5-2019

Investigating Virus Clearance via pH Inactivation During Biomanufacturing

Wenbo Xu

UNIVERSITY OF ARKANSAS®

Investigating Virus Clearance via pH Inactivation During Biomanufacturing

An undergraduate Honor Thesis by Wenbo Xu
submitted to the
Department of Biomedical Engineering
College of Engineering
University of Arkansas

Honor Advisor: Dr. Xianghong Qian
Submitted on April 26, 2019

Table of Contents

Abstract

In the processing of biopharmaceuticals, viral clearance and viral safety are important for the development of monoclonal antibodies. Murine xenotropic leukemia virus (XMuLV) is one of the retroviruses, recommended by Food and Drug Administration (FDA) as a model virus for viral clearance via inactivation from therapeutics derived from Chinese hamster ovary cells (CHO). A robust and effective method was investigated to clear or inactivate endogenous viruses by low pH inactivation. The effects of different conductivity and inactivated time on XMuLV clearance was determined. Acetate buffer was prepared with different conductivity, and 2% XMuLV was spiked into acetate buffer. XMuLV virus particles could be effectively inactivated in acetate buffer at pH 3.6. According to $TICD_{50}$ assay, the inactivation time of around 60 minutes was enough to clear all the viruses with more than 4 logs reduction value (LRV). Also, 50 mM acetate buffer has the most rapid inactivation process. $TICD_{50}$ assays were able to determine the XMuLV virus titer within 95% confidence level, by using 8 replicates and 10-fold series dilution factor.

1. Introduction

Biological and biochemical products have high risks of contamination from chemical impurities, bacteria, fungi, and the most dangerous one is from viruses. Viral contamination will affect raw materials, cell lines, cell culture process, and downstream processing (1). Thus, viral clearance and viral safety were critical for products produced by mammalian cells such as monoclonal antibodies (mAbs) because mammalian cells are highly sensitive to viral contamination by adventitious viruses. For example, Chinese hamster ovary cells (CHO) should express non-infectious retroviruses or retroviral-like particles during the production of therapeutics (2-3). As applying a virus inactivation method during downstream production, it could effectively eliminate concerns about underlying infectious, adventitious or dangerous agents. Commonly, two dedicated, virus clearance steps, such as viral removal and viral inactivation are included in the purification process. But virus inactivation processes are stable processes and less sensitive to minor factors than the viral removal process. As a result, virus inactivation is generally robust and effective (2,4).

One of the effective inactivation methods is low pH inactivation, performed by spiking enveloped viruses such as XMuLV, pseudorabies Virus, and herpesviruses into the low pH solution, which provides at least 4 logs of virus clearance for these viruses (7-8). For mAbs and Fc fusion proteins production, acidified buffer always follows the inactivation, and these enveloped viruses should be hold statically in for at least over 30 minutes to completely damage the surface glycoproteins and prevent the viruses infecting the cells (8,9). At the end of inactivation holding times, the acidified buffer with viruses is neutralized to pH 7 (9,10).

In the process of viral inactivation, XMuLV is used as inactivation model virus, produced by Mus Dunni cells and mink lung cells (5). As XMuLV is similar to gamma retrovirus, a nonmurine cell line PG-4 cell line is used to propagate the virus. PG-4 cells were astrocyte, moloney murine sarcoma virus transformed, using for detection and quantitation of replication competent retroviruses. PG-4 cells are resisted to infection and focus formation by ecotropic murine leukemia virus (5-6).

2. Material and Methods

PG-4 cells, McCoy's 5A medium, fetal bovine serum (FBA) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Penicillin/streptomycin solution (10,000 U/mL) was purchased from ATCC (Manassas, VA), stored at - $20^{o}C$. Sterile phosphate buffer saline without calcium and magnesium (PBS), and sodium acetate trihydrate ($CH_3COONa+3H_2O$) were purchased from VWR Scientific (Radnor, PA). 0.25% trypsin- 0.53 mM EDTA solution(1X), trypan-blue (0.4%), and acetic acid (CH_3CO_2H) were purchased from Millipore Sigma (Burlington, MA) and kept frozen at - $20^{o}C$. Sterile deionized water (DI water) was collected through water Pro/RO reverse osmosis plus deionization purification system by Labconco Corp (Kansas City, MO). L-Glutamine in220mM solution (292 mg/mL-Glutamine in 0.85% NaCl) was purchased from VWR scientific (PA). Tris base ($C_4H_{11}NO_3$) was purchased from G-biosciences (St. Louis, MO).

All the experiments were safely run inside the laminar flow cabinet. Different size of the serological pipettes and pipette tips as well as 15 and 50mL, sterile centrifuge tubes, micropipette and sterile disposable pipette tips, 25 and 75 cm^2 sterile cell culture flasks and 96-well flat-bottom cell culture plate (sterile) were used in cell recovery, cell passaging and $TCID_{50}$ assay. All equipment was sprayed by 70% IPA before entering the hood.

2.1 PG-4 Cell Line Recovery and Passaging

2.1.1 PG-4 Cell Recovery

McCoy's 5A medium, L-Glutamine (LG), FBS were warmed up at $37^{o}C$ in a cell culture incubator with 5% CO_2. Penicillin were thawed at room temperature without light under aluminum foil. Afterwards, a total of 20 mL of cell medium was prepared by adding in 20 mL of McCoy's 5A medium with 1% LG, 2% FBS, and 0.01% penicillin was prepared in a 50 mL sterile centrifuge tube. A total of 5 mL of prepared medium was transferred into a 25 cm^2 cell culture flask. A vial of frozen PG-4 cells was taken from the liquid nitrogen tank and thawed with $37^{o}C$ water bath, agitating back and forth with no more than 3 minutes. The warmed cell suspension was transferred into a 15 mL of medium drop by drop and mixed gently. After that, it was centrifuged at 1500 RPM for 5 minutes. A clear wall of cells attached on the centrifuge tube could be observed. The supernatant was removed from the other side of the wall carefully. The rest of 5 mL medium was resuspended into the cell pallet and transferred into 25 cm^2 flask. Finally, cells were kept in the cell culture incubator.

2.1.2 PG-4 Cell Passaging

A total of 30 mL of passaging medium was prepared in a 75 cm^2 sterile cell culture flask with the same contents as the cell recovery medium. Afterwards, the old cells were taken and observed under the inverted light microscope to check if the cells reached 50-60% confluence. Using a 10 mL serological pipette to aspire all the old medium from the 25 cm^2 flask for the cells to be passaged. 10 mL of sterile warm PBS was used to wash out the cell debris and remaining old medium. After that, 2.5-3 mL trypsin-EDTA (TE) was added to cell monolayers to detach the

cells. After incubating with TE for 3-5 minutes, cells were observed under microscope to check if all the cells are single and floating. Then, 5 mL of complete medium was aspirated into the cell to disperse the cells. The cell suspension was then transferred into a 15 mL centrifuge tube and centrifuged at 130 xg for 5 minutes. After removing the supernatant, cells were suspended in 5 mL of fresh complete medium. A designated number of cells were counted and transferred for the next passage with 20mL of medium remained in the flask (passage ratio at 1:4 to 1:10).

3. XMuLV Virus Inactivation in Low-pH Acetate Buffer

3.1 Acetate Buffer Preparation

20, 50 and 100 mM acetate buffer solutions were prepared using 100 mL with DI water, appropriate amount of sodium acetate and acetic acid, at low pH 3.6 (Table 1). After pH and conductivity were measured, the acetate buffers were equilibrated in an incubator at 18 °C for 2 hours.

Table 1. Initial condition for preparing acetate buffers at different concentration

Buffer Concentration(mM)	**Sodium Acetate (mg)**	**Acetate Acid (μL)**
20mM	17.61	107.08
50mM	44.03	267.07
100mM	88.05	535.41

3.2 Virus Propagation on PG-4 Cells and Virus Inactivation

After 2 hours of incubating, 100 mL acetate buffer was filtered with Millex-GP syringe filter. The filtered buffer was transferred into 100mL baker. A 40 mL filtered buffer was divided into tubes, containing 10 mL of buffer for each tube. These buffers were used to measure pH and conductivity after filtering. They were also used to test the amount of 1 M tris needed in order to increase the pH to 7. Then, this predetermined volume of 1M tris buffer was added into each of

the six tubes for neutralizing virus-buffer sample later (Table 2). The rest 60 mL of buffer was spiked with 2% XMuLV virus. As the beginning of the kinetic study, 1.2 mL XMuLV virus was spiked into 60 mL buffer and mixed well. After 1 minute, two of 10 mL inactivated virus-buffer were transferred into the tubes, one for pH and conductivity control, one for determining the virus titre with 1M tris to terminate the inactivation process. Then, after 5, 15, 30, 60 minutes, each 10 mL of inactivated virus-buffer sample was pipetted into tubes contained with 1 M tris. Thereafter, inactivated XMuLV stock was diluted by 10-fold series dilution factor for titer determination using $TCID_{50}$ assay. After seeding PG-4 cells for 24 hours, the cells were inoculated with diluted XMuLV stock by 8 replicates in 96 well plates. The cocultivation would last for 7 to 8 days. On day 7, the samples were ready for reading according to $TCID_{50}$ assay.

Table 2. Initial pH and conductivity of acetate buffer for inactivation, and 1M tris added to neutralize the pH and conductivity

Buffer Concentration (mM)	**Initial pH**	**Initial Conductivity ($\mu S/cm$)**	**1M Tris Neutralized (μL)**	**Final pH**	**Final Conductivity ($\mu S/cm$)**
20	3.60	256	185ul	7.00	1141
50	3.61	773	490ul	7.01	2853
100	3.60	1100	970ul	7.00	4450

3.3 $TCID_{50}$ Assay to Determine Virus Titre

On day 1, 100 μl newly-made cell suspension was added to each well of 96-wells plate and incubated at 37ºC overnight. On day 2, after cells seeding were complete and reach the 20% confluency, all inactivated virus samples were assayed for XMuLV infectivity by using $TCID_{50}$ assay (3). Before $TCID_{50}$ titration, all samples were diluted 10-fold series with fresh medium to eliminate cytotoxic effects. PG-4 cells were infected by 100 μl of diluted samples on 96-well plates, including the inactivated samples and original virus stock. Virus titre, and 95%

confidence level for the log titre of the virus stock were determined by method of Spearman-kärber (4).

4. Results and Discussion

After 7-8 days, the plates were ready for evaluation; by distinguishing health PG-4 cells (Fig. 1a) or infected PG-4cells (Fig. 1b), the wells would be recorded as negative or positive. Also, PG-4 cells were infected by inactivated XMuLV viruses at 1, 5, 15, 30 and 60 minutes (Fig 1c-1g). For inactivation time below 15 minutes, there were plenty of regions under infection by XMuLV. However, as the inactivation time increased, the infected region got smaller. Until 60 minutes, it was no infected PG-4 cells exiting. Based on figure 1, inactivation time around 60 minutes is enough to clear XMuLV virus, and $TCID_{50}$ assay was not able to detect infection over 60 minutes.

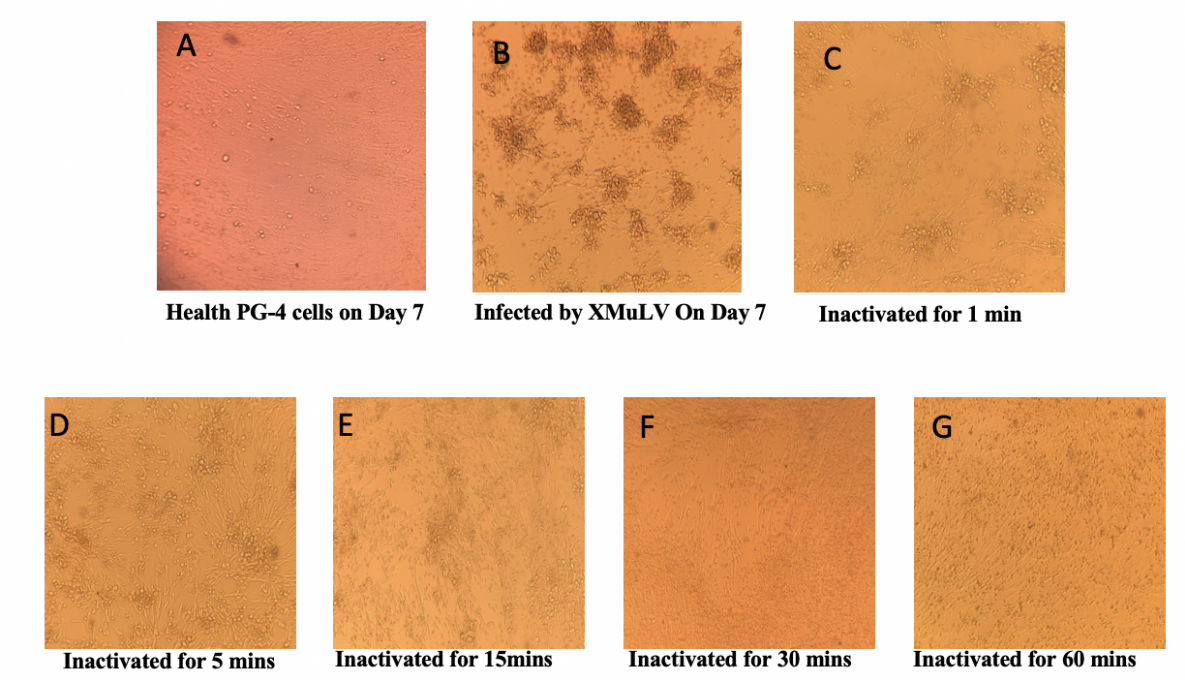

Figure 1 Infected PG-4 cells on day 7. A) Negative control- health PG-4 cells. B) Positive Control- infected by XMuLV virus. C)-D) infected PG-4 cells by inactivated XMuLV virus at different time.

The pH and conductivity of control samples after being inactivated were measured to make sure the pH was still around the same range as the buffer, and pH slightly increased around 3.68 or 3.7 (Table 3). Also, virus tire and LRV were calculated with different inactivation kinetics and shown in Table 4.

Table 3. pH and conductivity ($\mu S/cm$) measured after virus was spiked into 20, 50, and 100 mM acetate buffer

20mM	pH	conductivity
1min	3.7	276
5mins	3.68	270.8
15mins	3.64	264.9
30mins	3.68	270.8
60mins	3.68	270.7

50mM	pH	Conductivity
1min	3.7	776
5mins	3.68	772
15mins	3.70	778
30mins	3.69	773
60mins	3.68	765

100mM	pH	Conductivity
1min	3.7	730.2
5mins	3.69	723.4
15mins	3.69	723.2
30mins	3.68	725.5
60mins	3.68	725.4

Standard deviation and 95% confidence level indicated the accuracy and repeatable of $TCID_{50}$ assay. Table 4 was shown sample with 20 mM acetate buffer, the average original virus stock titre was 5.92 logs/mL, during each inactivation time, the titre was decreased till 1.58logs/mL. Until 60mins, $TCID_{50}$ assay was not able to determine the infection of XMuLV virus. Also, the pooled standard deviation for all samples were less than 0.5 logs and resulted in 95% confidence level less than 0.5 logs, and the variation within the assay didn't exceed ± 0.5 logs. Also, samples with 50mM and 100mM acetate buffer, and the original virus stock titre for both was calculated as 6.00 logs/mL, and for each inactivation kinetics, the average titre was decreased. Still, for 50mM kinetics, the pooled standard deviation for all samples was less than 0.5 logs and resulted in 95% confidence level less than 0.5 logs, and the variation within the assay didn't exceed ± 0.5 logs. Thus, initial precision experiments are proved that those assays were able to determine the XMuLV virus titre within 95% confidence level, by using 8 replicates and 10-fold series dilution factor. However, for 100mM kinetics, the inactivation time at 5

minutes, the standard deviation of 0.26 resulted in a 95% confidence level of 0.52 logs, and the variation within the assay exceeded ± 0.5 logs, thus, for more accurate determination, this kinetics with inactivation time at 5 minutes, should be repeated.

Table 4. Log total virus in 10mL, Log reduced value (LRV), 95% confidence level for 20mM, 50mM, and 100mM Acetate buffer.

	20mM		50mM		100mM	
Time (Mins)	**Total Virus (logs)**	**LRV**	**Total Virus (logs)**	**LRV**	**Total Virus (logs)**	**LRV**
0	6.92±0.64	0.00	7.00±0.78	0.00	7.08±0.72	0.00
1	4.42±0.5	2.50	3.69±0.18	3.33	4.25±0.50	2.83
5	3.92±0.3	3.00	3.08±0.30	3.92	3.17±0.52	3.17
15	3.33±0.42	3.58	2.92±0.30	4.08	3.08±0.30	4.00
30	2.83±0.28	4.08	2.83±0.24	4.17	2.67±0.18	4.41
60	2.58±0.08	4.33	2.50±0.00	4.50	2.58±0.08	4.50

Log reduced value was used to determine the number of viruses eliminated from the inactivation kinetics. LRV was calculated by the equation $LRV = log_{10}((C_F * V_f)/(C * V))$ (11). C_f and V_f refer as original virus titre and original virus volume, in here is 10mL. C and V refers as filtrate virus titre and filtrated virus volume, still 10mL. After calculation, LRVs was determined by plotting the each LRV kinetics curve with inactivation time. With long inactivation time, the curve was increased sharply and stay flat. At the beginning of 1-minute inactivation, 50mM inactivation kinetics had the most rapid increase on LRV, and for 20mM kinetics, it had smallest increase on LRVs (Fig. 2a) In addition, 50mM buffer has the most decreased on total amount of virus in 2.5 logs/10mL sample volume, and 20mM, 100mM has the same decreased value, reaching 2.58 logs/10mL (Fig. 2b). Accordingly, for all of kinetics, around 60-minutes inactivation, there were no more XMuLV virus being detected, and LRVs reached 4.50, which showed that most of XMuLV viruses were inactivated by the acetate buffer at pH 3.6

with different conductivity. Thus, the final LRVs were greater than 4.0-4.5. Also, the inactivated kinetics were independent on the changing conductivity.

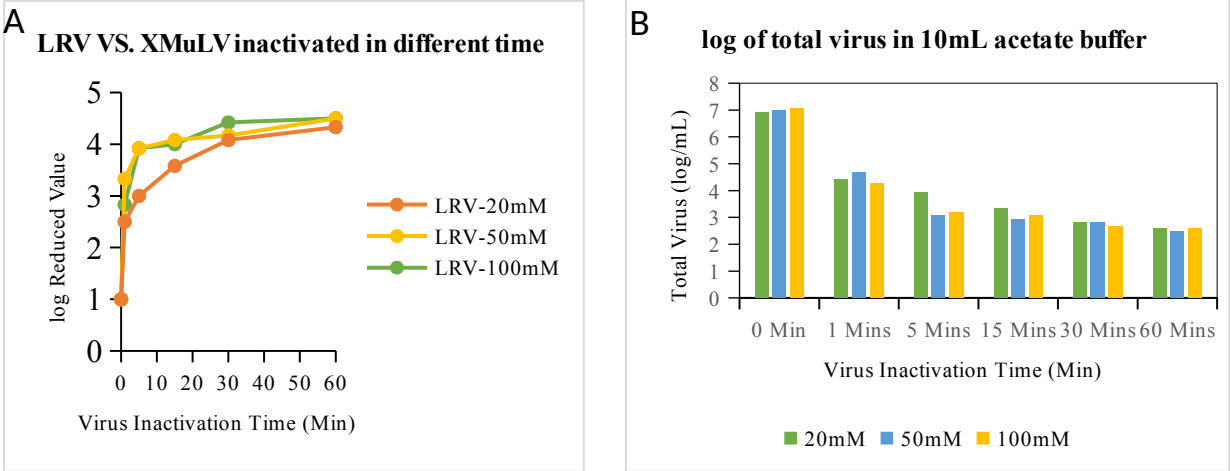

Figure 2 A) Trends for log reduced value with XMuLV virus being inactivated in different time. B) Error bar for log total virus in 10mL inactivation buffer with different time

5. Conclusion

The purpose of this project was to investigate the virus clearance method with low pH and determine if conductivity and inactivation duration had any effective impact on virus clearance. The entire work was performed by PG-4 cell line passaging and $TCID_{50}$ assay to determine the virus titre and LRV (log reduced value). At pH 3.6, all of the kinetics had completed the inactivation process around 60-minutes. Also, standard deviation resulted in 95% confidence level within 0.5 logs, which proved that this inactivation kinetic was effective and accurate. There were no more XMuLV being detected by $TCID_{50}$ assay until 60 minutes. For 60 minutes inactivation, it was enough to obtain > 4 logs LRVs.

In summary, XMuLV virus particles could be effectively inactivated in acetate buffer with different conductivity at pH 3.6. Also, inactivation efficiency was independent on changing conductivity and dependent on holding duration. However, based on data, 50mM acetate buffer

had the best inactivation on XMuLV virus particles. Furthermore, the kinetics over 60 minutes should be tested with other assays to check whether all the viruses got cleared.

6. Acknowledgment

I deeply appreciate Dr. Xianghong Qian's support and advising during the entire project. Also, I really want to thank Namila and Shuting Chen for helping me measuring pH and conductivity. This work funding was provided by REU project from National Science Foundation.

Reference

1. Sandle, Tim. “Current Methods and Approaches for Viral Clearance.” *American Pharmaceutical Review*, 30 Sept. 2015.
2. Chinniah, Shivanthi, et al. “Characterization of Operating Parameters for XMuLV Inactivation by Low PH Treatment.” *Biotechnology Progress*, American Chemical Society (ACS), 5 Nov. 2015.
3. Connell-Crowley, Lisa, et al. “Cation Exchange Chromatography Provides Effective Retrovirus Clearance for Antibody Purification Processes.” *Biotechnology and Bioengineering*, John Wiley & Sons, Ltd, 23 Aug. 2011.
4. Sofer, Gail. “Inactivation of Viruses.” *Biopharma Nternational*, June 2003.
5. Ma, Rong, et al. “Growth Curve of Murine Xenotropic Leukemia Virus-Related Virus Grown in Chinese Hamster Ovary Cells.” *Journal of the Chinese Medical Association*, vol. 77, no. 1, 2014, pp. 44–48., doi:10.1016/j.jcma.2013.09.005.
6. Yuen, P.h., et al. “Restriction of Moloney Murine Leukemia Virus Replication in Moloney Murine Sarcoma Virus-Infected Cells.” *Virology*, vol. 132, no. 2, 1984, pp. 377–389., doi:10.1016/0042-6822(84)90043-6.
7. Miesegaes, George, et al. “Analysis of Viral Clearance Unit Operations for Monoclonal Antibodies.” *Biotechnology and Bioengineering*, 2010, doi:10.1002/bit.22662.
8. Brorson, Kurt, et al. “Bracketed Generic Inactivation of Rodent Retroviruses by Low PH Treatment for Monoclonal Antibodies and Recombinant Proteins.” *Biotechnology and Bioengineering*, vol. 82, no. 3, 2003, pp. 321–329., doi:10.1002/bit.10574.
9. Shukla, Abhinav A., et al. “Downstream Processing of Monoclonal Antibodies—Application of Platform Approaches.” *Journal of Chromatography B*, vol. 848, no. 1, 2007, pp. 28–39., doi:10.1016/j.jchromb.2006.09.026.
10. Joshi, Varsha, et al. “Avoiding Antibody Aggregation during Processing: Establishing Hold Times.” *Biotechnology Journal*, vol. 9, no. 9, 2014, pp. 1195–1205., doi:10.1002/biot.201400052.
11. Ederveen, Joke. “A Practical Approach to Biological Assay Validation.” May 2010.