# Large-Scale Beta-Propiolactone Inactivation of HIV for Vaccines

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Beta-propiolactone (ßPL) has been used extensively to inactivate viruses for both human and veterinary vaccine production (**1, 2**). Human vaccines against influenza, and rabies have been successfully developed and safely administered using ßPL-inactivated forms of whole viruses associated with each disease (**4, 5**). Experimental candidates for poliomyelitis, severe acute respiratory syndrome (SARS), and human immunodeficiency virus (HIV) have additionally been produced with this inactivation technology and are currently being evaluated in animal models and human clinical trials (**3, 6, 7**). Although effective inactivation processes have been devised for each of these vaccines, the scale at which the ßPL inactivation process has been performed remains a limiting factor for production quantities of final drug products for commercialization.

Here we demonstrate a highly efficient and reproducible manufacturing process for production of an HIV-1 therapeutic vaccine candidate. Our process features a unique industrial scale ßPL viral inactivation that will support product demands of commercialization. Stainless steel 1000-L tanks are used in a safe, multitank transfer process that maximizes the capacity for ßPL inactivation. Physical and chemical parameters of the system were monitored through performance qualification studies and verified as reproducible through three successive production runs. This novel operation yields complete viral inactivation meeting US FDA guidelines while retaining antigenicity and immunogenicity of the viral proteins (**8**). The scale of ßPL viral inactivation outlined here may also assist in meeting the world demand for manufacture of other vaccines, such as the influenza vaccine that has proven problematic in recent years.

**Product Focus:** Vaccines

**Process Focus:** "Upstream" processing (between production and purification)

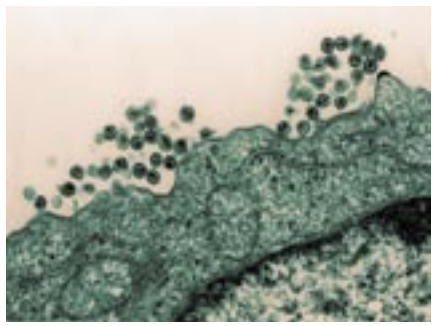
**Who Should Read:** QA/QC, process development, manufacturing, and regulatory affairs

**Keywords:** Beta-propiolactone, viral clearance/safety, inactivated vaccines, process optimization, scale-up

**Level:** Basic/overview

## Cell Culture and Harvest

The HIV-infected cell line we used in these studies is a human T lymphoma cell line (HuT 78) chronically infected with the HZ321 strain of HIV-1. We thawed vials of the manufacturer's working cell bank (MWCB) and initiated cultures in Roswell Park Memorial Institute medium (RPMI 1640) with 20% fetal bovine serum (FBS), and subsequently expanded them into T-Flasks. As the infected cells further divided, culture volumes were expanded into roller bottles in RPMI 1640 with 10% FBS and manually sparged with 5% $CO_2$/balance air.



**Colorized scanning electron micrograph of intact HIV-1 (HZ321) particles budding from a human T-cell (HUT 78)**
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Throughout the cell growth period, we monitored cell density, percent viability, and residence time (the number of days a culture is in a particular expansion vessel) as parameters for evaluating culture performance. During the final expansion in 250-L tanks toward a final volume of 800 L, FBS content was further reduced to 5%. Soluble virus was separated from intact cells and cellular debris using a 1.2-µm cellulose ester filter and transferred to a 1000-L stainless steel tank. Throughout the cellular expansion process, cultures were maintained at temperatures of 37 °C ± 2 °C. From initial thaw of MWCB to harvest of the final expansion, this cell culture production cycle took 33–38 days.

## Inactivation

Pooled filtrate containing the infectious virus in-process material

was cooled within the first 1000-L stainless steel tank to 5 °C, and the pH was adjusted to 7.30 ± 0.1 using NaOH. ßPL was then introduced to a final dilution of 1:2000 (v/v) and circulated for one hour. To ensure complete viral inactivation, the filtrate was then transferred to a second 1000-L vessel and incubated under continuous circulation at 5 °C (pH 7.00 ± 0.5) for an additional 23 hours. The transfer between tanks is designed to facilitate consistent ßPL–virus interaction throughout the entire lot of material, prohibiting untreated material to advance into the purification steps.

Following inactivation, treated material was gradually heated over a period of 24 hours to 37 °C ± 0.2 °C within a pH range of 7.00 ± 0.5. The temperature was then maintained for an additional five hours (Table 1). This increase in temperature facilitates the complete hydrolysis of any remaining ßPL into an isomer of lactate and beta-propionic acid derivatives, both of which are innocuous compounds. Focusing on this requirement, validation of the use of ßPL for viral inactivation is a critical safety issue.

Throughout the inactivation process, we removed samples for qualification analysis. Infectivity samples came from the vessel starting at the zero hour time point before addition of ßPL. They were immediately neutralized with sodium sulfite and maintained at –70 °C pending analysis. Sodium sulfite facilitates immediate hydrolysis of ßPL while maintaining viral integrity for analysis. Gas chromatography samples were removed from the vessel starting at the same zero hour time point, then immediately extracted with chloroform and maintained at 2–8 °C pending analysis. Chloroform processing allows ßPL isolation and prevents degradation of the compound.

**ßPL Inactivation of High-Titer Viral Spike:** To further define the total capacity of ßPL inactivation, an additional high-titer HIV-1 spike was added to a sample of in-process material. At the 12-hour point following ßPL addition, a 100-mL sample was removed from the 1000-L GMP manufacturing tank and transferred to our quality control laboratory. The material was spiked with an equal volume of high-titer infectious virus. The material was then subjected to identical conditions as the manufacturing lots, including the gradual temperature increase after 24 hours to hydrolyze the ßPL (Table 2). Samples of 1 mL were taken at specific time points and analyzed for their viral titers and extent of ßPL hydrolysis. This spike study demonstrates that the amount of ßPL used for GMP manufacturing is sufficient for inactivating the virus found in the cell culture harvest and capable of inactivating more virus than is actually present, which illustrates a significant margin of safety.

## Results

We performed three process qualification runs at the commercially adaptable 1000-L production scale. To assess safety, we measured the extent of ßPL hydrolysis through gas chromatography preparations of in-process materials to ensure eradication of the material from our final drug preparation. Complete hydrolysis was achieved by 48 hours in all manufacturing lots, displaying linear decay over time (Figure 1). Our QC spike studies demonstrated similar patterns of complete hydrolysis achieved by 42 hours (Figure 2).

**Table 1:** ßPL decay as a function of time (Lots 8118-1, 8118-2, and 8118-3)

| Time (post ßPL) | Temperature (°C) | | | pH | | | ßPL Concentration (ppm) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Lot 1 | Lot 2 | Lot 3 | Lot 1 | Lot 2 | Lot 3 | Lot 1 | Lot 2 | Lot 3 |
| 0 h | 5.3 | 5.3 | 5.3 | 6.7 | 6.72 | 6.79 | | | |
| 1 h | 5.3 | 5.2 | 5.3 | 7.21 | 7.23 | 7.26 | 233.97 | 255.41 | 247.24 |
| 2 h | 5.3 | 5.3 | 5.3 | 7.19 | 7.21 | 7.22 | 222.47 | 239.81 | 232.61 |
| 4 h | 5.3 | 5.3 | 5.3 | 7.17 | 7.18 | 7.2 | 215.13 | 225.68 | 216.91 |
| 6 h | 5.2 | 5.3 | 5.3 | 7.16 | 7.17 | 7.18 | 203.52 | 217.58 | 210.32 |
| 8 h | 5.3 | 5.3 | 5.3 | 7.13 | 7.15 | 7.16 | 192.71 | 209.95 | 203.61 |
| 10 h | 5.3 | 5.3 | 5.3 | 7.12 | 7.13 | 7.14 | 176.01 | 197.47 | 194.3 |
| 12 h | 5.4 | 5.8 | 5.4 | 7.1 | 7.11 | 7.13 | 168.97 | 183.96 | 179.44 |
| 14 h | 5.3 | 5.2 | 5.3 | 7.09 | 7.1 | 7.11 | 164.44 | 168.57 | 174.09 |
| 18 h | 5.3 | 5.3 | 5.2 | 7.06 | 7.07 | 7.08 | 143.95 | 151.97 | 149.41 |
| 24 h | 5.3 | 5.3 | 5.3 | 7.02 | 7.04 | 7.04 | 121.38 | 142.73 | 123.46 |
| 30 h | 17.2 | 17.1 | 17.2 | 6.84 | 6.87 | 6.87 | 79.43 | 92.63 | 85.48 |
| 36 h | 27.5 | 27.5 | 27.5 | 6.64 | .667 | 6.66 | 19.47 | 21.31 | 22.58 |
| 42 h | 34.5 | 34.4 | 34.4 | 6.56 | 6.6 | 6.58 | 0.00 | 1.99 | 1.82 |
| 48 h | 36.9 | 36.9 | 37.0 | 6.56 | 6.6 | 6.57 | 0.0 | 0.0 | 0.0 |
| 53 h | 37.2 | 37.0 | 37.2 | 6.56 | 6.61 | 6.6 | 0.0 | 0.0 | 0.0 |

**Table 2:** ßPL decay as a function of time (Lots 7108-1, 7108-2, and 7108-3

| Time Point | Temperature (°C) | | | ßPL Concentration (ppm) | | |
|---|---|---|---|---|---|---|
| | Lot 1 | Lot 2 | Lot 3 | Lot 1 | Lot 2 | Lot 3 |
| 12 h (spike) | 5.4 | 5.8 | 5.4 | 85.94 | 81.1 | 88.72 |
| 24 h (spike) | 5.3 | 5.3 | 5.3 | 55.57 | 53.63 | 57.58 |
| 36 h (spike) | 27.5 | 27.5 | 27.5 | 10.69 | 9.35 | 13.04 |
| 42 h (spike) | 34.5 | 34.4 | 34.4 | 0.0 | 0.0 | 0.0 |
| 48 h (spike) | 36.9 | 36.9 | 37.0 | 0.0 | 0.0 | 0.0 |
| 53 h (spike) | 37.2 | 37.0 | 35.8 | 0.0 | 0.0 | 0.0 |

## Materials and Methods

Beta-propiolactone at 98–99% purity (Cat#P-5648) was obtained from Ferak Berlin GmbH (www.ferak.de). Stainless steel tanks (1000 L) came from Feldmeier Equipment, Inc. (www.feldmeier.com). The HuT 78 cell line (cutaneous T lymphocytes) is ATCC#TIB-161 from American Type Culture Collection (www.atcc.org). Custom-made RPMI-1640 medium with defined fetal bovine serum (Cat#SH3A044) came from HyClone Laboratories, Inc. (www.hyclone.com). Source leukocytes are from New York Biologics, Inc. (www.nybiologics.com). And the HIV-1 p24 antigen EIA (Cat#626391) was obtained from Beckman Coulter, Inc. (www.beckmancoulter.com).

**Table 3:** ßPL inactivation of HIV-1 (Lots 8118-1, 8118-2, and 8118-3)

| Hour Post ßPL | 8118-1 | | 8118-2 | | 8118-3 | |
|---|---|---|---|---|---|---|
| | Titer* | RCVPs** | Titer* | RCVPs** | Titer* | RCVPs** |
| 0 | 6.4 | $2.51 \times 10^6$ | 4.3 | $2.00 \times 10^4$ | 4.8 | $6.31 \times 10^4$ |
| 1 | 2.4 | $2.51 \times 10^2$ | 3.6 | $3.98 \times 10^3$ | 3.6 | $3.98 \times 10^3$ |
| 2 | 2.8 | $6.31 \times 10^2$ | 3.1 | $1.25 \times 10^3$ | 3.0 | $1.00 \times 10^3$ |
| 4 | 1.9 | $7.94 \times 10^1$ | 2.8 | $6.31 \times 10^2$ | 2.7 | $5.01 \times 10^2$ |
| 6 | 1.9 | $7.94 \times 10^1$ | 1.9 | $7.94 \times 10^1$ | 0.8 | 6.3 |
| 8 | 1.5 | $3.16 \times 10^1$ | 1.5 | $3.16 \times 10^1$ | 0.8 | 6.3 |
| 12 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 | 0 | 0 | 0 |
| 36 | 0 | 0 | 0 | 0 | 0 | 0 |
| 53 | 0 | 0 | 0 | 0 | 0 | 0 |

*Titers are expressed as $\log_{10}$ $TCID_{50}$/mL ** Replicate-competent viral particles

**Assays:** We determined the effectiveness of ßPL at 1:2000 (v/v) to inactivate HIV-1 activity by using infectivity assay analysis and p24 ELISA quantification. The "Sampling Details" box discusses some regulatory compliance issues involved (9–12).

In the infectivity assay, phytohemagglutin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) were incubated with in-process material over a 14- to 21-day period to determine the presence of replication-competent virus particles. Through a series of media splits—performed on days 4, 7, 11, and 18—this assay demonstrated an ability to remove background levels of inactivated antigen while allowing expansion of live virus within the cell-based system. The sensitivity of this assay was amplified by a day-14 supernatant transfer to a new lot of PBMCs.

We analyzed the infectivity supernatants by p24 ELISA and scored them based on a positive (+) or negative (–) system, calculating titers of the material by the Spearman–Karber formula:

$$M = xk + d\,(0.5 - [(1/n) \times \Sigma r])$$

where $xk$ is the dose of highest dilution, $n$ is number of replicates per dilution, $d$ is the spacing between dilutions, and $\Sigma r$ is the sum of negative responses. The 50% endpoint is then converted to $10x$ by $x = (M)(\log_4)$. The $\log_{10}$ $TCID_{50}$/mL is antilog ([$10x$] × [100.70]).

Our ßPL process demonstrated highly reproducible and controlled HIV-1 inactivation through all qualification runs (Table 3). In all three lots of GMP material containing initial viral titers of >4.3 $\log_{10}$, infectivity assays showed complete inactivation of viral activity by the 12-hour time point (Figure 3).

Similar results were found in the parallel QC spike study, where the additional challenge with 5.5 $\log_{10}$ infectious virus displayed reproducible inactivation curves across all three trials (Table 4). Our ßPL inactivation process demonstrated a margin of safety in producing complete inactivation of that excess viral load by the 42-hour time point (Figure 4). We calculated the cumulative range of inactivation within our process to be between 9.1 $\log_{10}$ and 11.5 $\log_{10}$ (Table 5). Additional inactivation may be presumed based on the fact that the ßPL is diluted 1:2 (v/v) within the spike study. The highest titer of a manufacturing lot in this study was 6.4 $\log_{10}$, producing a minimum margin of safety for viral inactivation of 2.7 $\log_{10}$.

We performed additional infectivity testing on a 25-dose equivalent of purified material at both the post-ßPL stage and again following gamma irradiation. Complete inactivation was demonstrated in all three qualifications (data not shown).

## Discussion

Inactivated whole virus vaccines have historically induced successful immunity to diseases in both humans and animals. To make safe and effective whole-virus vaccines, numerous procedures using both physical and chemical means have been developed. Here we have demonstrated a large-scale, commercially adaptable ßPL inactivation process for HIV-1.

### Sampling Details

**Sample Sizing:** It is important to note that sampling of ßPL-inactivated harvest material does not reflect the total sampling requirements of in-process material for detection of residual infectious virus. Our ßPL inactivation is followed by extensive concentration and purification of the in-process material, which is then treated with a second viral inactivation step: gamma irradiation. Larger volume sampling is required after ßPL with other inactivated vaccines, e.g., the rabies vaccine, where inactivation is achieved through a single step. The FDA, EMEA, and WHO recommend testing the equivalent of no less than 25 doses of final drug product. Within our process, sampling of each production lot of ßPL-inactivated material is coupled with testing of both downstream purified material (before gamma irradiation) and gamma irradiated material. That represents a concentrated form of the final drug product. The equivalent of 25 doses is inoculated into cell cultures and tested at both pre- and post-gamma stages, essentially doubling the recommended volumes for safety testing outlined by the governing bodies.

**Representative Sample:** Lot-to-lot comparison of data values generated across three successive validation/production runs provides a "representative sample" from our process. ßPL hydrolysis values are nearly identical for both the production runs and spiking studies, and viral titer discrepancies can be attributed to the expected variability of the validated infectivity assay. In both cases, a substantial margin of safety has been demonstrated.

Complete viral inactivation was achieved through a two-tank–transfer ßPL inactivation step engineered to prohibit advancement of untreated viral particles into the downstream purification process.

The ability of ßPL to demonstrate complete viral inactivation was further defined in a high-titer spike study. The inactivation of added high-titer virus spikes demonstrated a significant level of safety in this manufacturing process. Although ßPL itself has been assessed by the US Occupational Safety and Health Administration (OSHA) as a potential human carcinogen, we have demonstrated an ability to completely hydrolyze the compound into two nontoxic degradation products: an isomer of lactate and beta-hydroxypropionic acid, which is naturally found in the human body as a product of fat metabolism (**13**).

Thus, ßPL demonstrates an advantage over other chemical inactivators (e.g., formaldehyde), which can produce residues in final drug products that may be harmful to recipients. ßPL displays a complement of additional advantages over other chemical agents, validating its use within the biopharmaceutical industry. Its ability to react with nucleic acids allows ßPL to serve as an internal bactericide and fungicide, additionally inactivating possible host-cell DNA contamination (**14**). Also, ßPL has demonstrated the ability to maintain the antigenicity and immunogenicity of viral proteins—as witnessed by the success of the poliomyelitis, influenza, and rabies vaccines (**15**). Although further gamma irradiation provides an extra margin of safety in our vaccine manufacturing process, ßPL demonstrates potential for use in a single-step complete inactivation process for a wide range of viruses.

**Table 4:** ßPL inactivation of HIV-1 (Lots 7108-1, 7108-2, and 7108-3)

| Hour Post ßPL | 7108-1 | | 7108-2 | | 7108-3 | |
|---|---|---|---|---|---|---|
| | Titer* | RCVPs** | Titer* | RCVPs** | Titer* | RCVPs** |
| 12 | 5.1 | $1.26 \times 10^5$ | 4.8 | $6.31 \times 10^4$ | 5.1 | $1.26 \times 10^5$ |
| 18 | 3.6 | $3.98 \times 10^3$ | 3.7 | $5.01 \times 10^3$ | 5.1 | $1.26 \times 10^5$ |
| 24 | 2.7 | $5.02 \times 10^2$ | 3.3 | $2.00 \times 10^3$ | 4.0 | $1.00 \times 10^4$ |
| 30 | 1.0 | $1.01 \times 10^1$ | 1.2 | $1.58 \times 10^1$ | 1.3 | $2.00 \times 10^1$ |
| 36 | 0 | 0 | 0 | 0 | 0.8 | 6.3 |
| 42 | 0 | 0 | 0 | 0 | 0 | 0 |
| 48 | 0 | 0 | 0 | 0 | 0 | 0 |
| 53 | 0 | 0 | 0 | 0 | 0 | 0 |

*Titers are expressed as $\log_{10}$ $TCID_{50}$/mL ** Replicate-competent viral particles

**Table 5:** Cumulative ßPL inactivation of HIV-1

| Lot | GMP 8118 | Spike 7108 | Cumulative |
|---|---|---|---|
| 628 | 6.4 | 5.1 | 11.5 |
| 629 | 4.3 | 4.8 | 9.1 |
| 630 | 4.8 | 5.1 | 9.9 |
| **Average** | **5.2** | **5.0** | **10.2** |

*Titers are expressed as $\log_{10}$ $TCID_{50}$/mL

**Figure 1:** ßPL hydrolysis

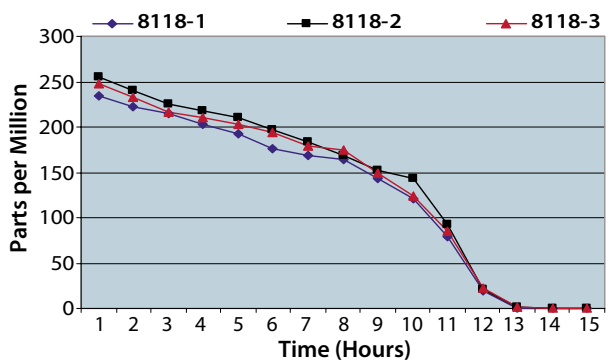



**Figure 2:** ßPL hydrolysis in the spiking study

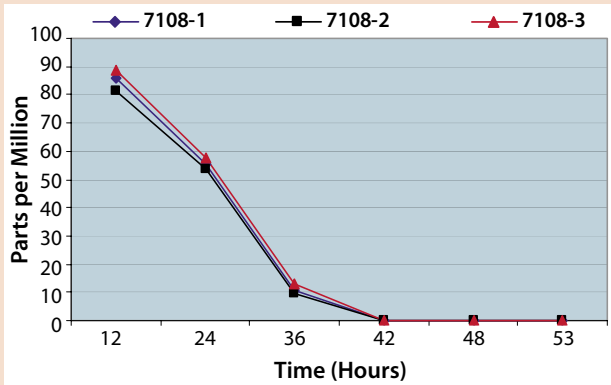



**Figure 3:** ßPL inactivation of HIV-1

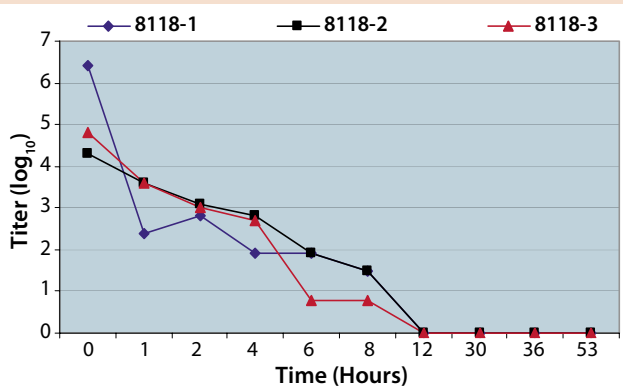
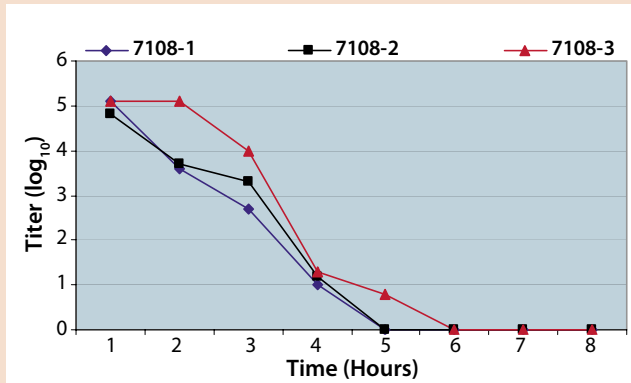



**Figure 4:** ßPL inactivation of HIV-1 in the spiking study



We have described a novel manufacturing process for producing an inactivated HIV virus particle as a candidate for a therapeutic HIV vaccine. This ßPL procedure is both scalable and highly effective. Although applied here to HIV, the technology may aid in meeting worldwide production needs for other vaccine products as well.

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