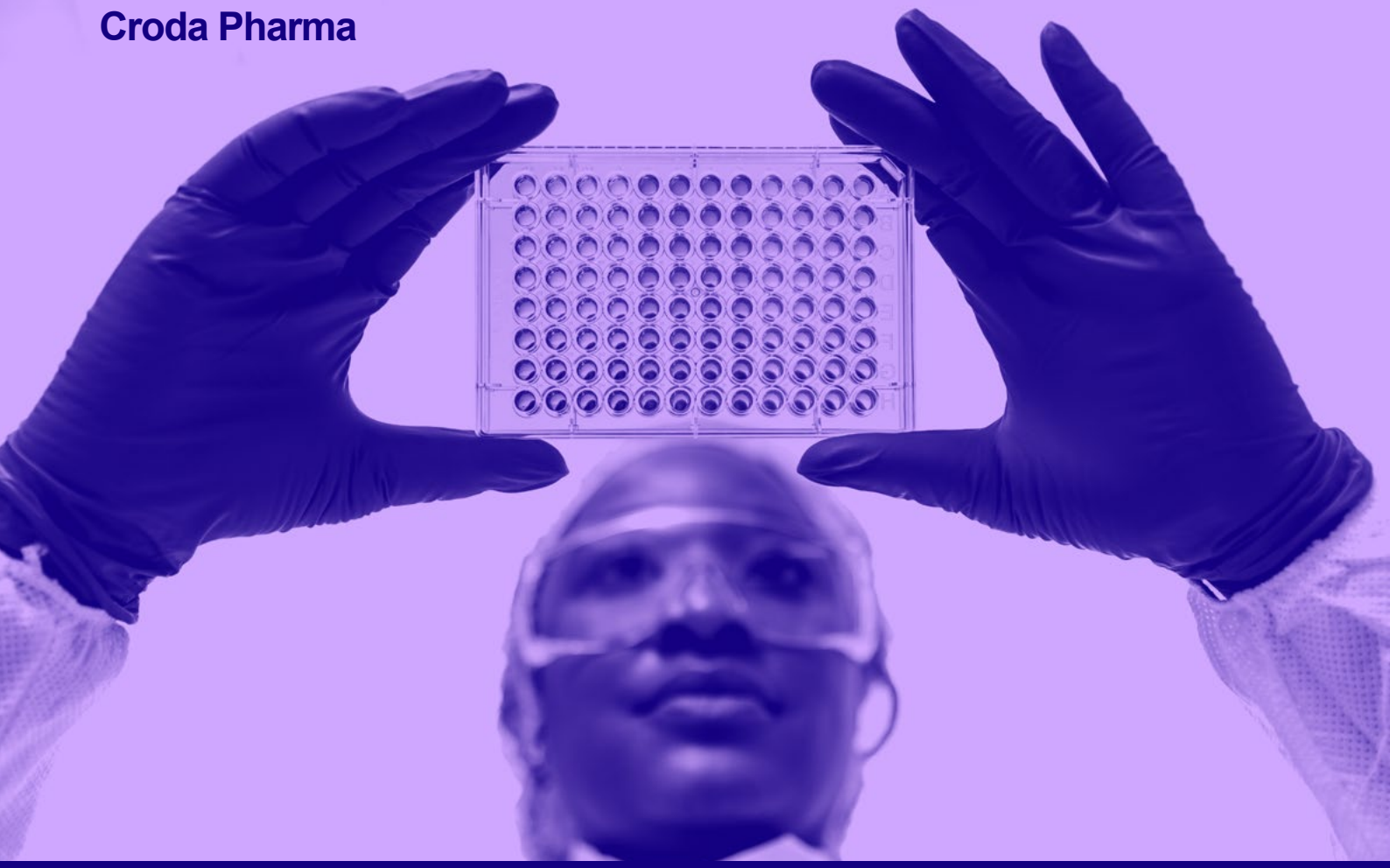
Croda Pharma



Virodex™ TXR-1 and TXR-2: Safe and effective viral inactivation and cell lysis

The REACH* addition of Triton™ X-100 to the candidate list of Substances of Very High Concern (SVHC), and its subsequent ban in Europe, left biopharmaceutical manufacturers seeking viable alternatives to this essential bioprocessing chemical.

Explore the Virodex™ range for viral inactivation and cell lysis: REACH-compliant, sustainable materials that have been optimised for use in the biomanufacturing of recombinant proteins, blood fractionation products, viral vector-based therapies and more.

Features and benefits:

- Sustainable, REACH-compliant, and cGMP EXCiPACT manufactured
- Compendial grade, with known parenteral applications**
- Known use of chemistries by industry for viral inactivation[1]
- Equivalent performance to Triton™ X-100, with better performance than three other replacements on the market
- No risk of nitrosamine formation
- Improved biologic quality and lower biomanufacturing risk, enabling faster speed to market
- Offered in a range of pack sizes, including single-use
- Comes with additional COA testing: bacterial endotoxin, Total Aerobic Microbial Count (TAMC), and Total Yeast and Mould Count (TYMC) to support use in sterile manufacture
- Analytical methods available to confirm detergent removal in finished biopharmaceutical products

*EU Registration, Evaluation, Authorization and Restriction of Chemicals
**Virodex™ TXR-1 chemistry has known parenteral applications

CRODA

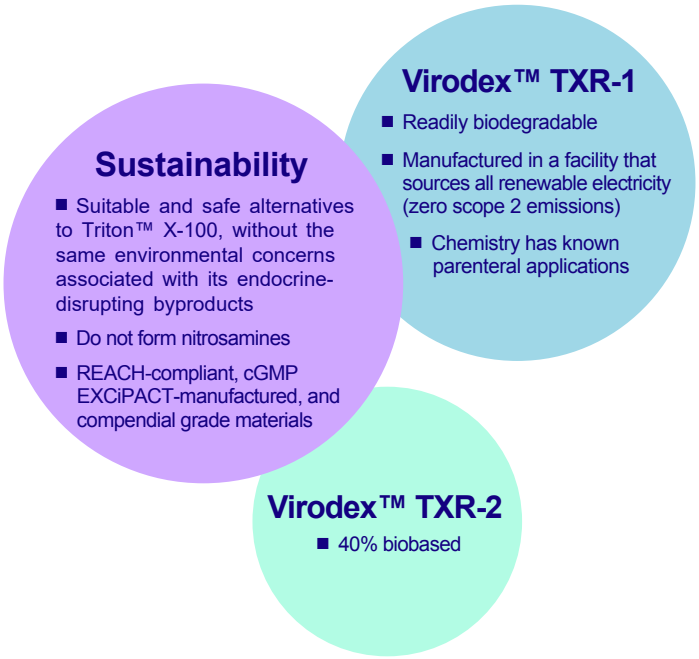
Smart science to improve lives™

Why consider alternatives to Triton™ X-100?

Triton™ X-100 is a non-denaturing, non-ionic detergent that has been widely used for decades in various industries, including biopharmaceutical manufacturing. Triton™ X-100 is used to inactivate lipid-enveloped viruses, such as hepatitis viruses B and C and human immunodeficiency virus (HIV), in plasma derivatives and biopharmaceuticals produced by mammalian cell culture. Triton™ X-100 is also frequently employed as a cell lysis agent to release therapeutic molecules from cells. With evidence of their toxicity to aquatic life via endocrine disrupting activity, phenol ethoxylates were added to the to the European Chemicals Agency (ECHA) candidate list of Substances of Very High Concern (SVHC) under the REACH regulation in 2012.[2] Substances on this list are of very high concern due to their potential impacts on human health and the environment. The sunset date for the ban of Triton™ X-100 in Europe occurred on January 4th, 2021, prohibiting its use for most applications unless granted an exemption.[3,4]

Sustainable, compendial, cGMP EXCiPACT-manufactured, REACH-compliant chemicals

In response to concerns about potential future restrictions on its use, biopharmaceutical manufacturers are turning to alternatives to Triton™ X-100. When searching for replacements, biomanufacturers look for a product that is more sustainable, and is free of the same safety or environmental concerns associated with Triton™ X-100. This is the basis on which we have developed our **Virodex™** range of detergents for viral inactivation and cell lysis: sustainable, compendial, cGMP EXCiPACT-manufactured, and REACH-compliant chemicals.



Croda Pharma's Virodex™ range for viral inactivation and cell lysis

Under our **Virodex™** range, we currently offer two distinct, well-studied chemistries: **Virodex™ TXR-1** and **Virodex™ TXR-2**. Both products provide efficient viral inactivation and cell lysis by similar mechanisms, and with equivalent performance to Triton™ X-100. **Virodex™** detergents are offered in single-use packs to meet the volume requirements for viral inactivation and cell lysis applications and come with additional COA testing for bacterial endotoxins, TAMC, and TYMC. Continue reading to learn how our sustainable, compendial, REACH-compliant, cGMP EXCiPACT-manufactured chemicals are used in the manufacturing of biologic drugs.

Identification of Triton™ X-100 replacements

Virus inactivation is a key step in the downstream processing of biopharmaceutical products to eliminate potentially harmful viruses or viral contaminants that could infect patients, ensuring the safety and efficacy of the final biopharmaceutical formulation. Detergent treatment aims to achieve a 10,000- to 100,000-fold reduction in viable virus particles (i.e., a log 4 to log 5 reduction). To identify next-generation detergents for biopharmaceutical manufacturing applications, 31 detergents belonging to 11 detergent classes were screened for equivalent virus inactivation activity to Triton™ X-100. This was achieved using xenotropic murine leukaemia virus (XmuLV), an established model lipid-enveloped virus frequently used to assess detergents for virus inactivation. This research identified two detergents, **Virodex™ TXR-1** and **TRX-2**, that exhibited equivalent performance characteristics to Triton™ X-100.

Virodex™ detergents for viral inactivation

To characterise the virus inactivation functionality of **Virodex™ TXR-1** and **TRX-2** the potency and kinetics of virus inactivation were assessed using the XmuLV model of lipid-enveloped virus inactivation. In this assay, XmuLV preparations were treated with detergent prior to infecting a sensitive cell line (*Felis catus* PG-4 ATCC CRL-2032). The viral titer of detergent-treated and untreated XmuLV preparations was determined and the reduction in infectious viral particles was calculated using the equation:

$$Log\ 10\ \frac{Viral\ titer\ untreated}{Viral\ titer\ treated} = Log\ reduction\ factor\ (LRF)$$

Potency determination: dose response assay

To assess the potency of the detergents for viral inactivation (i.e., the minimum concentration of detergent required to achieve maximum virus inactivation) XmuLV was treated with detergents at 0.2%, 0.1%, 0.05%, 0.025% and 0.00625% for 2 h at 22°C prior to infecting *F. catus* PG-4 cells. As shown in Table 1, both **Virodex™ TXR-1** and **Virodex™ TXR-2** were as potent as Triton™ X-100, achieving maximum virus inactivation at 0.025%.

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

Table 1. Potency of XmuLV inactivation. The minimum concentration of detergent required to achieve the maximum level of viral inactivation (LRF3) after detergent treatment for 2 h at 22°C.

Virus inactivation kinetics

The time required to achieve a LRF of 4 to 5 is an important characteristic of a detergent used in downstream processing applications. To determine the kinetics of virus inactivation by **Virodex™ TXR-1** and **Virodex™ TXR-2**, inactivation of XmuLV was assessed at three time points (1 min, 15 min, 120 min). Both **Virodex™** detergents showed equivalent virus inactivation as Triton™ X-100 after a 15 min treatment time (Figure 1).

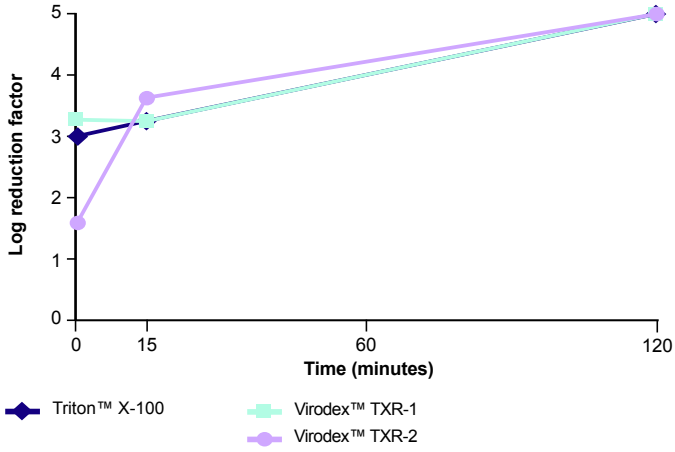


Figure 1. Virus inactivation kinetics. XmuLV was treated with each detergent at a concentration of 0.1% at 22°C and aliquots were removed at 1 min, 15 min and 120 min and used to infect *F. catus* PG-4 cells.

Virodex™ detergents for cell lysis

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 both 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 (Figure 2 and Figure 3). The lowest detergent concentration required to achieve lysis of CHO-K1 cells was identical between the **Virodex™** detergents and Triton™ X-100 (Table 2; Figure 2).

In the case of HEK-293T cells, **Virodex™ TXR-1** lysed the cells at a concentration 4-fold lower than Triton™ X-100, while **Virodex™ TXR-2** was equivalent to Triton™ X-100 (Table 2; Figure 3).

Lowest concentration (%) for Cell Lysis		
Detergent	**CHO-K1**	**HEK-293T**
Virodex™ TXR-1	0.01563	0.01563
Virodex™ TXR-2	0.01563	0.0625
Triton™ X-100 control	0.01563	0.0625

Table 2. Minimum concentration of detergent at which lysis was achieved for CHO-K1 and HEK-293T cell lines. Cell lines were treated with samples at concentrations ranging from 1% to 0.000015% for 2 h at 22°C and then stained with Alexa Fluor 488-conjugated wheat germ agglutinin and Hoechst 3328 and imaged using a BioTek Cytation 5 multimode imaging plate reader.

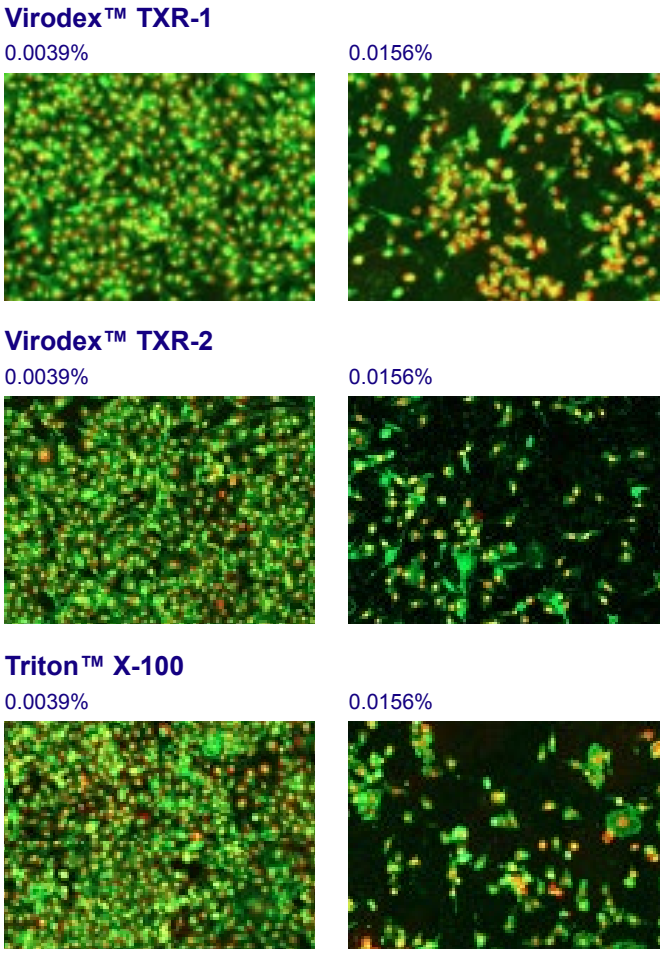


Figure 2. Lysis of CHO-K1 cells. Cells were treated with detergents at concentration of 1% down to 0.000015% for 2 h at 22°C and then stained with Alexa Fluor 488-conjugated wheat germ agglutinin (green - membrane) and Hoechst 3328 (red - nucleus) stains. Cell imaging was performed using a Cytation 5 multimode imaging plate reader (BioTek). Lysis is indicated by the disintegration of cell membranes and the nucleus at concentrations greater than 0.0039%.

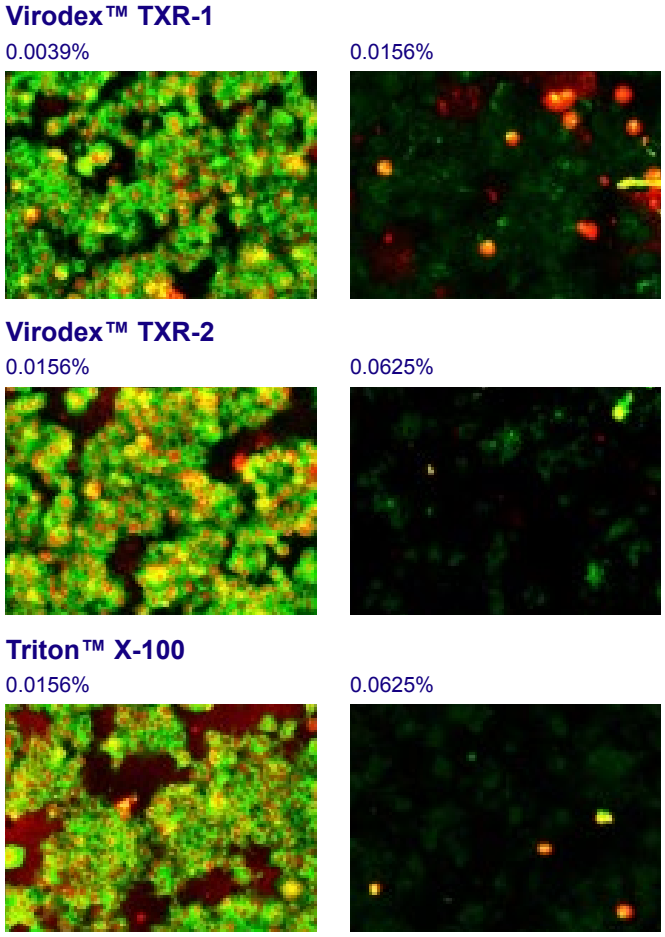


Figure 3. Lysis of HEK-293T cells. Cells were treated with detergents at concentrations of 1% down to 0.000015% for 2 h at 22°C and then stained with Alexa Fluor 488-conjugated wheat germ agglutinin (green - membrane) and Hoechst 3328 (red - nucleus) stains. Cell imaging was performed using a Cytation 5 multimode imaging plate reader (BioTek). Lysis is indicated by the disintegration of cell membranes and the nucleus at concentrations greater than 0.0156% for Triton™ X-100 and Virodex™ TRX-2, and at concentrations above 0.0039% for Virodex™ TRX-1.

Protein compatibility

Biopharmaceutical bioprocesses are frequently used to manufacture protein-based products such as enzymes, growth factors and immunoglobulins. When using a detergent as a lysing agent, or when treating protein-containing products (e.g., plasma) to inactivate lipid-enveloped viruses, it is essential that the protein products are not denatured as the secondary and tertiary structures of the proteins are essential for their biological function. Triton™ X-100 has excellent protein compatibility, and any alternative should not denature proteins at the use rates employed in bioprocessing applications.

To evaluate the ability of **Virodex™** detergents to lyse cells while preserving the integrity and activity of the target protein product, the protein-denaturing properties of the detergents were assessed using secreted embryonic alkaline phosphate expressed (SEAP) by HEK-293 cells.

The SEAP enzyme is a truncated form of human placental alkaline phosphatase generated by deleting the glycosylphosphatidylinositol anchor sequence. When expressed in cells, SEAP is secreted into the cell culture supernatant, where it dephosphorylates its substrate forming a purple product that can be monitored spectrophotometrically. Non-ionic detergents like Triton™ X-100 typically exhibit low protein denaturing activity, so the common anionic detergent sodium dodecyl sulphate (SDS) was used as a positive control. Cells expressing SEAP were treated with the detergents at concentrations ranging from 0.156% to 2.5% for 2 hours at 37°C. After 2 hours, an aliquot of the cell culture medium was mixed with detection reagent and the enzymatic activity was monitored by measuring the absorbance at 620 nm.

As expected, SDS had a profound effect on protein stability, reducing SEAP activity by 75% at 0.625%, with complete inactivation at all concentrations ≥ 1.25% (Figure 4). In contrast, **Virodex™ TXR-1** and **TXR-2** caused no reduction in SEAP activity at concentrations up to 2.5%. The protein compatibility of the **Virodex™** series of detergents was very similar to Triton™ X-100 and they provide equivalent performance at typical application rates (0.1%-1%) used in biopharmaceutical manufacturing processes (Figure 4).

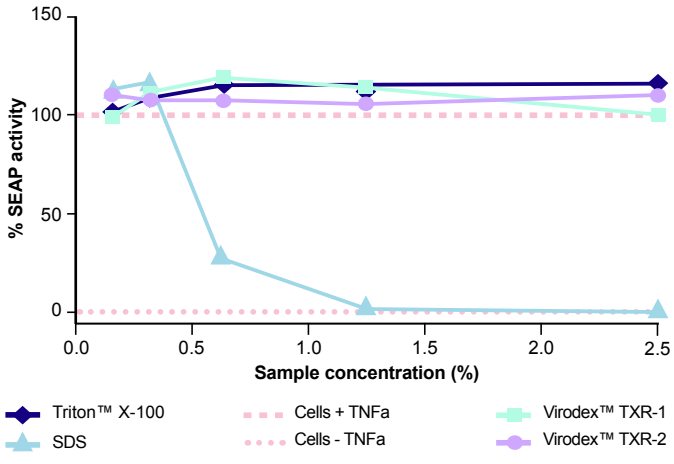


Figure 4. Activity of SEAP after detergent treatment. Expression of SEAP by HEK-293 cells was induced by treatment with tumor necrosis factor alpha (TNFa) and 100% activity was defined as SEAP activity in untreated controls (green dotted line). Base-line SEAP activity (0%) was determined using cells that were not induced with TNFa (purple dotted line). Following TNFa activation (24 h) cells were treated with detergents for 2 h at 37°C and then an aliquot of the cell culture medium was mixed with HEK-Blue Detection medium (Invivogen) and incubated for 24 h at 37°C and 5% CO^2. Enzymatic activity was monitored by measuring the absorbance at 620 nm using a Cytation 5 plate reader (BioTek).

Virodex™ performance against competitors

To assess the performance of the **Virodex™** detergents relative to other products on the market, we compared the virus inactivaion activity of the **Virodex™** detergents to three competitor products.

Competitor 1, an alkyl polyglucoside detergent, attained an equivalent level of virus inactivation (LRF3) as Triton™ X-100 and the **Virodex™** detergents but required a concentration four-fold higher to achieve this benchmark (Figure 5). 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).

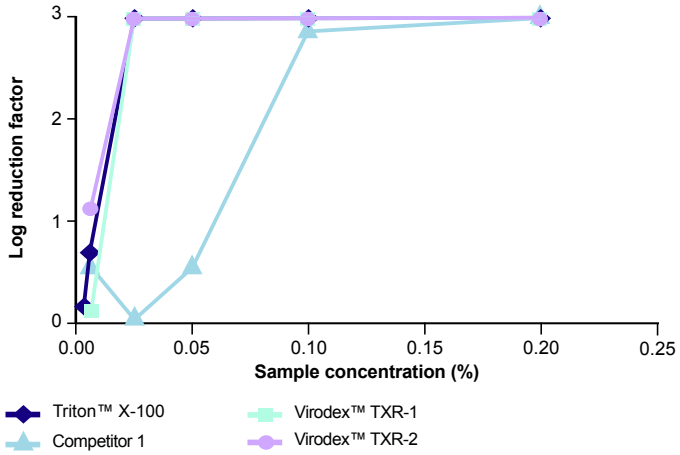


Figure 5. Virus inactivation potency determination. XmuLV was treated with each detergent at concentrations ranging from 0.0125% - 0.2% at 22°C for 120 min and used to infect *F. catus* PG-4 cells. The maximum achievable LRF in this assay was 3.

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%.

Under these conditions Competitor 2 was only able to achieve a LRF of 1 (Figure 6). The cytotoxicity of Competitor 2 was consistent with the manufacturer's safety data sheet which indicated that the product is corrosive and an aquatic toxin.

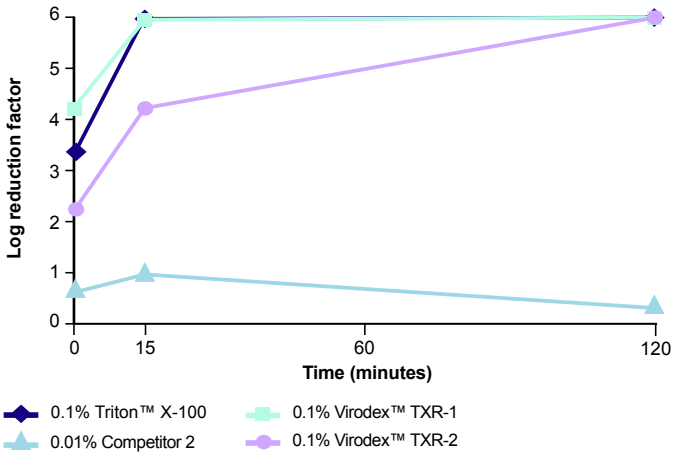


Figure 6. Virus inactivation kinetics of Virodex™ TXR-1 and TXR-2 compared to Triton™ X-100 and Competitor 2. XmuLV was treated with each detergent at a concentration of 0.1%, except for Competitor 2, which was tested at 0.01%. Detergent treatments were conducted at 22°C and aliquots were removed at 1 min, 15 min and 120 min and used to infect *F. catus* PG-4 cells. The maximum achievable LRF in this assay was 6.

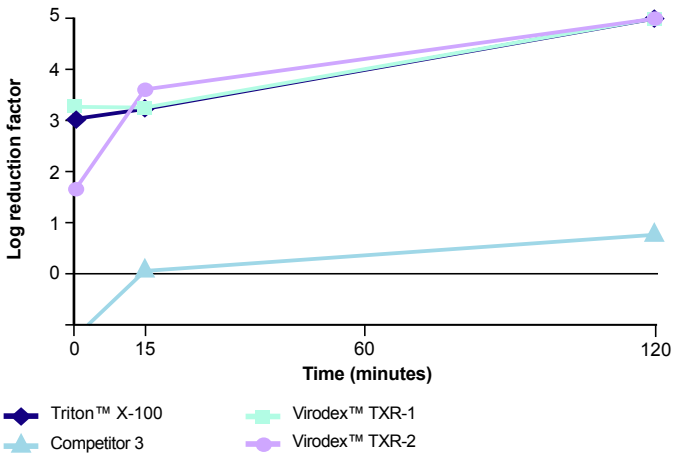


Figure 7. Virus inactivation kinetics of Virodex™ TXR-1 and TXR-2 compared to Triton™ X-100 and Competitor 3. XmuLV was treated with each detergent at a concentration of 0.1%. Detergent treatments were conducted at 22°C and aliquots were removed at 1 min, 15 min and 120 min and used to infect *F. catus* PG-4 cells. The maximum achievable LRF in this assay was 5.

In comparison to Competitor 3, **Virodex™ TXR-1** and **TXR-2** achieved a LRF of 5 while the competitor product did not reach an LRF of 1 even after 2 h (Figure 7). Based on these comparisons, the **Virodex™ TXR-1** and **TXR-2** detergents clearly outperform these competitors for the virus inactivation applications tested.

Batch-to-batch consistency

Product consistency is an essential criterion for bioprocessing reagents to ensure consistent, reproducible performance between batches. To assess the consistency of the **Virodex™** detergents we tested the virus inactivation kinetics of three distinct lots of **TXR-1** and **TXR-2**. The three batches of **TXR-1** exhibited nearly identical virus inactivation kinetics to each other (Figure 8). Slight variation was observed between the three batches of **TXR-2** (Figure 9); however, all three batches achieved a LRF greater than 4 after 15 min and a maximum LRF of 6 after 120 min.

This data demonstrates the excellent batch-to-batch consistency of the **Virodex™** detergents for virus inactivation applications, showing that you can trust the **Virodex™** range of detergents for your critical bioprocessing needs.

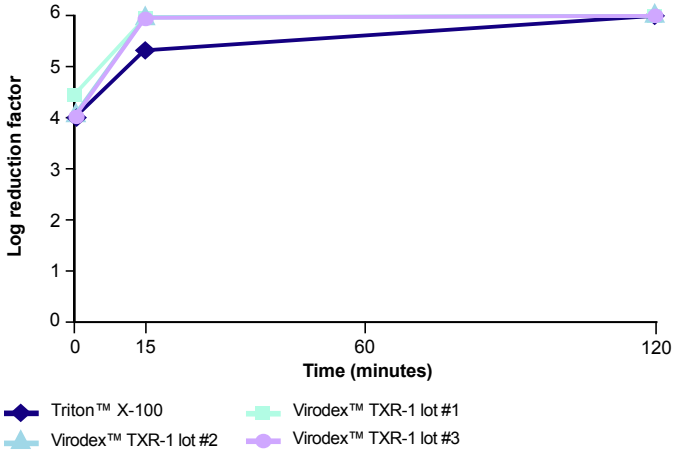


Figure 8. Virus inactivation kinetics of three batches of Virodex™ TXR-1 compared to Triton™ X-100. XmuLV was treated with each detergent at a concentration of 0.1%. Detergent treatments were conducted at 22°C and aliquots were removed at 1 min, 15 min and 120 min and used to infect *F. catus* PG-4 cells. The maximum achievable LRF in this assay was 6.

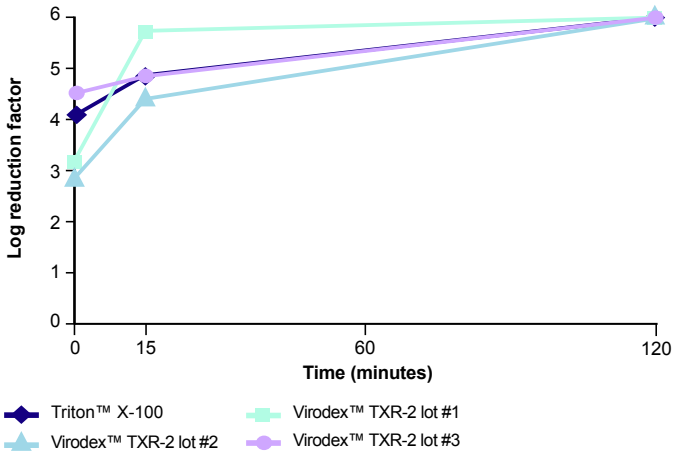


Figure 9. Virus inactivation kinetics of three batches of Virodex™ TXR-2 compared to Triton™ X-100. XmuLV was treated with each detergent at a concentration of 0.1%. Detergent treatments were conducted at 22°C and aliquots were removed at 1 min, 15 min and 120 min and used to infect *F. catus* PG-4 cells. The maximum achievable LRF in this assay was 6.

Virodex™ analytical detection

Reliable and sensitive detection and quantification methods are essential to confirm detergent removal in finished biopharmaceutical finished products. Croda has 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) (Figure 10). 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** (Figure 11).

To demonstrate detection and quantification using a different chromatography system and detector, a second method was developed for **Virodex™ TXR-1** employing UHPLC chromatography coupled with a charged aerosol detector (CAD). This method achieved a 0.5 parts per million (ppm) LOD, a 1 ppm LOQ and a linear quantification response between 0.5 ppm – 10 ppm (R^2 - 0.9994) (Figure 12). These results clearly demonstrate sensitive and accurate detection and quantitation of **Virodex™ TXR-1** and **TXR-2** using two different detection methods commonly found in biopharmaceutical manufacturing laboratories.

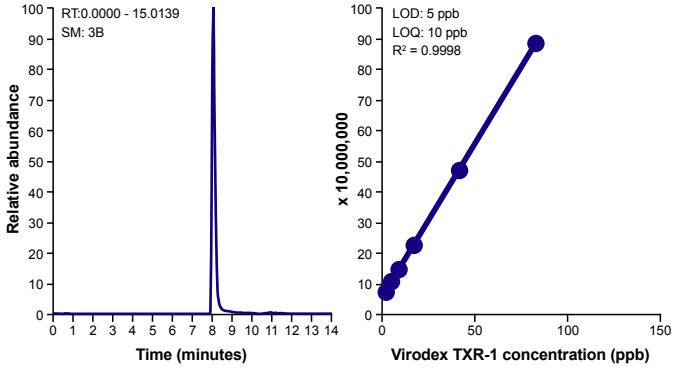


Figure 10. UHPLC-MS detection and quantification of Virodex™ TXR-1. The total ion chromatogram showing separation of Virodex™ TXR-1 is shown on the left. The standard curve generated by calculating the area under the curve for the characteristic mass of Virodex™ TXR-1 is shown on the right. Chromatography and detection were performed using a Thermo Scientific UHPLC and Orbitrap MS with an Oasis Max 2.1 x 20 mm column 30 µm (Waters). A gradient of 4mM ammonium formate in water and 4mM ammonium formate in 90:10 IPA:water was used. The flow rate was 0.25 mL/min and the injection volume was 10 µL.

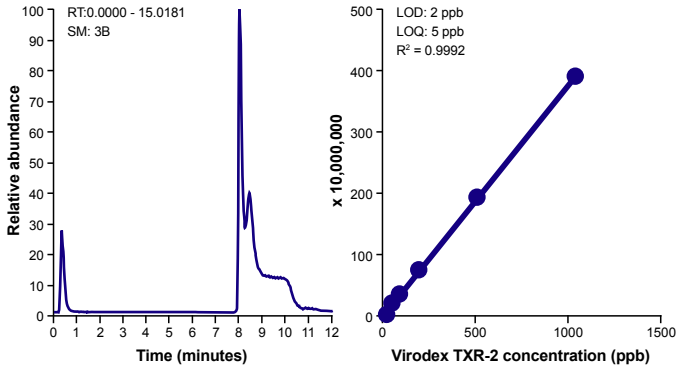


Figure 11. UHPLC-MS detection and quantification of Virodex™ TXR-2. The total ion chromatogram showing separation of Virodex™ TXR-2 is shown in the left pane. The standard curve generated by calculating the area under the curve for the characteristic mass of Virodex™ TXR-2 eluting at 8.0 min is shown on the right. Chromatography and detection was performed using a Thermo Scientific UHPLC and Orbitrap MS with an Oasis Max 2.1 x 20 mm column 30 µm (Waters). A gradient of 4mM ammonium formate in water and 4mM ammonium formate in 90:10 IPA:water was used. The flow rate was 0.25 mL/min and the injection volume was 10 µL.

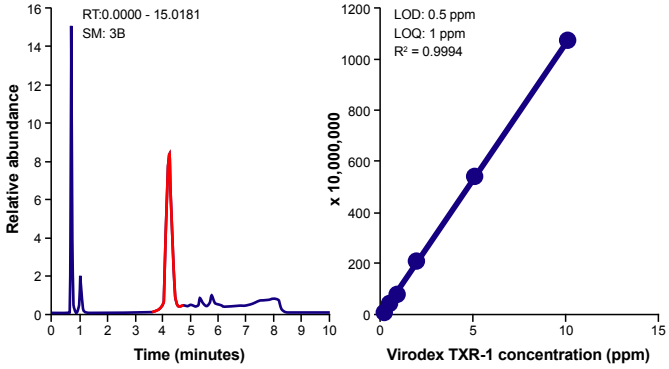


Figure 12. UHPLC-CAD detection and quantification of Virodex™ TXR-1. The CAD chromatogram showing separation of Virodex™ TXR-1 is shown in the left pane. The standard curve generated by calculating the area under the curve for the peak in red is shown on the right. Chromatography and detection was performed using a Waters UHPLC and Thermo Scientific CAD with an Acclaim Surfactant Plus 3.0 x 100 mm column 3 µm (Thermo Scientific). A gradient of 4mM ammonium formate in water and acetonitrile was used. The flow rate was 0.6 mL/min and the injection volume was 10 µL.

Revolutionise your bioprocess with Virodex™

Are you ready to revolutionise your bioprocess with **Virodex™**? Request samples today of our sustainable, compendial, cGMP EXCiPACT-manufactured, and REACH-compliant detergents to achieve efficient viral inactivation and cell lysis, and meet all of your bioprocessing needs.

Contact a Croda Pharma sales representative today or ask to speak with one of our technical or regulatory experts. Rely on our unmatched regulatory support to guide you through the impact of the Triton™ X-100 ban to ensure seamless compliance.

References

[1] Hunter, A. K. et al. Identification of compendial nonionic detergents for the replacement of Triton X-100 in Bioprocessing. Biotechnology Progress 38, (2022). [2] 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).

[3] Substance information ECHA. Available at: https://echa.europa.eu/substance-information/-/substanceinfo/100.123.919 (Accessed: 15 June 2023).

[4] Annex XIV Authorisation list (2022) ECHA. Available at: https://www.echa.europa.eu/authorisation-list (Accessed: 19 May 2023).

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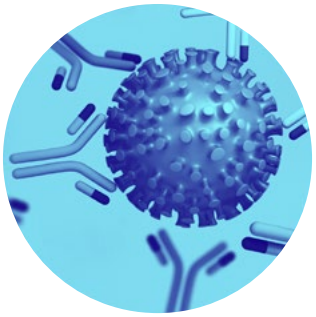
Small Molecule Delivery

Protein Delivery

Nucleic Acid Delivery



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Europe, Middle East & Africa: pharma.emea@croda.com
Asia Pacific: pharma.asia@croda.com
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