# Sustainable compendial grade GMP detergent substitutes for Triton™ X-100 in bioprocessing applications

Katherine McQuillan, Kritika Bajaj, Garima Sinha, Maria Broilo, Brian Malys, Miquel Mir, Sreejit Menon, Stephen Rumbelow, James Humphrey and Bradley Haltli

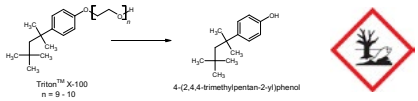
Croda International Plc & Associates

## Abstract

Triton™ X-100 (octyl phenol ethoxylate) has been used for decades as a detergent for viral inactivation in bioprocessing applications and the treatment of human or animal derived plasma, along with numerous cell lysis applications at laboratory scale and in cell-based biopharmaceutical production. While the ability of Triton™ X-100 to perform in these applications has never come into question, the environmental impact of this material ultimately led to its ban in Europe as of January 2021, with the exception of existing pharmaceutical applications allowed under a public exemption or for small scale laboratory research use[1]. This ban has led to significant interest in finding alternative detergents which are biodegradable, GMP compliant and pharmaceutically acceptable for use in the manufacturing of new cell-derived drug products in Europe. Alternatives to Triton™ X-100 must perform equally in regard to cell lysis and protein compatibility in order to ensure global applicability in a wide range of bioprocessing applications. Employing xenotropic murine leukemia virus (XmuLV) and the *Feline catus* PG4 cell line as a model lipid-enveloped virus – host system, we compared the viral inactivation properties of over 31 different detergents belonging to 11 chemical classes. This analysis identified ten detergents that exhibited an equal log reduction in XmuLV viability compared to Triton™ X-100. We then extended the characterization of these detergents to compare their minimum virucidal concentrations, kinetics of virus inactivation, cell lysis capabilities against three cell lines (PG4, CHO K1 and HEK293) and protein compatibility using alkaline phosphatase. Two detergents exhibited equivalent performance to Triton™ X-100 in all categories. This study identified two chemically distinct detergent alternatives to Triton™ X-100 which are biodegradable, GMP compliant and have an established track record of pharmaceutical use and compendial compliance. These detergents represent exciting new options in the biopharmaceutical manufacturing tool kit.

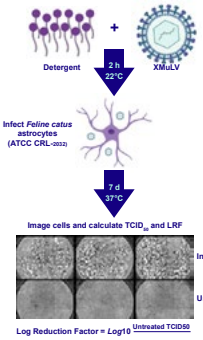
## Introduction

Triton™ X-100 is a 4-(1,1,3,3-tetramethylbutyl)-phenyl ethoxylate detergent that is used widely in the biopharmaceutical industry for virus inactivation and cell lysis applications. Triton™ X-100 degrades to endocrine disrupting by-products making it an aquatic reproductive toxin[2]. This led to its inclusion in the European Chemical Agency's candidate list of substances of very high concern of the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation[3]. Triton™ X-100 was banned for use in the EU on Jan 4, 2021 with a few exemptions, requiring biopharmaceutical manufacturers to find alternative detergents. Replacements for Triton™ X-100 should be eco-friendly and functionally equivalent or superior to Triton™ X-100. Alternatives to Triton™ X-100 have been developed[4] but there is room in the market to explore detergents from Croda's portfolio that can act as suitable replacements.




To identify Croda detergents with equivalent or superior performance to Triton™ X-100, 31 detergents belonging to 11 structural classes were evaluated for their virus inactivation properties and compared to Triton™ X-100 and three other industry benchmarks. To profile the virus inactivation activity of the detergents, assays utilizing XMuLV were developed as this virus is commonly used as a model for human retroviruses[5]. This study was conducted in two phases. In the first phase we screened 31 detergents to identify the highest performing detergents. In the second phase we compared two prioritized detergents to industry benchmarks. The results of this work identified **Virodex TXR-1** and **TXR-2** as exciting new products for virus inactivation and cell lysis applications in the bioprocessing and biopharmaceutical industries.

## Viral inactivation testing

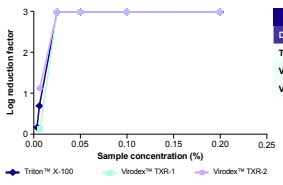



| Log reduction factor | % viral clearance |
|---|---|
| 0 | 0% |
| 1 | 90% |
| 2 | 99% |
| 3 | 99.9% |
| 4 | 99.99% |
| 5 | 99.999% |
| 6 | 99.9999% |
| 7 | 99.99999% |
| 8 | 99.999999% |
| 9 | 99.9999999% |
| 10 | 99.99999999% |

Virus inactivation was assessed using XMuLV. Tissue culture infectious dose (TCID) was measured to determine the viable viral particles after treatment with the detergent. The reduction in viable viral titer achieved by detergent treatment was calculated by determining the log reduction factor (LRF). A LRF of 3 was used for the initial screening assay. A LRF of 4 is an acceptable target for detergent based virus inactivation. However, LRF of 8 – 10 is the target for a finished product.

## Detergent screening and potency testing

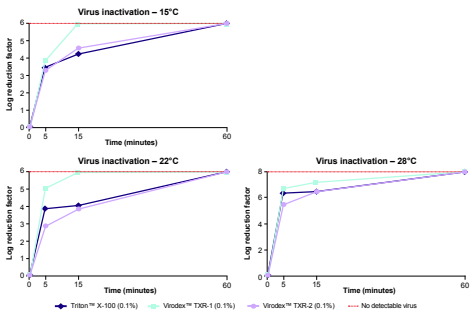
Thirty-one detergent materials belonging to 11 different chemical classes were screened for their viral inactivation capabilities. Two chemically distinct products (**Virodex™ TXR-1** and **Virodex™ TXR-2**) that exhibited the same level of virus inactivation activity as Triton™ X-100 were identified. **TXR-1** and **TXR-2** achieved maximum virus inactivation (LRF3) at the same concentration (0.025%) as Triton™ X-100 after a 2 h detergent treatment at 22 °C.



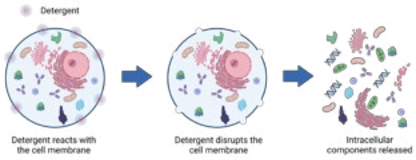

| Virus inactivation potency | |
|---|---|
| **Detergent** | **Concentration (%)** |
| **Triton™ X-100 control** | 0.025 |
| **Virodex™ TXR-1** | 0.025 |
| **Virodex™ TXR-2** | 0.025 |

## Kinetics of viral inactivation

The time required to achieve a LRF of 4 is an important characteristic of a virus inactivation step in a biomanufacturing process. A LRF of 4 is the industry standard virus inactivation target. Virus inactivation was assessed at three temperatures (15°C, 22°C and 28°C) to determine the effect of temperature on virus inactivation perfromance. At all temperatures, both **Virodex™** detergents showed equivalent or better virus inactivation kinetics than Triton™ X-100 after a 15 min exposure, with **Virodex™ TXR-1** exhibiting more rapid virus inactivation than Triton™ X-100 at 15°C and 22°C. At all temperatures, both **Virodex™** detergents achieved an LRF greater than 3 after a 5-minute treatment time, and after 60 min, the LRF increased to 6-8, exceeding the industry standard target of 4.

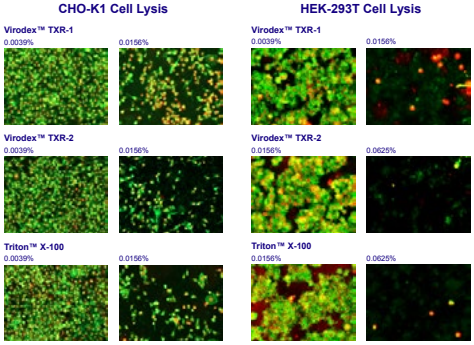



## Cell lysis of two mammalian cell lines



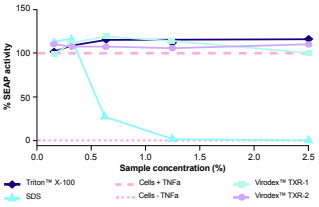

In the production of biopharmaceuticals, Triton™ X-100 is used to lyse cells to extract biomolecules such as therapeutic proteins or viral vectors from production cell lines. For this reason, the effectiveness of **Virodex™** detergents for cell lysis was evaluated in two industrially relevant cell lines, Chinese Hamster Ovary (CHO-K1) and Human Embryonic Kidney (HEK-293T) cells. Cultured cells were treated with each detergent over a wide range of concentrations (0.00015% - 1%) for 2 h at 22°C. To assess lysis, cells were stained with fluorescent stains that stain the cell membrane and nucleus and cellular lysis was assessed by fluorescent microscopy. Lysis was evident by disintegration of the cell membrane (green) and nucleus (red) in acquired images.

| Lowest concentration (%) for Cell Lysis | | |
|---|---|---|
| **Detergent** | **CHO-K1** | **HEK-293T** |
| **Virodex™ TXR-1** | 0.0156 | 0.0156 |
| **Virodex™ TXR-2** | 0.0156 | 0.0625 |
| **Triton™ X-100 control** | 0.0156 | 0.0625 |

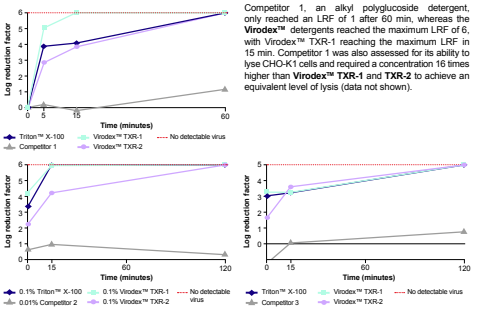



## Protein compatibility of Virodex™ TXR-1 and TXR-2

Biopharmaceutical bioprocesses are frequently used to manufacture protein-based products such as enzymes, protein therapeutics, growth factors and immunoglobins. Detergent treatments used to lyse cells or inactivate lipid-developed viruses must not denature protein products because they will typically lose their biological activity once denatured. The protein compatibility of **Virodex™ TXR-1** and **TXR-2** was investigated by assessing their effect on the activity of the secreted embryonic alkaline phosphatase (SEAP) enzyme using a HEK-293 reporter cell line. **Virodex™ TXR-1** and **TXR-2** do not affect SEAP activity at concentrations up to 2.5% and their protein compatibility is equivalent to Triton™ X-100. This is consistent with the non-ionic nature of **Virodex™** detergents. In comparison, an anionic detergent (sodium dodecyl sulfate - SDS) shows strong enzyme inactivation at concentrations > 0.3% .




## Virodex™ detergents outperform competitor products

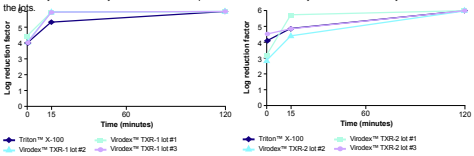



Competitor 1, an alkyl polyglucoside detergent, only reached an LRF of 1 after 60 min, whereas the **Virodex™** detergents reached the maximum LRF of 6, with Virodex™ TXR-1 reaching the maximum LRF in 15 min. Competitor 1 was also assessed for its ability to lyse CHO-K1 cells and required a concentration 16 times higher than **Virodex™ TXR-1** and **TXR-2** to achieve an equivalent level of lysis (data not shown).

Competitors 2 and 3 were tested in separate head-to-head kinetic virus inactivation experiments with the **Virodex™** detergents and Triton™ X-100. Due to potent cytotoxicity against the *F. catus* PG4 cell line used in the virus inactivation assay, Competitor 2 could not be tested at 0.1% and was instead tested at the non-toxic concentration of 0.01%. In both comparisons, the **Virodex™** detergents out-performed the competitors.

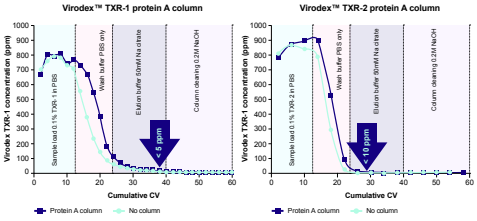
## Performance consistency

Three lots of **Virodex™ TXR-1** and **TXR-2** were tested in the kinetic virus inactivation assay to assess the consistency of their activity. All lots showed exceptional lot-to-lot consistency with LRFs essentially identical between the lots.




## Virodex™ removal: Protein A column affinity

In the manufacture of biotherapeutic proteins, it is typical to use chromatographic methods to purify the drug substance. A common resin used in the downstream processing of mAbs is the Protein A resin, with antibody specificity. In mAb manufacture utilising Triton™ X-100, this detergent is washed from the drug product alongside other waste materials prior to mAb elution. **Virodex™ TXR-1** and **Virodex™ TXR-2** were tested for their affinity to a Protein A column* verses no column using typical loading, washing, elution and cleaning buffers. Neither **Virodex™ TXR-1** or **Virodex™ TXR-2** were found to have any affinity to the Protein A resin, and thus, the resin can be effectively used to remove **Virodex™** detergents from mAb solutions.

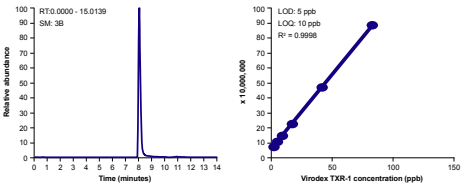



*MabCapture C™ MiniChrom Column 1 ml, ex Thermo Fisher Scientific

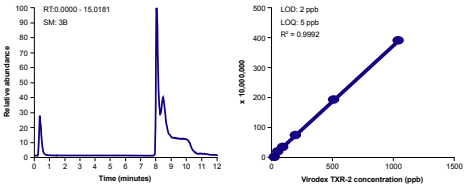
## Virodex™ analytical detection

Reliable and sensitive detection and quantification methods are essential to confirm detergent removal in finished biopharmaceutical finished products. Croda developed a chromatography method that detects and quantifies **Virodex™ TXR-1** and **TXR-2** at low parts per billion (ppb) concentrations using standard reversed-phase ultra-high-pressure chromatography and mass spectrometry (UHPLC-MS) methodology.

For **Virodex™ TXR-1** the method achieved a 5 ppb limit of detection (LOD), a 10 ppb limit of quantification (LOQ) and a linear quantification response between 5 ppb and 100 ppb (R2 - 0.9998).




The same method achieved a 2 ppb LOD, a 5 ppb LOQ and a linear quantification response between 2 ppb – 1000 ppb ($R^2$ - 0.9992) for **Virodex™ TXR-2**.




## Freeze-thaw stability of Virodex™

Stability under freeze-thaw conditions is essential for bioprocessing detergents, which may be subjected to repeated cycles of freezing and thawing during storage, transportation, or processing. The below data shows that **Virodex™** detergents remain freeze-thaw stable after 14 cycles of freezing and thawing, and thus preserve their integrity under these conditions. During each cycle, solutions were frozen at a temperature of -20°C for 12 hours and thawed at 40°C for 12 hours.

| Solutions tested | Results after 14 cycles |
|---|---|
| **Virodex™ TXR-1, 10.0% w/w** | Clear solution, unchanged after 14 cycles |
| **Virodex™ TXR-1, 1.0% w/w** | Clear solution, unchanged after 14 cycles |
| **Virodex™ TXR-2, 10.0% w/w** | Clear solution, unchanged after 14 cycles |
| **Virodex™ TXR-2, 1.0% w/w** | Hazy solution, unchanged after 14 cycles |

## Conclusion

Of the 31 detergents screened for viral inactivation, two detergents (**Virodex™ TXR-1** and **TXR-2**) exhibited equivalent or superior performance to Triton™ X-100 in all head-to-head comparisons. This data is consistent with previous research evaluating Triton™ X-100 alternatives[6]. **Virodex™** detergents also outperformed competitor products in terms of safety and virus inactivation. **Virodex™** detergents are effectively removed in mAb manufacture using traditional Protein A chromatography. Sensitive ppb-level detection and quantification methods have been developed for industry standard instrumentation to aid customers in quality control analyses of finished products. **Virodex™** detergents also show no sign of degradation after repeated freeze-thaw cycles. The data presented here illustrates that the **Virodex™** detergents are exceptional alternatives to Triton™ X-100 for biopharmaceutical and bioprocessing applications.

## References

1. Annex XIV Authorisation list (2022) ECHA. Available at: https://www.echa.europa.eu/authorisation-list (Accessed: 19 May 2023).
2. Substance information ECHA. Available at: https://echa.europa.eu/substance-information/-/substanceinfo/100.123.919 (Accessed: 15 June 2023).
3. Candidate list of substances of very high concern for authorisation (2012) ECHA. Available at: https://echa.europa.eu/candidate-list-table/-/dislist/details/0b0236e1807db570 (Accessed: 19 May 2023).
4. Farcet, J-B. et al. (2019) Development of a Triton X-100 replacement for virus inactivation in biotechnology processes. Eng. Rep.. 1: e12078.
5. Wang et al. (2023) Validation: Current approaches and considerations for viral clearance in cell and gene therapy. BioPhorum. https://doi.org/10.46220/2023OST004
6. Hunter, A. K. et al. (2022) Identification of compendial nonionic detergents for the replacement of Triton X-100 in Bioprocessing. Biotechnol. Prog. 38: e3235
7. Cellular diagrams Created with BioRender.com

02/24 CPPT002v3 EN