thereby yielding rapid and accurate snapshot of the state of infection.

Monitoring of GFP fluorescence and virus propagation in batch cultures of infected HEK293 cells

At MOI 10 the highest production of adenoviruses in attached and suspension cells was at 48 h post infection (Figure 5 and 6). In both cases a correlation exists

Figure 6. Virus propagation in HEK293 cell line grown in suspension. Cells were infected at a) 50 MOI and b) 10 MOI. Cells were grown in serum free medium. Cell density was measured by standard trypan blue method. Titres were obtained by plaque assay and flow cytometry. GFP fluorescence intensity was obtained by flow cytometric method. Virus propagation was carried out for 5 days.

between plaque assay and that obtained by the flow cytometric assay validated herein. During the 5 days post infection the medium was not replenished and thus the consumption of nutrients and the accumulation of metabolites subsequently caused cell death. The decrease in adenovirus titre after 48 h post infection may thus be due to the release of the viruses from dying and damaged cells. The adenovirus titre obtained with cells infected at MOI 10 was higher than that obtained with infected cells at MOI 50. This may be explained by multiple infection at high virus-cell

ratio since multi-infected cells are more likely to die sooner than those infected with single virus.

It is expected that increasing the amount of virus in culture (MOI) can intensify the process of cell infection. Thus, by increasing the MOI, a reduction in the time of cell infection can be achieved. Figure 5 indicates that if cells were infected at high MOI they are likely to produce high viral titre at an early stage. As a consequence of cell infection at higher MOI the viable cell number and virus titre started falling immediately after infection.

No significant difference was found between the T-flasks and the spinner flasks with respect to the effect of MOI on infectivity. The equivalence of infection would imply the absence of a bulk diffusion limitation during the infection stage and that the mechanisms of attachment, fusion with the plasma membrane and engulfment operated similarly with both attached cells in T-flasks and supplemented cells in spinner flasks.

In all experiments GFP intensity was shown to increase as infectivity increased up to 48 h post infection indicating a good correlation between the two parameters. It is probable that the loss of correlation at later stages of infection resulted from increasing culture heterogeneity and different rates of release of GFP and virus from dying or membrane damaged cells.

The final titre will depend on two parameters; GFP intensity and viability, where

$$\text{Titre} = \text{Production per cell} \times$$

$$\text{Number of viable GFP producing cells} \quad (9)$$

Only highly GFP fluorescing cells were considered as viable and GFP intensity was averaged over this discrete population to provide a unique estimate of viral production per viable producing cell.

Conclusion

Flow cytometry has proved to be a useful tool for the rapid quantification of a biological titre of adenovirus vectors carrying the GFP reporter gene. Flow cytometric titres were found to be proportional to plaque assay titres. The flow cytometric assay was validated by the standard method plaque assay for titres ranging from 10^6 to 10^{10} pfu ml⁻¹. The large number of events that can be handled by flow cytometry guarantees the reliability and the reproducibility of the method. Moreover, the assay is inexpensive for laboratories equipped with flow cytometry facilities. A large number of experiments can be screened in the shortest period of time, making possible the reliable determination of adenoviral titres in gene therapy research. The technique of titration has the potential to be used with suspension cells and it could thus provide a more reliable assessment of viral titre as a practical preliminary to the infection of productive cell cultures.

Results presented in this paper have demonstrated the application of flow cytometry as a rapid and objective tool for routine measurement of viable cell density, virus titre and GFP intensity in batch cultures of HEK

293 cells infected with adenovirus expressing GFP protein. The method facilitates a determination of the best time to harvest the viruses having the highest titre at high cell densities. Production protocols for adenovirus can thus be optimised to obtain the highest yields even when different cell lines and MOI are utilised.

Acknowledgements

The authors acknowledge the support of Rhône-Poulenc Industrialisation, France (VG) and CON-ACYT, Mexico (JANV).

References

- Blanche F, Cameron B, Barbot A, Ferrero L, Guillemin T, Guyot S, Somarriba S & Bisch D (2000) An improved anion-exchange HPLC method for the detection and purification of adenoviral particles. *Gene Ther* 7: 1055–1062.
- Cooper PD (1961) The plaque assay of animal viruses. *Adv Virol Res* 8: 319–378.
- Côté J, Bourget L, Garnier A & Kamen A (1997) Study of adenovirus production in serum-free 293SF suspension culture by GFP-expression monitoring. *Biotechnol Prog* 13: 709–714.
- Domínguez J, Lorenzo MDM & Blasco R (1998) Green fluorescent protein expressed by a recombinant vaccinia virus permits early detection of infected cells by flow cytometry. *J Immunol Methods* 220: 115–121.
- Eykhoit RL, Murray DM & Marvin KW (2000) Accelerated titrating of adenoviruses. *Biotechniques* 28: 870–873.
- Galbraith DW, Anderson MT & Herzenberg LA (1999) Flow cytometric analysis and FACS sorting of cells based on GFP accumulation. *Methods Cell Biol* 58: 315–341.
- Kay MA, Liu D & Hoogerbrugge PM (1997) Gene Therapy Proc Natl Acad Sci USA 94: 12744–12746.
- Luria SE, Darnell JE Jr, Baltimore D & Campbell A (1978) Titration of viruses. In: *General Virology* (pp. 21–32) John Wiley & Sons, New York.
- Maizel JV, White DO & Scharff MD (1968) The polypeptides of adenovirus – I. Evidence for multiple protein components in the virion and a comparison of types 2, 7a and 12. *Virology* 36: 115–125.
- March KL, Madison JE & Trapnell BC (1995) Pharmacokinetics of adenoviral vector-mediated gene delivery to vascular smooth muscle cells: modulation by poloxamer 407 and implications for cardiovascular gene therapy. *Hum Gene Ther* 6: 41–53.
- Mittereder N, March KL & Trapnell BC (1996) Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J Virol* 70: 7498–7509.
- Murakami P & McCaman MT (1999) Quantitation of adenovirus DNA and virus particles with the PicoGreen fluorescent dye. *Anal Biochem* 274: 283–288.
- Nadeau I, Jacob D, Perrier M & Kamen A (2000) 293SF Metabolic flux analysis during cell growth and infection with an adenoviral vector. *Biotechnol Prog* 16: 872–884.
- Nielsen LK, Smyth GK & Greenfield PF (1992) Accuracy of the end point assay for virus titration. *Cytotechnology* 8: 231–236.

- Nyberg-Hoffman C, Shabram P, Li W, Giroux D & Aguilar-Cordova, E (1997) Sensitivity and reproducibility in adenoviral infectious titer determination. *Nat Med* 3: 808–811.
- Philipson L (1961) Adenovirus assay by the fluorescent cell-counting procedure. *Virology* 15: 263–268.
- Pinteric L & Taylor J (1962) The lowered drop method for the preparation of specimens of partially purified virus lysates for quantitative electron micrographic analysis. *Virology* 18: 359–371.
- Russell WC (2000) Update on adenovirus and its vectors. *J Gen Virol* 81: 2573–2604.
- Shabram PW, Giroux DD, Goudreau AM, Gregory RJ, Horn MT, Huyghe BG, Liu X, Nunnally MH, Sugarman BJ & Sutjipto S (1997) Analytical anion-exchange HPLC of recombinant type-5 adenoviral particles. *Hum Gene Ther* 8: 453–465.
- Subramanian S & Srienc F (1996) Quantitative analysis of transient gene expression in mammalian cells using the green fluorescent protein. *J Biotechnol* 49: 137–151.
- Wang Y & Huang S (2000) Adenovirus technology for gene manipulation and functional studies. *DDT* 5: 10–16.
- Weaver LS & Kadan MJ (2000) Evaluation of adenoviral vectors by flow cytometry. *Methods* 21: 297–312.
- Zhao R, Natarajan A & Srienc F (1999) A flow injection flow cytometry system for on-line monitoring of bioreactors. *Biotech Bioeng* 62: 609–617.