

Inactivation of Adenovirus Type 5 by Caustics

Risat Jannat, David Hsu,[†] and Gargi Maheshwari*

Fermentation and Cell Culture, Bioprocess R&D, Merck Research Laboratories, West Point, Pennsylvania 19486

Adenovirus shows significant promise as a vehicle for transfer of therapeutic genes into humans. Based on the importance of this viral vector, it is critical that adequate decontamination procedures are implemented during its large-scale production in multiproduct manufacturing facilities to prevent cross-product contamination and to reduce the risk of personnel exposure. Liquid decontamination procedures based on caustics are easily implemented in a manufacturing setting and are not corrosive to stainless steel surfaces at the concentrations found to inactivate viral proteins and nucleic acids. In this study, we have conducted small-scale experiments to determine the effectiveness of caustic inactivation procedures on adenovirus type 5 and have evaluated the robustness of the process to different sample matrices and adenovirus constructs. We find that the pH of a sample post-addition of caustic solution is a more accurate indicator of the effectiveness of the caustic than its concentration. We have demonstrated that a greater than 6 log reduction in the potency of adenovirus type 5 may be obtained upon exposure of the sample to sodium hydroxide and CIP-100 at concentrations greater than 0.09 M and 0.9%, respectively, at times greater than 10 min.

Introduction

Adenovirus vectors provide an effective method for gene delivery *in vivo* and are currently being explored for use in gene therapy and vaccine applications in a number of recent studies (Limbach and Paoletti, 1996; Zhang, 1999). As a result of their high commercial potential, large manufacturing facilities are being designed for their production. With the rising cost of operating a biological manufacturing facility and an increase in the number of multivalent products, there is a trend in the industry to design multiproduct manufacturing facilities. To prevent cross-product contamination during the manufacture of biopharmaceuticals in such settings, regulatory agencies have emphasized the implementation and validation of stringent decontamination procedures (Sofer, 1995). The safety of personnel working in such manufacturing facilities is also an important consideration owing to the immunogenic potential of live adenovirus constructs (Jooss and Chirmule, 2003; Luff, 1992; Sofer, 2003).

To design viral inactivation procedures that can be implemented during large-scale production, small-scale studies must first be performed to demonstrate their effectiveness. Caustic solutions have previously been recommended for reducing the infectivity of viruses and other pathogenic organisms (Borovec et al., 1998; Boschetti et al., 2003; Derbyshire and Arkell, 1971; Lancz, 1976; Ye et al., 2003). Boschetti et al. showed 4.7 log reduction of Minute Virus of Mice after 1 min of exposure to 0.1 M sodium hydroxide. Previous studies have shown that sodium hydroxide at 60 °C resulted in approximately a

5 log reduction in human hepatitis A virus infectivity after 16 min of treatment (Borovec et al., 1998). Additionally, literature indicates that a 3–5 log reduction in adenovirus type 3 infectivity is obtained following treatment with calcium hydroxide over a period of many hours at room temperature (Derbyshire and Brown, 1979). The ability of caustic solutions to degrade proteins and nucleic acids, the two primary constituents of viruses, is also well documented (Crathorn and Shooter, 1982; Gulich et al., 2002; Shooter, 1976). There is limited published literature on the effect of caustics on adenovirus and their kinetics of inactivation. We have investigated inactivation of adenovirus type 5 with potassium hydroxide, sodium hydroxide, and CIP-100. CIP-100 is an alkaline cleaning agent commonly employed in clean-in-place systems within biopharmaceutical manufacturing facilities and is composed of potassium hydroxide, surfactants, and chelating agents. The potential for using CIP-100 to both clean and decontaminate process equipment in a single step motivated us to explore this particular caustic agent. A PCR-based infectivity assay was used to measure the initial inactivation kinetics of a highly concentrated adenovirus sample as previously reported (Maheshwari et al., 2004). The extent of caustic inactivation of adenovirus at optimal conditions was then confirmed using a cytotoxicity-based end-point assay. We found that the effectiveness of potassium hydroxide, sodium hydroxide, or CIP-100 is a function of the final pH of the sample and that the degree of inactivation at optimal conditions is independent of the adenovirus construct used or the matrix content of the adenovirus sample. We also observed a greater than 6 log reduction in adenovirus infectivity at NaOH and CIP-100 concentrations greater than 0.09 M and 0.9%, respectively, at times greater than 10 min as opposed to previously published results of a 3–5 log reduction following hours

* To whom correspondence should be addressed. Tel: 215-652-6756. Fax: 215-993-4884. E-mail: gargi_maheshwari@merck.com.

[†] Current address: City of Hope National Medical Center and Beckman Research Institute Center for Biomedicine and Genetics, 1500 E. Duarte Rd., Duarte, CA 91010.

of exposure of a caustic at room temperature (Derbyshire and Brown, 1979).

Materials and Methods

Quantitative PCR-Based Potency Assay. One of the assays used to measure adenovirus infectivity was a quantitative PCR-based assay as previously reported (Lewis et al., 2001; Maheshwari et al., 2004). Briefly, 293 cells were cultured in 96-well tissue culture plates (Falcon, Bedford, MA) at a density of 6.25×10^4 cells/cm² using MEM- α medium supplemented with 5% FBS, penicillin (100 units/mL), and streptomycin (100 μ g/mL). Cells were maintained in 96-well plates at 37 °C with 5% CO₂ in a humidified incubator for 24 h prior to infection. Each sample was then infected by dispensing virus at the appropriate dilution into four replicate wells. Following infection, cells were incubated at 37 °C and 5% CO₂ in a humidified incubator for 48 h. Total DNA was harvested and purified using the QIAmp DNA Blood Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. After two wash steps, the nucleic acid was eluted from the membrane into a buffer. Viral DNA was amplified using quantitative PCR with adenovirus-specific primers and probes (Applied Biosystems, Foster City, CA). PCR reactions were carried out in an ABI Prism 7700 (Applied Biosystems), and raw data was collected using Sequence Detector 1.6.3 software. The potency of each sample was then read off a semilogarithmic standard curve using purified adenovirus type 5 of known potency. To evaluate the variability of the Q-PCR-based potency assay, the root variability of the assay was calculated using the natural log transformed values of the reported titer of a single untreated adenovirus sample that had been run in over 50 independent assays. Using these data, the root variability of the assay was determined to be approximately 18% (data not shown). The 95% confidence interval for the titration of this untreated sample was found to be $\pm 40\%$.

Endpoint Dilution Assay. A cytotoxicity-based endpoint dilution assay was used as a more sensitive measurement of virus infectivity. Briefly, 293 cells were cultured in 96-well tissue culture plates at a density of 3.13×10^4 cells/cm² using MEM- α medium supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μ g/mL). Cells were maintained in 96-well plates in a humidified incubator at 37 °C with 5% CO₂ for approximately 48 h prior to infection. Infections were performed by removing the culture medium from the 293 cell monolayers and dispensing virus at the appropriate dilution into each well. After the addition of virus, plates were stored in a humidified incubator at 37 °C and 5% CO₂ for approximately 2 h to allow virus to adsorb onto the cells. Medium containing 10% FBS was then added into each well and the plates were incubated in a humidified incubator at 37 °C with 5% CO₂ for 12 days to allow development of cytopathic effect. At 12 days post-infection, cell viability in each well was measured by staining with Cell Titer 96 Aq_{ueous} One Solution (Promega, Madison, WI). The absorbance of each plate at 490 nm was then read using a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA). A minimum of 60 wells were inoculated with medium containing 10% FBS as negative controls. A sample well with a spectrophotometric reading less than 50% of the negative control median value was considered positive for adenovirus infection. This 50% cutoff value was established by independent method validation studies, which demonstrated a correlation between the endpoint dilution assay and a quantitative PCR-based infectivity assay (Mahesh-

wari et al., 2004). Sample titer was then calculated assuming a single infectious particle per positive well. A sample titer was calculated only when fewer than 50% of the wells at a given dilution scored positive. The following equation was used to calculate sample titers generated from the endpoint dilution assay:

$$\text{endpoint dilution assay titer} = \left[\frac{\text{no. wells positive for adenovirus infection (IU)}}{\frac{\text{infection volume}}{\text{well}} \times \text{no. wells infected}} \right] \times \text{sample dilution factor}$$

To evaluate the variability of the endpoint dilution assay, the root variability was calculated using the log transformed values of the reported titer of a single untreated adenovirus sample which had been run in 21 independent assays. Using these data, the standard deviation was determined to be 0.27 log infectious titer. The 95% confidence interval for the titration of this untreated sample was found to be ± 0.57 log infectious titer (Michelson and Schofield, 1996). This potency assay has a limit of detection of 30 IU/mL, which is the titer that would be measured if only a single positive well was detected.

Selection of Virus Samples. The inactivation profiles of multiple adenovirus type 5 constructs with unique DNA sequences were evaluated in this study. These constructs were generated by replacing the E1 gene of an adenovirus type 5 vector with various HIV-1 transgenes. In addition, virus samples from two different steps of an adenovirus production process were selected for analysis to evaluate the effect of a range of physicochemical parameters such as protein and nucleic acid concentration and process buffers on the robustness of thermal inactivation. Virus-infected cell broth was concentrated approximately 20-fold and the sample was designated as "unpurified concentrated cell lysate". This sample was tested to determine whether the presence of high protein and nucleic acid in the sample matrix had an effect on adenovirus inactivation. Samples were also taken after the final purification steps into a proprietary buffer, which were designated as "purified virus samples". When compared to the purified samples, the unpurified concentrated cell lysate matrix was found to contain greater than 50-fold higher protein concentrations (as measured by a colorimetric protein quantitation assay) and greater than 100-fold higher nucleic acid concentrations (as measured by a fluorescent nucleic acid staining method).

Chemical Inactivation Procedure. Virus samples were inactivated by addition of a caustic solution (KOH, NaOH (Fisher Scientific, Pittsburgh, PA), CIP-100 (Steris Corporation, Mentor, OH)) of known pH and concentration at room temperature (10% final volume). The final pH of the caustic solution following virus addition was recorded. After the desired time of treatment, each sample was neutralized by addition of equimolar HCl, vortexed, collected by brief centrifugation using a microfuge, diluted into a stabilizing buffer and stored at -70 °C. The final pH of neutralized samples was typically between 6.8 and 7.4. The samples were then thawed at room temperature prior to assay. Complete neutralization of the caustic agent and removal of interference with the assay procedure resulting from the neutralized chemical was confirmed by adding untreated virus at known concentrations into neutralized caustic solutions. No statistically significant differences in the titers of the

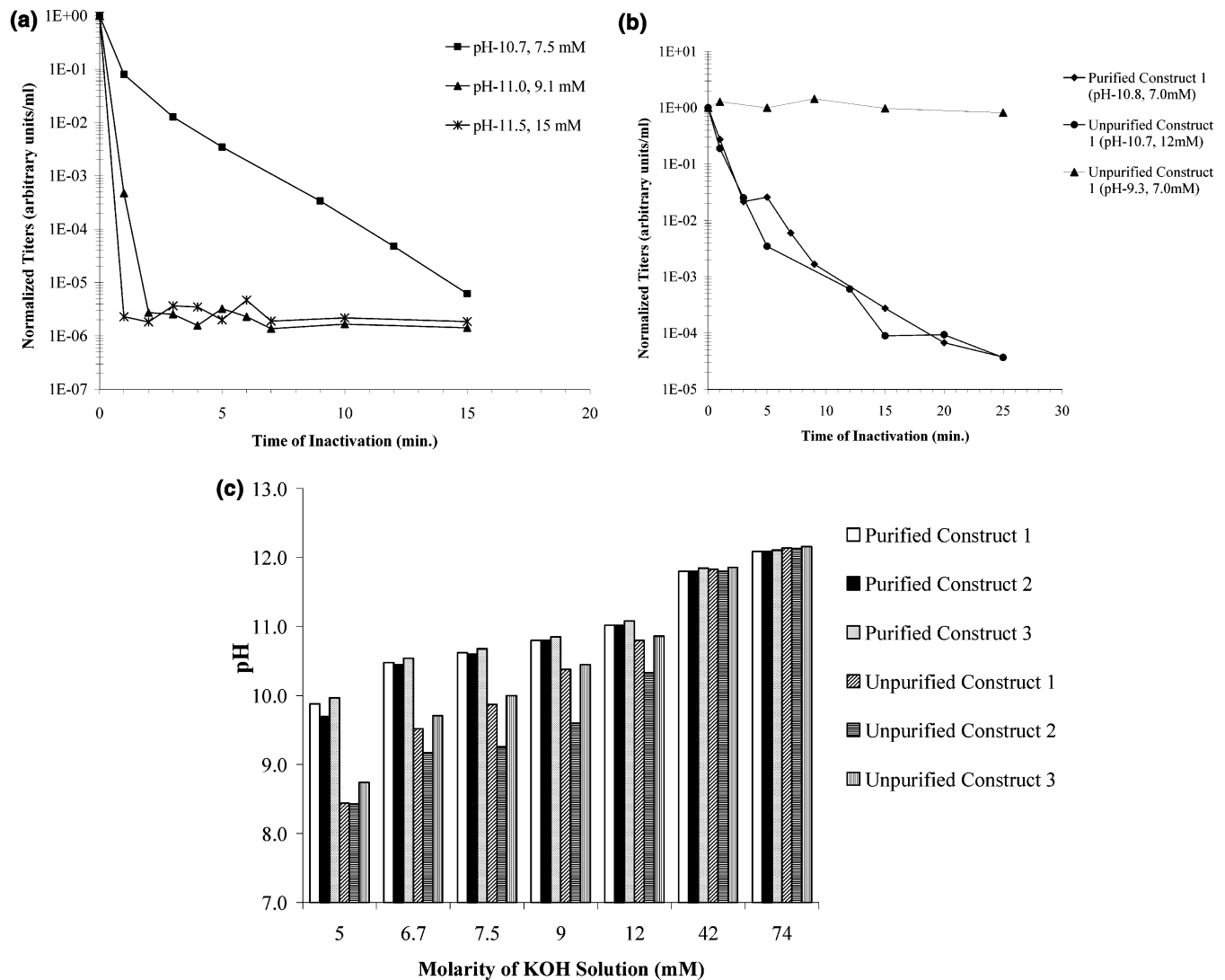


Figure 1. (a) Effect of increasing molarity and pH on inactivation of purified adenovirus with solutions of KOH. Purified adenovirus samples at pH 10.7 (■), 11.0 (▲) and 11.5 (∗) were inactivated for varying lengths of time, stored at -70°C , and subsequently assayed using the PCR-based potency assay as described in Materials and Methods. The sample titers have been normalized to a control. Normalized titer values less than 5×10^{-4} arbitrary units/mL fall below the limit of quantification of the PCR-based assay. (b) Effect of pH and molarity on inactivation of unpurified and purified adenovirus with solutions of KOH. Unpurified construct 1 (◆) and purified construct 1 (▲) were inactivated with a 7 mM KOH solution for different times. Unpurified construct 1 was also inactivated with a KOH solution at 12 mM (●). Samples were assayed using the PCR-based potency assay as described in Materials and Methods. The resulting titers have been normalized to a control. Normalized titer values less than 5×10^{-4} arbitrary units/mL fall below the limit of quantification of the PCR-based assay. (c) Effect of sample matrix and DNA sequence on the final pH in KOH solutions of varying molarity; 5–74 mM KOH was added into purified (bars 1, 2, and 3) and unpurified samples (bars 4, 5, and 6). The final pH of each sample was measured and plotted against the corresponding molarity of the KOH solution. The three adenovirus type 5 constructs tested were produced using similar processes and differ only in the inserted HIV-1 transgene sequence.

control samples were noted by either the quantitative PCR-based assay or endpoint dilution assay (data not shown).

Results

Inactivation with Hydroxides Is pH-Dependent.

To determine an optimal caustic concentration for quantifying adenovirus inactivation kinetics, initial experiments were performed with solutions of potassium hydroxide at concentrations between 7.5 and 15 mM and pH between 10.7 and 11.5. We found that the infectivity of a purified adenovirus construct is reduced to the detection limit of the PCR-based assay within 3 min using KOH concentrations greater than 9 mM while the kinetics of inactivation were captured using a 7.5 mM KOH solution (Figure 1a). Based on these data, a KOH

concentration of 7 mM was chosen to determine whether an unpurified adenovirus sample would exhibit a similar rate of inactivation. We found that when 7 mM KOH was added to the unpurified adenovirus sample matrix, almost no reduction in infectivity was noted after 20 min of exposure (Figure 1b). We also observed that the pH of this sample was at 9.3 whereas the pH of the purified adenovirus sample with 7 mM KOH was at 10.7. When the pH of the unpurified adenovirus sample was increased to 10.7 using 12 mM KOH, identical kinetics of inactivation were observed for the purified and unpurified adenovirus samples at this pH. These results suggest that inactivation of adenovirus with caustics is dependent on the final pH of the solution rather than the concentration of the caustic solution. To further examine the role of pH in hydroxide-based inactivation, the final pH of a

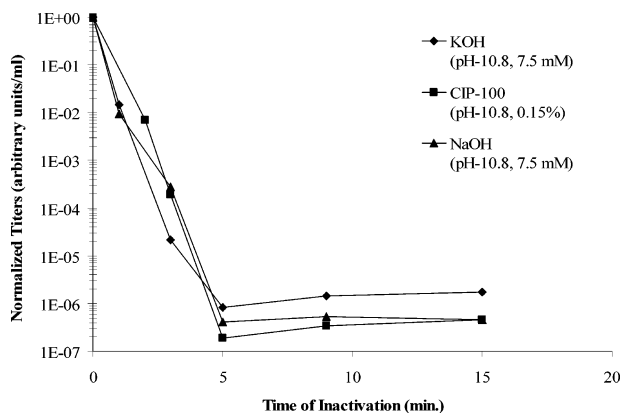


Figure 2. Comparison of the effect of KOH (◆), CIP-100 (■), and NaOH (▲) solutions at similar pH on the inactivation of purified adenovirus. Purified adenovirus was inactivated with KOH, CIP-100 and NaOH solutions at a pH of 10.8 for different times, frozen at -70°C and assayed using the PCR-based potency assay. The titers of each sample have been normalized to an untreated purified control. Normalized titer values less than 5×10^{-4} arbitrary units/mL fall below the limit of quantification of the PCR-based assay.

range of purified and unpurified sample types were measured using varying hydroxide concentrations. As shown in Figure 1c, there is significant variation in the final pH of different unpurified samples in KOH solutions of pH less than 12 possibly due to a difference in their matrices. As would be expected, the final pH of different purified samples does not vary significantly over the range of KOH concentrations that were evaluated. These results suggest that process additives and culture conditions can significantly affect the final pH obtained during inactivation.

Hydroxide-Based Chemicals Exhibit Similar Inactivation Profiles at a Given pH. To determine if inactivation with three different caustics agents would be similar at a given pH, inactivation of a purified virus sample using KOH, CIP-100, and NaOH was performed at pH 10.8. Similar inactivation profiles were observed for each of the three caustic agents tested provided the pH fell within a similar range (Figure 2). We also found that differences in transgene DNA sequence did not impact the inactivation kinetics of adenovirus type 5 constructs. No significant difference in the inactivation profiles of two different adenovirus type 5 constructs with a different gene sequence inserted at the E1 region was observed upon treatment with KOH at 6 mM which corresponded to a pH of 10.6 (Figure 3).

Maximum Log Reduction Values Obtained for Inactivation with NaOH and CIP-100. Based on the data from the previous experiments, we tested the effectiveness of high concentrations of caustics for inactivation of adenovirus. We used an endpoint dilution assay for this study since it has a lower limit of quantitation. We also verified the sensitivity of the assay procedure to determine whether the assay was able to detect low levels of infective adenovirus particles in a background of inactivated adenovirus and cellular debris. Untreated virus at known concentrations was spiked into dilutions of purified and unpurified samples inactivated by NaOH (0.09 M for 10 min) and CIP-100 (0.9% for 10 min). No interference with the measurement of the spiked sample was observed in the various matrices (data not shown). These results confirmed that the endpoint dilution assay is able to accurately measure infectious titers as low as 30 IU/mL in samples with potentially $>10^6$ inactivated adenovirus particles/mL. Table 1 shows

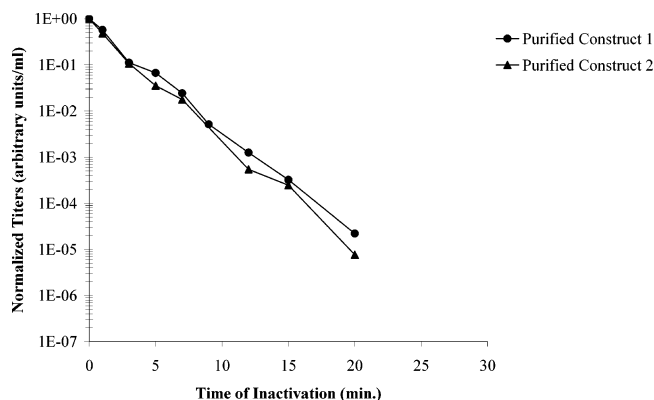


Figure 3. Effect of DNA sequence of adenovirus-based construct on inactivation kinetics with 6 mM KOH (pH 10.6). Purified adenovirus constructs were inactivated with a 6 mM solution of KOH for different times, frozen at -70°C and assayed using the PCR-based potency assay. The titers of each sample have been normalized to an untreated purified control. Normalized titer values less than 5×10^{-4} arbitrary units/mL fall below the limit of quantification of the PCR-based assay.

Table 1. Log Reduction Values Obtained Using NaOH and CIP-100 Inactivation with Various Adenovirus Type 5 Constructs and Sample Matrices^a

sample type	calcd log reduction value	
	0.09 M NaOH for 10 min	0.9% CIP-100 for 10 min
purified construct 1	6.7	7.3
purified construct 2	>7.9	>8.0
purified construct 3	7.8	7.8
unpurified construct 1	6.5	6.2
unpurified construct 2	6.0	6.5
unpurified construct 3	7.2	6.6

^a Samples were inactivated with 0.09 M NaOH or 0.9% CIP-100 for 10 min, and the resulting titer was measured using the endpoint dilution assay as described in Materials and Methods. Log reduction values were calculated by normalizing the measured titer by the potency of the appropriate untreated adenovirus sample.

that a greater than 6 log reduction in adenovirus type 5 infectivity was observed following a 10 min exposure of the sample to either 0.09 M NaOH or 0.9% CIP-100. Similar log reduction values were obtained for adenovirus type 6 using the same conditions (data not shown). As noted previously, no significant differences were observed in log reduction values when different sample matrices or constructs were used, provided the pH was greater than 11 following addition of caustic solution. The differences in the log reduction values of the various samples are more a reflection of the differences in their starting titers.

Discussion

In this study, we have shown that adenovirus inactivation with hydroxides is pH dependent and that inactivation with different hydroxide-based chemicals (NaOH, KOH, and CIP-100) at a similar pH produces similar inactivation profiles. These results point to the importance of considering the impact of process buffers, which may have a significant impact on pH, when designing chemical inactivation procedures for a range of sample matrices. Furthermore, we have demonstrated that unpurified and purified adenovirus may be inactivated by greater than 6 log following treatment with 0.09 M NaOH or 0.9% CIP-100 for 10 min. To generate confidence in the accuracy of the log reductions obtained for each caustic inactivation condition, we have performed ap-

appropriate control experiments to verify the absence of interference from the inactivated sample matrix, which increases in proportion to the log reduction value.

We also find that the log reductions obtained did not vary significantly with the sample matrix content or the type of adenovirus construct used provided the pH of the samples were at similar levels. For future studies, it may be acceptable to use the inactivation data generated for a particular construct to provide guidance for procedures used for other constructs with similar DNA sequences.

Based on the results of this study, we propose that caustic inactivation procedures for adenovirus use 0.09M NaOH and 0.9% CIP-100, which result in a final sample pH greater than 12, at contact times greater than 10 min. These results have been verified with adenovirus concentrations as high as 1×10^9 to 1×10^{10} IU/mL.

If the process sample matrix contains buffering agents that may significantly impact the final pH during inactivation, higher concentrations of NaOH and CIP-100 may be required to ensure maximum log reduction values are achieved. Use of either NaOH or CIP-100 at these relatively low concentrations can easily be implemented in a manufacturing setting without significant modification or corrosion of equipment.

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