Determination of adenovirus concentration using Biacore T200

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Determination of adenovirus concentration using Biacore™ T200

This application note demonstrates the performance of two assays for determination of adenovirus concentration based on surface plasmon resonance (SPR). In one assay, virus particles were allowed to bind via the fiber protein (spikes extruding on the virus capsid) to recombinant human coxsackie adenovirus receptor (CAR) protein immobilized on a Biacore sensor chip. In a second assay, Factor X (FX) protein was immobilized on a Biacore sensor chip to allow binding of virus particles via the virus hexon protein (dominating capsid protein). Bound virus particles were detected using the Biacore T200 system. These sensitive assays, not only provided reproducible results, indicating robust performance, but also showed strong correlation with standard assays for adenovirus quantitation. In comparison with traditionally used assays, the described Biacore assays provided the benefit of significantly reduced assay and hands-on time, enabling their use in determination of virus content in critical steps of an adenovirus production process.

Introduction

As viral vectors have a potential use in vaccine and therapeutic applications such as gene therapy, cell therapy, oncolytic cancer immunotherapies and tumor vaccines, they are expected to become high-value products. Common viral vector systems include adenovirus, adeno-associated virus, and lentivirus, of which adenovirus constitutes the most widely used vector system in clinical trials. One of the most studied adenovirus vectors is the first generation of recombinant adenovirus serotype 5 (AdV5), making this a suitable model system for enhancing upstream production and improving downstream purification processes.

This work is part of a project for the development of an AdV5 production process (1), ranging from upstream production to a purified sterile-filtered bulk product (Fig 1). Previous work has demonstrated HEK293 cell growth and virus production in HyClone™ CDM4HEK293 medium (2), using both single-use Xcellerex™ XDR-10 and ReadyToProcess WAVE™ 25 bioreactor systems (3, 4). Development of a virus release and filtration process as well as resin selection for downstream virus purification steps have also been described previously (5, 6).

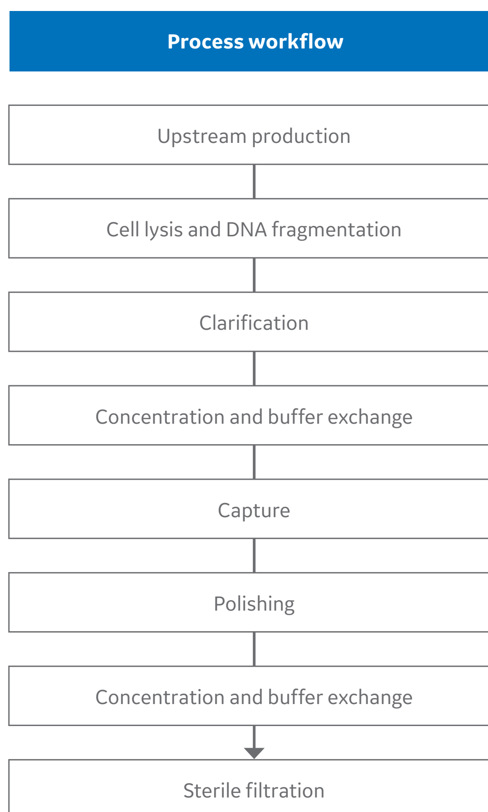

Fig 1. Overview of the steps of the adenovirus production process.

Analysis of virus particles and impurities to monitor the production process is time consuming and costly, and sometimes robustness and reproducibility is difficult to achieve. The assays can also be affected by sample composition (early or late process samples) or buffer components. Total virus particles is often assessed by qPCR for viral genome copies. Infectious virus particle titer is traditionally determined by various cell based assays (e.g., $TCID_{50}$) that can require more than a week until results. Here, we describe the use of SPR in determination of adenovirus concentration. Two assays were developed, one based on virus binding via the fiber protein to CAR and one based on virus binding via the hexon protein to FX. The principle of the assays is outlined in Figure 2.

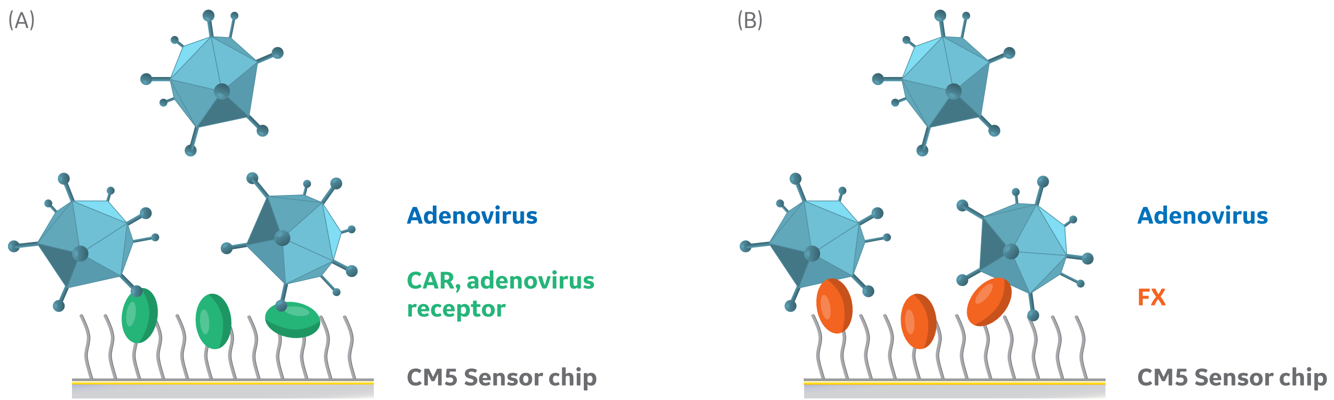

Fig 2. Principle of the two Biacore assays for determination of adenovirus concentration, where virus particles injected in a flow system bind to (A) the CAR receptor via the virus fiber protein or (B) FX via the virus hexon protein. In the separate assays, CAR or FX protein is immobilized on Sensor Chip CM5, and bound virus is detected using the Biacore T200 system.

Materials and methods

Adenovirus production

HEK293 suspension cells (HEK-293.2sus, ATCC) were infected with E1/E3-deleted recombinant AdV5 coding for the GFP reporter protein. Cell growth and adenovirus production as well as adenovirus release and filtration were performed as previously described (2, 5).

Adenovirus downstream samples

Samples were taken from different steps of the downstream adenovirus purification process (1). Briefly, cell harvest, clarified by normal flow filtration (NFF), was subjected to tangential flow filtration (TFF) (ultrafiltration and diafiltration [UF/DF]) followed by capture using Capto™ Q ImpRes anion exchange resin and polishing using Capto Core 700 resin. After the polishing step, a second TFF step was included for concentration, formulation, and final sterile filtration (SF). For fraction analysis, samples were taken from eluate pool after the capture step using the ReadyToProcess™ Adsorber Q nano anion exchange membrane capsule (6).

Analysis using the CAR assay

Total virus titer was analyzed using the Biacore T200 system at an analysis temperature of 25°C and a sample compartment temperature of 10°C, using HBS-EP+ as running buffer. CAR was immobilized to Sensor Chip CM5 using a standard amine coupling procedure, with CAR (12.5 µg/mL in 10 mM Na-acetate, pH 5.5) injected during 10 min, resulting in ca 2000 RU immobilized protein. Immobilization was performed in a single flow cell. As recommended for Biacore concentration determination assays, no reference surface was used. Virus binding to unmodified CM5 sensor surface was tested separately and found to be insignificant (not shown). A calibration curve was constructed using adenovirus reference material VR-1516 (ATCC), covering the range of 0.11–14 × 10^9 virus particles/mL. The calibration curve range was selected to obtain robust response levels and at the same time being able to dilute samples sufficiently to minimize the effect of sample matrices. Regeneration was performed with 10 mM glycine-HCl, pH 1.5. Two 30 s injections with a stabilization time of 60 s after the last injection was found to be optimal. Five startup cycles using a standard or virus sample plus regeneration were used. Standards and samples were injected in increasing concentration order at 5 µL/min for 400 s. One calibration curve was run before the samples and one after. Two different dilutions of each process sample were typically used (e.g., 200-fold and 100-fold) and concentration was calculated using the ***Trend calibration*** functionality of the Biacore T200 software.

Analysis using the FX assay

Virus hexon binding is reported to be Ca^{2+} dependent, why HBS-P+ buffer supplemented with 5 mM $CaCl_2$ was selected as running buffer and HBS-EP+ as regeneration solution (two 60 s injections followed by an extra wash with running buffer). Human FX (Haematologic Technologies Inc.) was immobilized to Sensor Chip CM5 using a standard amine coupling procedure, with FX (15 µg/mL in 10 mM sodium acetate, pH 5.0) injected during 20 min, resulting in ca 4000 RU immobilized protein. Similar assay parameters as for the CAR assay were used for the FX assay, apart from the differences in running buffer and regeneration solution.

Analyses by qPCR, $TCID_{50}$, IN Cell Analyzer, and Western blot

Total virus titer was analyzed in triplicate samples by qPCR using PureLink™ Viral RNA/DNA Mini Kit, TaqMan™ Universal PCR Master mix, and forward and reverse primers for hexon DNA and TaqMan MGB 6-FAM probe on the StepOnePlus™ Real-Time PCR System (all Applied Biosystems).

Infectious virus titer was analyzed by automated fluorescence microscopy of living cells using the IN Cell Analyzer 2200, after which images were analyzed for GFP signal (coded by virus) with a methodology similar to $TCID_{50}$ (1).

Virus protein concentrations were detected with fluorescent Western blot. A polyclonal anti-Adv5 primary antibody (Abcam), a Cy™3-labeled secondary antibody, and VR-1516 (ATCC) as standard were used for analysis of virus proteins.

Results and discussion

Performance of the CAR and FX assays are shown in Figure 3. The calibration curves demonstrate the stability of the assay. As shown by the near identical standard curves, with 27 samples runs in between, the assay can be used repeatedly with reproducible results (Fig 3B). This is further demonstrated in Figure 4 where calculated concentrations of 16 samples (stored at -20°C) from different steps in the process are shown. The same samples were thawed and diluted at two different occasions, with five days in between, and analyzed using the CAR assay. The same immobilized CAR surface was used at both occasions.

The Biacore assays are convenient to use and provide reproducible results for analysis of adenovirus in the different steps of a purification process (Fig 5). As shown for this adenovirus process, the assays are preferably used for monitoring of intact virus particles in the later stages of the purification process, when free virus have been removed. Accordingly, the Biacore assays show higher correlation with the qPCR virus titer after the capture step, when most of the free viral proteins were removed in the flowthrough. Levels of free virus and host cell proteins were still significant in the samples after the first TFF step, as a nominal molecular weight cut-off of M_r 300 000 was selected to avoid low virus recovery (5).

Variation in calculated concentrations was generally low using the Biacore CAR assay, with a coefficient of variability (CV) of < 5% for samples analyzed in duplicate, with each duplicate in two different dilutions. The FX assay showed a somewhat higher CV for some samples. However, both assays exhibited lower variation compared with qPCR over the purification process.

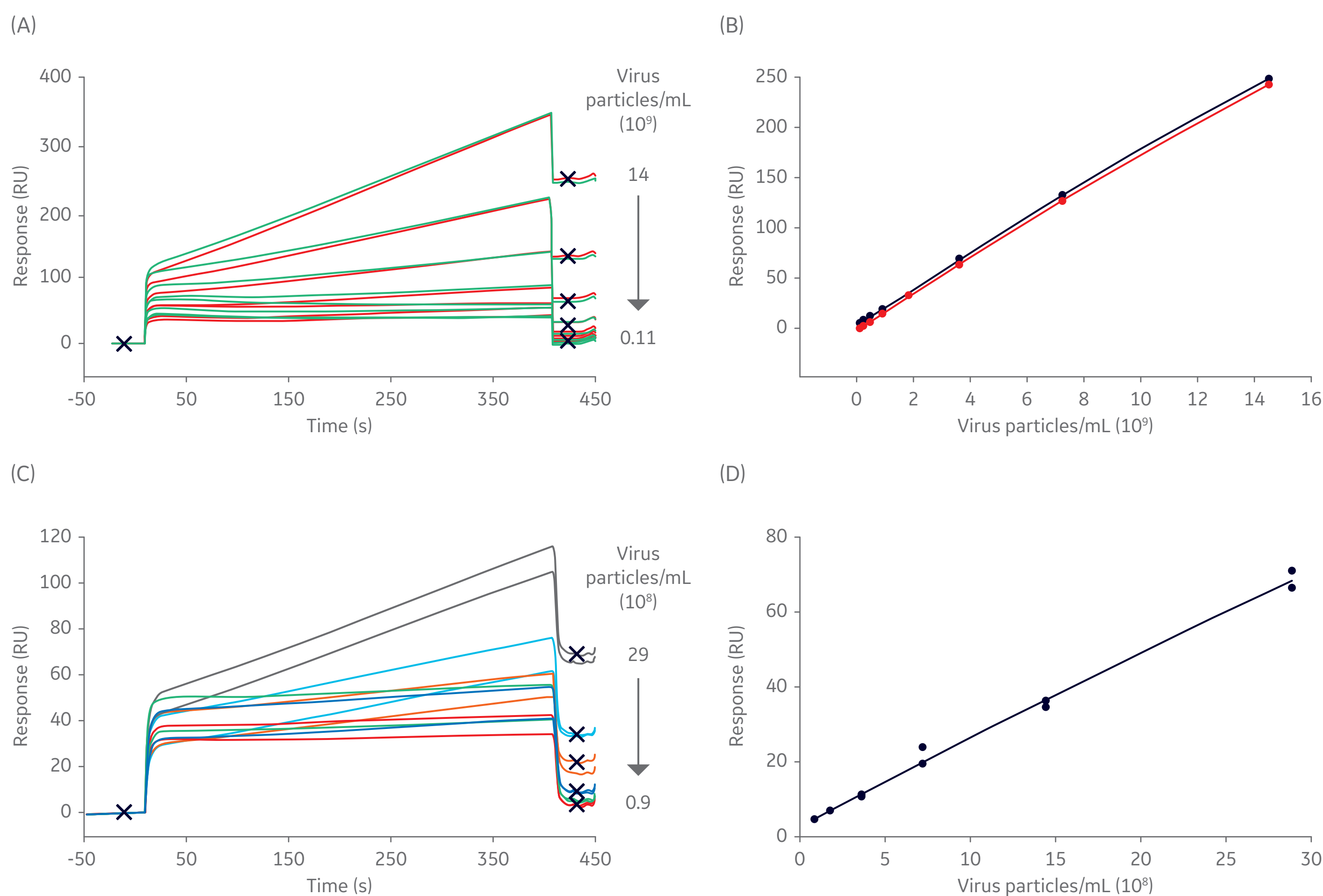

Fig 3. Performance of the CAR and FX assays. (A) Overlay plot showing ATCC adenovirus standards binding to immobilized CAR, with red sensorgrams originating from the first calibration series and green curves from the second. (B) Calibration curves for the CAR assay, run before (black line) and after (red line) 27 samples to demonstrate the robustness of the assay. (C) Sensorgrams from the FX assay, with each color referring to a specific concentration. (D) Average calibration curve from the FX assay. Report points after end of injections (X) were used for construction of the calibration curves in both assays.

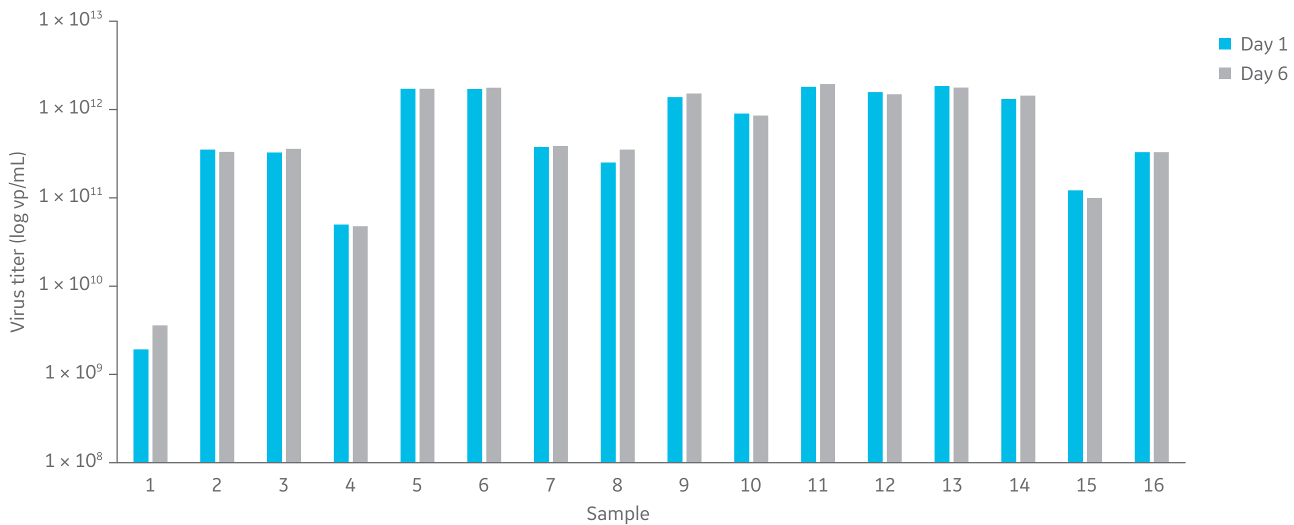

Fig 4. Determination of virus concentration using the CAR assay for 16 process samples (stored at -20°C) analyzed on the same CAR sensor surface at two different occasions five days apart.

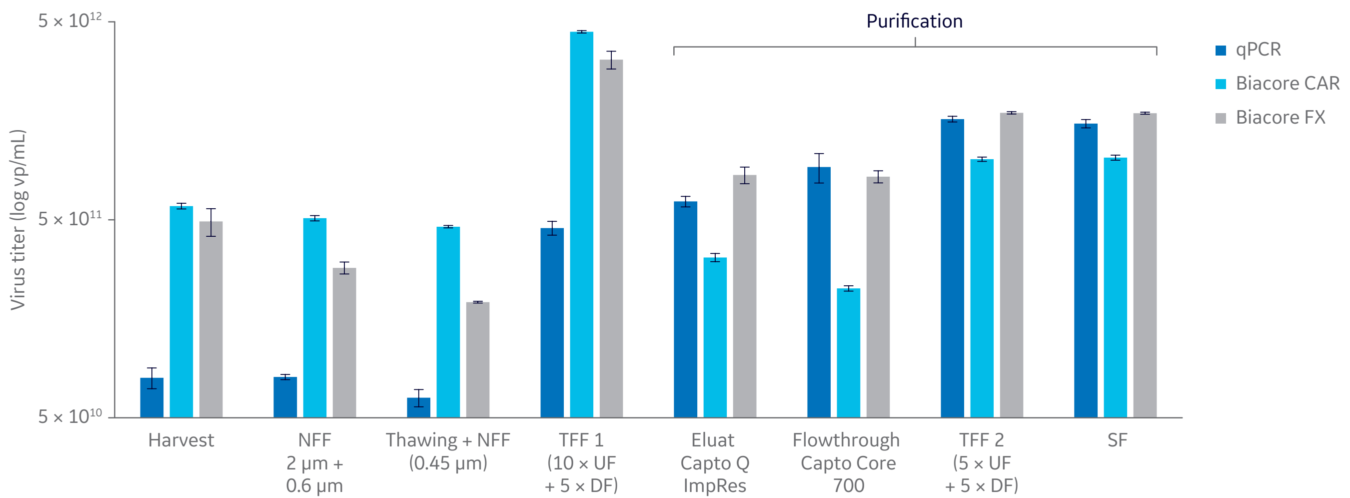

Fig 5. Comparison of virus titer determination using Biacore CAR assay, Biacore FX assay, and qPCR for samples from different steps of the adenovirus production process at 10 L scale.

Figure 6 shows results from calculation of virus recovery in different fractions of the adenovirus capture step using the ReadyToProcess Adsorber Q membrane. The virus fiber protein has been known to bind CAR, and Western blot signal for the fiber protein was compared with Biacore CAR binding signal in the flowthrough as well as in wash and elution fractions. The two methods result in similar patterns for calculated adenovirus concentrations, with the Biacore CAR assay exhibiting a higher sensitivity than Western blot, detecting virus also in the elution peak 3 fraction as shown in Figure 6.

As depicted in Figure 7, results from the Biacore assays correlate well with qPCR for determination of adenovirus concentrations. The CAR assay is targeting adenovirus fiber and could thus be used for determination of adenovirus quality and infectivity, as the fiber protein is mediating the attachment of adenovirus to the cell during infection (7).

A major advantage of the Biacore assays is the minimal preparation required for the assays and samples. The standard and samples are directly diluted into the running buffer. Immobilization of CAR or FX only involves dilution of the proteins in immobilization buffer, inserting the prepared solution and ready-to-use reagents, and then immobilization is an automated procedure taking about 30 min. Moreover, the immobilized surfaces showed high stability, and could be used for at least one week in this study. The high assay sensitivity allows for dilution of the samples to avoid any interference of the sample composition or buffer components in the assay.

Fig 6. (A) Step elution from ReadyToProcess Adsorber Q membrane. (B) Western blot analysis of fractions from flowthrough, wash, and elution peaks. Fiber protein signal comparison between (C) fluorescent Western blot and (D) Biacore CAR assay.

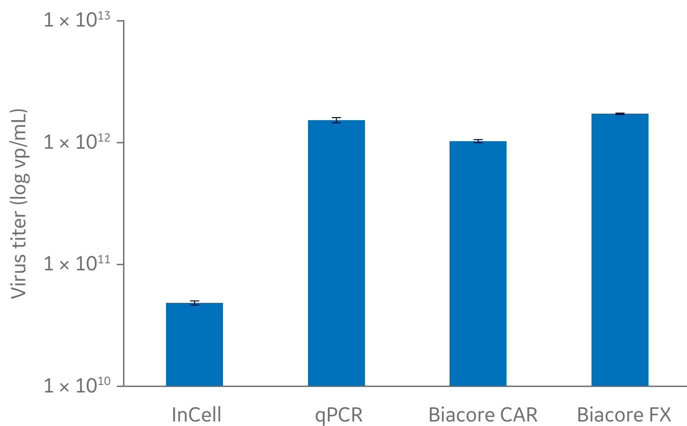

Fig 7. Calculated adenovirus concentrations in purified and concentrated bulk product, using different methods. IN Cell assay is showing infectious virus titer, whereas qPCR and the Biacore assays show total virus titer. Infections virus titer is expected to be lower than total virus titer. Regulatory requirements for the ratio of total to infections virus particles is < 30 (FDA).

Conclusion

The Biacore assays for determination of adenovirus concentration were robust, provided reproducible results, and correlated well with qPCR, while showing lower variation. The Biacore assays were shown to be significantly less laborious than established methods. Particularly, minimal assay and sample preparation is required, and the standard and samples are directly diluted into the running buffer before analysis. This in contrast to, for example, qPCR that contains laborious and time-consuming sample preparation steps. The use of assays for detection of fiber or hexon protein, or both, can be further explored for optimization of process conditions, enabling their use in monitoring virus titer, recovery, and function, as well as virus quality after purification steps. Biacore assays for other viral vectors could also be explored for potential for use in detailed virus interaction studies.

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Ordering information

Product	Description	Product code
Series S Sensor Chip CM5	Pack of 3	BR-1005-30
Amine Coupling Kit	Reagents for immobilization	BR-1006-50
HBS-P+	Analysis buffer	BR-1006-71
HBS-EP+	Analysis buffer	BR-1006-69
Biacore T200 system	SPR system for characterization of molecular interactions	28975001
Amersham WB goat anti-rabbit Cy3	150 µg/vial	29038276
IN Cell Analyzer 2200	Cell imaging system	29027886
ReadyToProcess Adsorber Q 150	8 mm membrane capsule	17372113
Capto Q ImpRes	25 mL	17547010
Capto Core 700	25 mL	17548101
ÄKTA pure 150	Chromatography system	29046665
ÄKTA pure 25	Chromatography system	29018225

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