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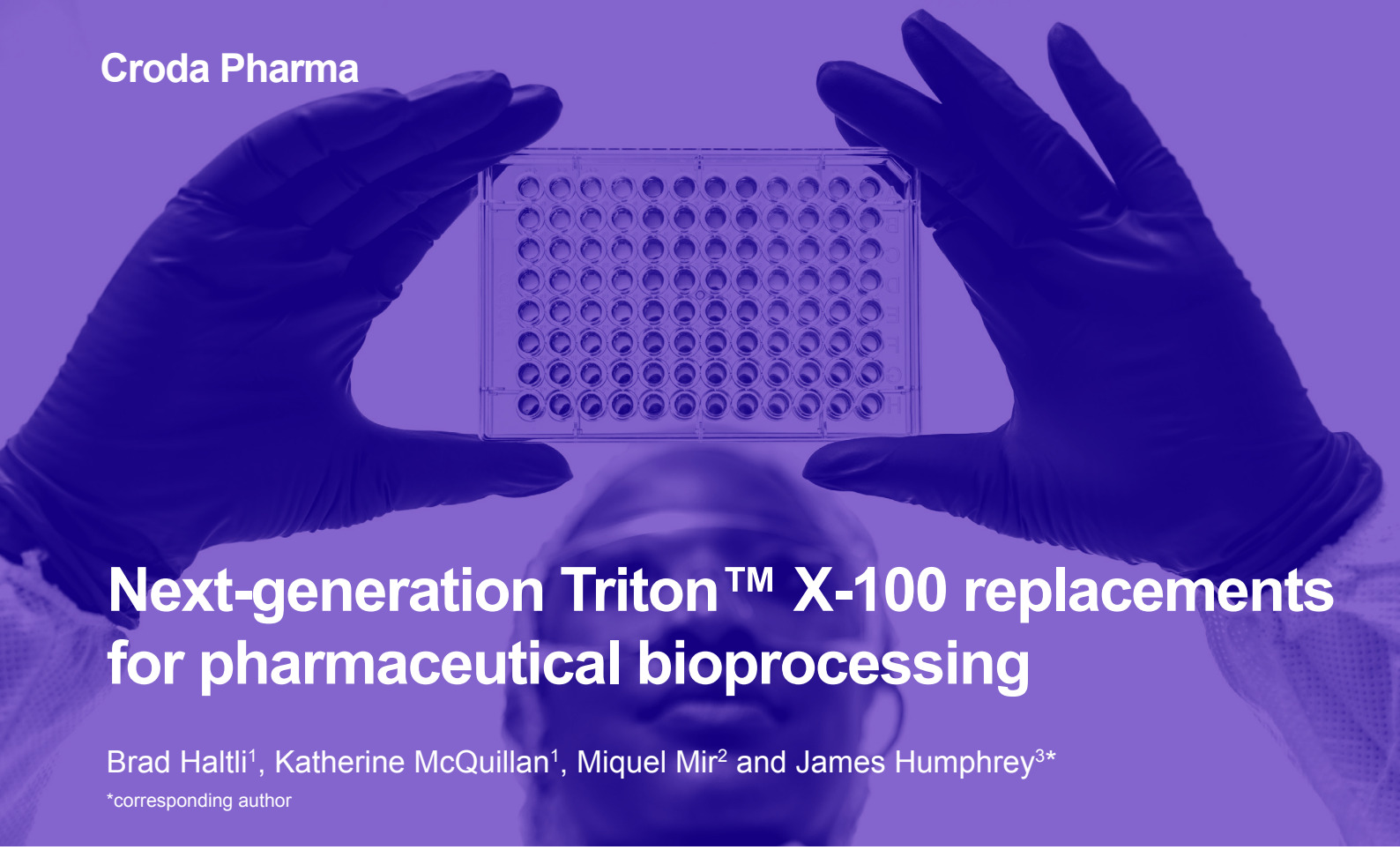

Next-generation Triton™ X-100 replacements for pharmaceutical bioprocessing

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Abstract

Triton™ X-100 (tert-octyl phenyl ethoxylate) is used extensively in the manufacture of biologics, plasma-derived products and cell and gene therapies (CGTs). This non-ionic detergent is used because it readily disrupts biologic membranes while leaving proteins unharmed. As a result, it is widely used for cell lysis applications and to inactivate adventitious lipid-enveloped viruses that may contaminate biologic manufacturing processes that employ human and animal cell lines. Degradation of Triton™ X-100 in the environment results in the formation of endocrine disrupting by-products that are toxic to aquatic organisms. As a result, the European Chemicals Agency (ECHA) added Triton™ X-100 to the list of Substances of Very High Concern (SVHC) in 2017, leading to a complete ban, except for a small number of publicly listed exemptions on the use of this chemical in the European Union as of 4th January 2021. The ban of Triton™ X-100 is anticipated to be adopted by other jurisdictions in the near future. As a result, there is an urgent need for new safe, sustainable and effective alternatives to Triton™ X-100. To address the needs of the biopharmaceutical industry we developed two chemically distinct detergents, **Virodex™ TXR-1** and **TXR-2**, that show equivalent or better cell lysis and virus inactivation (VI) activity than Triton™ X-100. The **Virodex™** detergents were also compared to three competitor products in VI tests and were shown to outperform the competitors in this important application. The **Virodex™** detergents are non-denaturing detergents that do not affect the structure of proteins, making them ideal reagents for processes that generate protein-based therapeutics or non-enveloped viral vectors used in CGT products. To facilitate their adoption in biopharmaceutical manufacturing processes, the **Virodex™** detergents are compendial-grade materials manufactured to cGMP EXCiPACT standard. Functional assessment of three batches of each detergent in VI tests demonstrated excellent batch-to-batch consistency for both products. To facilitate the detection and quantification of the **Virodex™** detergents in finished products and waste streams, we developed liquid chromatographic analytical methods with universal detectors capable of detecting and quantifying low parts-per-million or billion concentrations of the detergents. Analytical detection methods were utilised to assess the affinity of the Virodex products to protein A resin, no binding was seen demonstrating the ease of depletion in standard downstream processing. The **Virodex™** chemistries are not on the ECHA list of SVHC and have several positive sustainability characteristics that set them apart from Triton™ X-100. The results presented here clearly demonstrate that **Virodex™ TXR-1** and **TXR-2** are high performance, safe and sustainable replacements for Triton™ X-100.

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Introduction

Biological therapeutics, commonly referred to as “biologics” are a class of medicines that are manufactured via large-scale cultivation of bacteria, yeast, plant or animal cells. This diverse group of medicines includes vaccines, antibodies, immune modulators and growth factors, as well as products derived from human blood and plasma. Typically, biologics are purified proteins and are differentiated from conventional modalities which are small molecule chemicals produced synthetically or purified from plants or microorganisms.[1]

Manufacturing of biologics is a complex process that utilises living production systems which present challenges not encountered in the manufacturing of conventional medicines. Due to the extensive use of human and animal cell lines in biologic manufacturing, these products are susceptible to adventitious microbial contaminants. Blood and plasma-derived therapies may contain blood-borne pathogens and are also susceptible to contamination. Due to their small size and difficulty to detect, viral contaminants are of particular concern. Unfortunately, there are several examples in the twentieth century of viral contamination of released vaccines and blood-derived plasma which resulted in the infection of patients receiving these treatments[2]. These lessons resulted in increased regulation and the development of integrated strategies to ensure product safety. Current best practice dictates the use of three complimentary strategies (the “three pillars” approach) to prevent the release of products contaminated with viral pathogens and involves 1) section of raw materials with a low risk of viral pathogen content, 2) testing of cell banks and in-process samples for viral contaminants and 3) inactivation and removal of undetected adventitious or endogenous virus during downstream processing steps.[2]

Downstream processing (DSP) for biologic products can vary significantly but typically utilise one or more virus removal steps to mitigate the risk of viral contamination and ensure product safety. DSP protocols employ multiple orthogonal techniques to remove and inactivate viruses and viral particles through chromatographic purification and/or nanofiltration and virus inactivation (VI) by exposure to heat, low pH or detergent treatment (Figure 1). For biologics that are labile when exposed to heat or low pH conditions, treatment with non-ionic detergents is the preferred VI approach as non-ionic detergents do not denature proteins and can be removed from the finished product using commonly used chromatographic purification steps.[3]

Cell and gene therapy (CGT) products can utilise viral vectors to deliver altered genes to cells either in vivo or ex vivo to treat conditions attributed to genetic disorders. The viral vectors used for these therapies are manufactured using production cell lines, and as a result are susceptible to endogenous and adventitious viral contamination. Like protein-based biologics, the risk of viral contamination is mitigated in CGT products using the three pilar strategy where detergent-based VI is an important strategy for virus removal from non-enveloped viral vectors that are not sensitive to mild detergent treatment, such as adeno-associated viruses (AAVs). AAVs are produced in the cell and detergent treatment is used to both lyse the cells to release the AAV and inactivate adventitious enveloped viruses.[4]

The detergent most frequently used for VI and cell lysis in biologic and CGT manufacturing is tert-octyl phenol ethoxylate, which is more commonly known by the Dow Chemical Company trade name Triton™ X-100. Triton™ X-100 is a non-ionic surfactant with a hydrophilic polyethylene oxide chain and a hydrophobic alkylated aromatic group (Figure 2).[5] This detergent has found widespread use in pharmaceutical industries with diverse applications including endotoxin removal, VI, cell lysis, and membrane permeation.[6] However, as research on Triton™ X-100 advanced, concerns emerged regarding the safety of its degradation products. Like other alkyl phenolic compounds, the degradation products from Triton™ X-100 exhibit long-lasting estrogenic properties in fish, birds, and mammals, causing disruptions to their endocrine systems.[7] In response to these environmental and health concerns, the European Chemicals Agency (ECHA) took regulatory action and included Triton™ X-100 in the Authorisation List (Annex XIV) in 2017 under the EU REACH regulation, leading to a total ban from 2021 on its use in products manufactured in Europe and defined limits on those manufactured elsewhere but exported to Europe that contain Triton™ X-100.[8]

Despite these regulatory developments, some pharmaceutical manufacturers outside Europe continue to employ Triton™ X-100 in biologic and CGT manufacturing processes. However the latest version of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Humans Use (ICH) Guideline Q5A(R2) for the Viral Safety Evaluation of Biotechnology Products derived from Cell Lines of Human or Animal Origin.[9] clearly highlights concerns about the hazards posed by Triton™ X-100’s degradation products and recommends the adoption of other commercially available detergent alternatives possessing similar

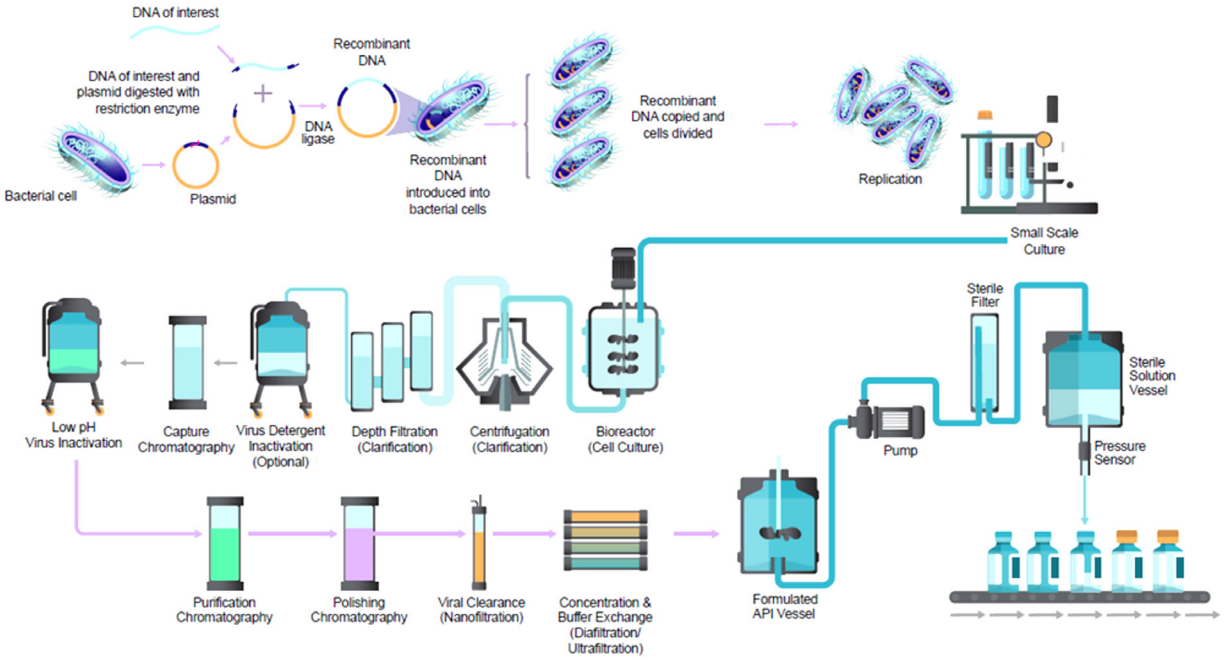

Figure 1. Overview of a model biopharmaceutical production process indicating steps where virus removal or inactivation may take place.

physicochemical properties and equivalent VI capabilities.[9] Given that ICH guidelines are recognized by health authorities in Europe (EMA), the United States (FDA), Japan (PMDA), and other countries, phasing out of Triton™ X-100 is anticipated in regions beyond Europe, likely leading to a global phasing out of Triton™ X-100 and potential ban in many key regions. To avoid time-consuming and costly modifications to approved manufacturing processes it is imperative that both global and regional biopharmaceutical and CGT manufacturers proactively adopt alternative detergents for VI and cell lysis to avoid filing for process changes in the future.

To enable the transition away from Triton™ X-100, alternative detergents should provide equivalent or better performance in DSP operations (cell lysis and VI), while possessing similar physiochemical properties to Triton™ X-100. Alternative detergents should be easily handled (i.e., not highly viscous) and readily water soluble under normal processing temperatures, be compatible with biopharmaceutical products and be readily removable by common DSP techniques. Sensitive analytical detection methods should also exist to quantify residues in finished products and waste streams. To facilitate their rapid adoption in cGMP manufacturing processes Triton™ X-100 alternatives should be available as compendial and cGMP grades. Detergent alternatives should also be sustainable and free of potential safety or environmental concerns. 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 materials.

Identification of Triton™ X-100 replacements

As a leading manufacturer of detergents and pharmaceutical excipients, Croda set out to meet the growing need for Triton™ X-100 alternatives by evaluating our extensive catalog of detergents for performance in bioprocessing applications. As described above, VI is a key step in the DSP 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 structural classes were screened for VI and their performance was compared to Triton™ X-100.

VI was assessed using xenotropic murine leukaemia virus (XmuLV), which is an established model lipid-enveloped virus frequently used to assess detergents for VI.[9-12] The initial assessment of 31 detergents identified 13 which showed equivalent performance to Triton™ X-100 and reduced the level of viable viral particles by a factor of 1000, resulting in a calculated log reduction factor (LRF) of three within 120 min. One log reduction is equivalent to a ten-fold reduction in viable viral particles, thus an LRF of three reduces the number of viable viral particles by 1000-fold, or by 99.9 %. The detergents passing this criterion were then compared on the basis of potency of VI. Three samples belonging to the fatty alcohol ethoxylate (2) and the ethoxylated glyceride (1) classes achieved the maximum level of VI (LRF3) at 0.025 % (v/v), which was equivalent to the performance of Triton™ X-100. Of these three detergents, two were manufactured under cGMP standards and were available as compendial materials (Table 1). This selection process resulted in the prioritization of two detergents, **Virodex™ TXR-1** and **TRX-2**, for further characterization as candidate replacements for Triton™ X-100.

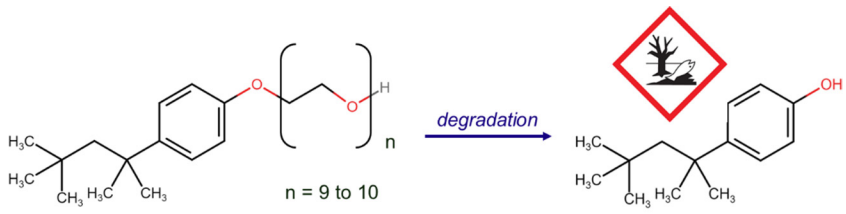

Figure 2. Triton™ X-100 and its toxic degradation product.

Detergent class	Samples tested	Primary VI assay	VI potency	cGMP	Compendial	Triton™ X-100 replacement
Alkyl amine oxide	1	1	-	-	-	
Alkyl polyglucoside	3	2	-	-	-	
Alkyl sarcosinate	1	1	-	-	-	
EO/PO polysorbate	1	0	-	-	-	
Ethoxylated glyceride	1	1	1	1	1	**Virodex™ TXR-2**
Fatty acid ethoxylate	1	-	-	-	-	
Fatty alcohol ethoxylate	9	7	2	1	1	**Virodex™ TXR-1**
PEG	1	-	-	-	-	
Polyglyceryl ester	1	-	-	-	-	
Polysorbate	10	1	-	-	-	
Triglyceride ethoxylate	2	-	-	-	-	
Total	**31**	**13**	**3**	**2**	**2**	

Table 1. Evaluation of Triton™ X-100 replacements from 31 candidate detergents. Thirty-one detergents belonging to 11 classes were assessed for VI in a primary assay where Triton™ X-100 achieved an LRF of three at 0.1 % (v/v). Samples exhibiting equivalent performance to Triton™ X-100 were then assessed for VI potency using a VI dose-response assay. VI was determined using the XmuLV model of lipid-enveloped VI. In this assay, XmuLV stocks were treated with detergents 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} (Viral $Titer_{untreated}$ / Viral $Titer_{treated}$)] = Log Reduction Factor. Three samples exhibited equivalent potency to Triton™ X-100 (LRF of 3 at 0.025 % v/v). Two materials were cGMP and compendial leading to the section of two detergents as the **Virodex™** series of bioprocessing reagents.

Virus inactivation kinetics

The time required to achieve a LRF of 4 to 5 (99.99-99.999 % VI) is an important characteristic of a detergent used in DSP applications. Temperature is also an important VI variable as DSP may occur above, or below, room temperature depending on the specific process parameters. To determine the kinetics of VI by **Virodex™ TXR-1** and **Virodex™ TXR-2**, inactivation of XmuLV was assessed at three time points (5 min, 15 min, 60 min) at three temperatures relevant to DSP (15 °C, 22 °C, 28 °C) (Figure 3).

At all temperatures the **Virodex™** detergents showed equivalent or superior performance to Triton™ X-100. Both **Virodex™** detergents achieved a LRF of 3 or greater within 5 min and reached the maximum level of VI (LRF of 6-8) after 60 min. **Virodex™ TXR-1** exhibited faster VI kinetics at 15 °C and 22 °C than Triton™ X-100 or **Virodex™ TXR-2**. These results clearly show that both **Virodex™** detergents possess exceptional VI activity, and exhibit equivalent or better VI activity than Triton™ X-100.

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 the cell membrane and nucleus, and cell lysis was assessed by fluorescent microscopy.

Lysis was evident by disintegration of the cell membrane (green) and nucleus (red) in the microscopic images (Figure 4). In the case of HEK-293T cells **Virodex™ TXR-2** and Triton™ X-100 lysed cells at the same concentration (0.0625 % v/v), while **Virodex™ TXR-1** lysed cells at a four-fold lower concentration (0.0156 % v/v) (Figure 4). The **Virodex™** detergents and Triton™ X-100 showed equivalent lysis of CHO-K1 cells, with lysis occurring at 0.0156 % (v/v) (Figure 4).

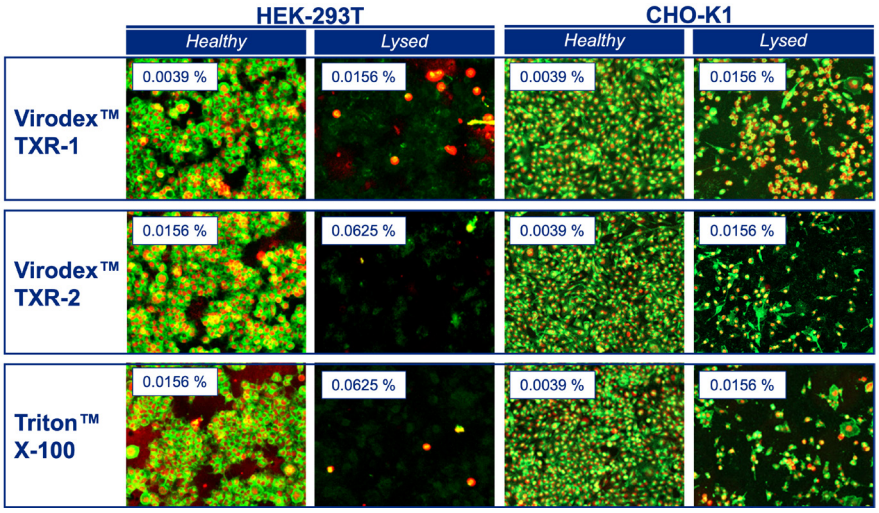

Figure 4. Lysis of HEK-293T and CHO-K1 cells. Cells were treated with detergents (0.00015 - 1 %) for 2 h at 22 °C and then stained with Alexa Fluor 488-conjugated wheat germ agglutinin (green - membrane) and Hoechst 3328 (red - nucleus) stains. Cell imaging was performed using a Cytation 5 multimode imaging plate reader (BioTek). The transition from healthy cells to a lysed state are indicated as shown in the images. Detergent concentrations are indicated in each image (% v/v).

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-293T 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 medium, where it dephosphorylates its substrate forming a purple product that can be monitored spectrophotometrically.[13] 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

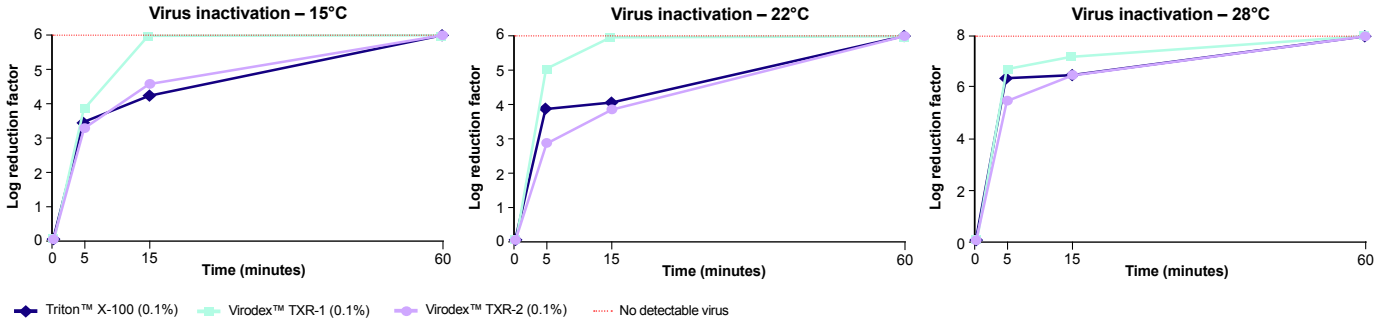

Figure 3. Inactivation kinetics of XmuLV by **Virodex™ TXR-1** and **Virodex™ TXR-2** and Triton™ X-100 at 0.1 % (v/v) at three different temperatures. The dotted red line at the top of the graph indicates the LRF above which no viable virus can be detected. XmuLV was treated with each detergent at a concentration of 0.1 % (v/v) at the indicated temperature and aliquots were removed at 5 min, 15 min and 60 min and used to infect *F. catus* PG-4 cells.

ranging from 0.156 % - 2.5 % for 2 h at 37 °C. After 2 h, 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 significant negative effect on protein stability, reducing SEAP activity by 75 % at 0.625 % and totally abolishing activity at concentrations ≥ 1.25 % (Figure 5). In contrast, **Virodex™ TXR-1** and **TXR-2** caused no reduction in SEAP activity at concentrations up to 2.5 %, showing very similar protein compatibility as Triton™ X-100 (Figure 5). The protein compatibility of **Virodex™ TXR-1** and **TXR-2** is consistent with their non-ionic nature. These results show that protein function is not negatively impacted at concentrations well above typical detergent application rates (0.1 % -1 %) used in biopharmaceutical manufacturing processes.

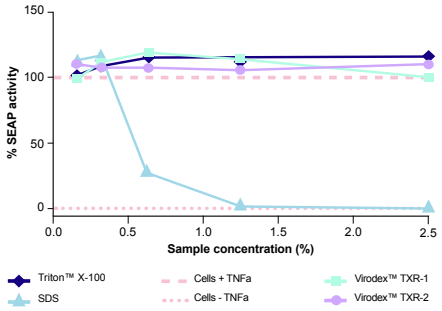

Figure 5. Activity of SEAP after detergent treatment. Expression of SEAP by HEK-293T cells was induced by treatment with tumor necrosis factor alpha (TNFa) and 100 % activity was defined as SEAP activity in untreated controls (pink dashed line). Base-line SEAP activity (0 %) was determined using cells that were not induced with TNFa (pink 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 VI activity of the **Virodex™** detergents to three competitor products at 22 °C. The comparison to Competitor 1, an alkyl polyglucoside detergent, is shown on the top panel in Figure 6. In this comparison all detergents were tested at a 0.1 % (v/v) concentration at 22 °C and monitored over 60 min. The grey line representing Competitor 1 doesn't reach the maximum LRF after 60 min. Competitor 1 was also tested for its ability to lyse CHO-K1 cells, and while it was capable of lysing the cells, it required a concentration 16 times higher than **TXR1** and **TXR2** to achieve lysis (data not shown).

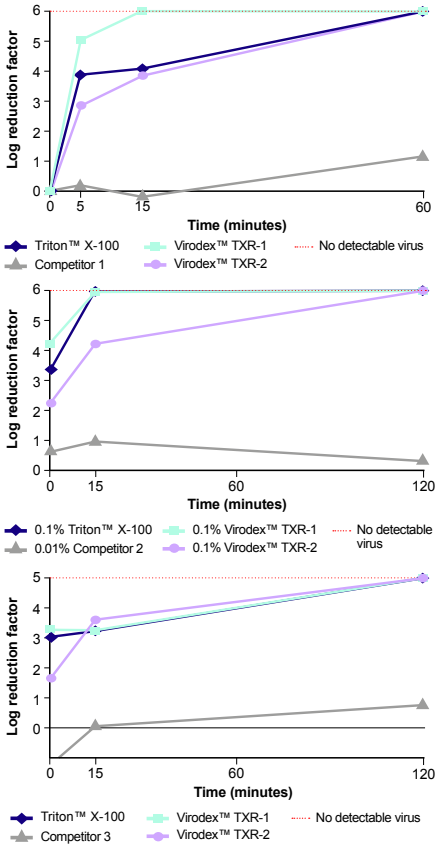

Figure 6. XmuLV VI by three competitors compared to **Virodex™ TXR-1** and **TXR-2** and Triton™ X-100. Competitor products, **Virodex™** detergents and Triton™ X-100 were tested at 22 °C at a concentration of 0.1 % (v/v), unless indicated otherwise. The dotted red line indicates the LRF at which no additional virus can be detected.

Competitor 2 is a detergent marketed for VI and the comparison to the **Virodex™** detergents is shown in the middle panel in Figure 6. Efforts to test Competitor 2 for VI were complicated by the toxicity of this detergent towards the astrocyte cell line used in the VI assay.

As a result of the toxicity of Competitor 2 we were only able to test VI for this material at 0.01 %. Under the tested conditions Competitor 2, the grey line, was unable to reach a log reduction factor greater than 1, while the **Virodex™** detergents, the aqua and purple lines, reached a LRF of 6. While this was not a head-to-head comparison, under the conditions tested and in terms of cytotoxicity profiles, the **Virodex™** detergents outperformed Competitor 2.The final comparison to Competitor 3 is shown on the bottom panel in Figure 6. This was a head-to-head comparison at identical concentrations to Triton™ X-100 and the **Virodex™** detergents at 0.1 %. Competitor 3, represented by the grey line wasn't able to reach a LRF of 1 after 120 min, while the **Virodex™** products and Triton™ X-100 achieved a 5 log reduction. Based on these comparisons we can confidently conclude that the **Virodex™** range of detergents clearly outperform these three competitor products for VI applications.

Virodex™ product 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 VI kinetics of three distinct lots of **TXR-1** and **TXR-2**. The three batches of **TXR-1** exhibited nearly identical VI kinetics to each other (Figure 7). Slight variation was observed between the three batches of **TXR-2** (Figure 7); 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 VI applications.

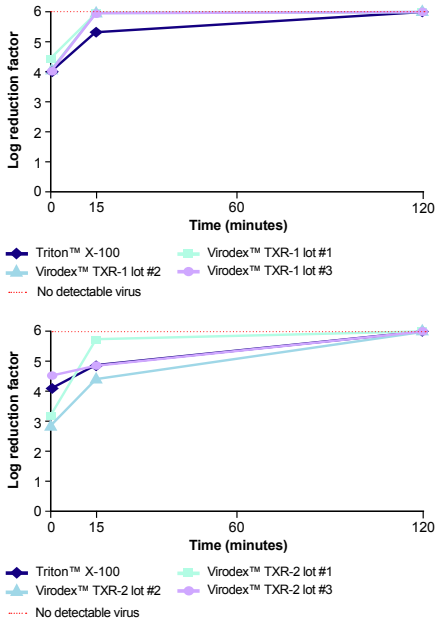

Figure 7. XmuLV VI kinetics of three batches of **Virodex™ TXR-1** and **TXR-2** compared to Triton™ X-100. XmuLV was treated with each detergent at a concentration of 0.1 % (v/v). Detergent treatments were conducted at 22 °C and aliquots were removed at 1 min, 15 min and 120 min and used to infect *F. catus* PG-4 cells. The dotted red line indicates the LRF at which no additional virus can be detected.

Virodex™ removal: Protein A column affinity

In the manufacture of biotherapeutic proteins, chromatography is typically used to purify the biologic drug substance. Protein A resin is commonly used to purify mAbs as protein A exhibits reversible and specific binding between the immobilized protein A ligand and antibodies. In mAb manufacturing processes utilising Triton™ X-100, the detergent is removed from the drug product alongside other impurities in washes applied prior to mAb elution. **Virodex™ TXR-1** and **Virodex™ TXR-2** were tested for their affinity to protein A affinity resin using MabCaptureC™ MiniChrom columns (Thermo Fisher) using typical loading, washing, elution and cleaning buffers. To assess protein A affinity, the elution profile of the **Virodex™** detergents from the MabCaptureC™ MiniChrom columns was compared to empty column controls and detergent concentrations in eluates were determined using an HPLC-ELSD method analogous to the HPLC-CAD method described in the following section. The elution profiles of **Virodex™ TXR-1** and **TXR-2** from empty column controls were nearly identical to the profiles obtained from the protein A resin columns (Figure 8). In chromatography runs with and without protein A resin, the concentration of **Virodex™ TXR-1** dropped to less than 5 ppm at a column volume (CV) of 38, while the concentration of **Virodex™ TXR-2** dropped to less than 10 ppm at a CV of 29. This data shows that neither **Virodex™** detergent have any affinity to the protein A resin and can effectively be removed from the biotherapeutic product during affinity chromatography purification.

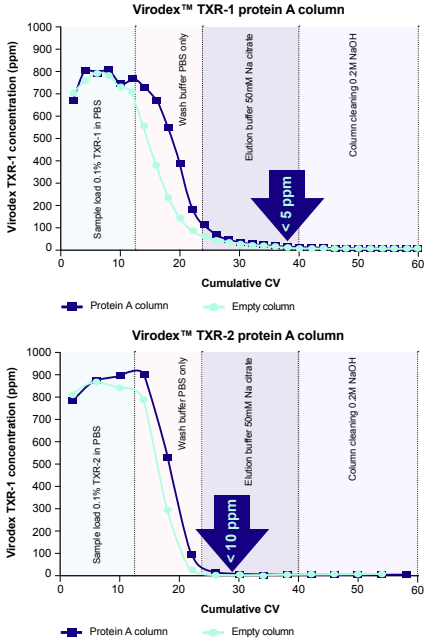

Figure 8. Protein A column affinity of **Virodex™ TXR-1** and **TXR-2**. Protein A resin affinity was assessed using MabCaptureC™ MiniChrom columns (ThermoFisher). **Virodex™** detergents in chromatography eluates was measured by HPLC-ELSD. The limit of quantification was 5 ppm for **Virodex™ TXR1** and 10 ppm for **Virodex™ TXR-2**. One CV was 1 mL.

Virodex™ analytical detection

Reliable and sensitive detection and quantification methods are essential to confirm detergent removal in biopharmaceutical finished products and in waste streams. Croda has developed chromatography methods that detect and quantify **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 (R^2 - 0.9998) (Figure 9). 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 10).

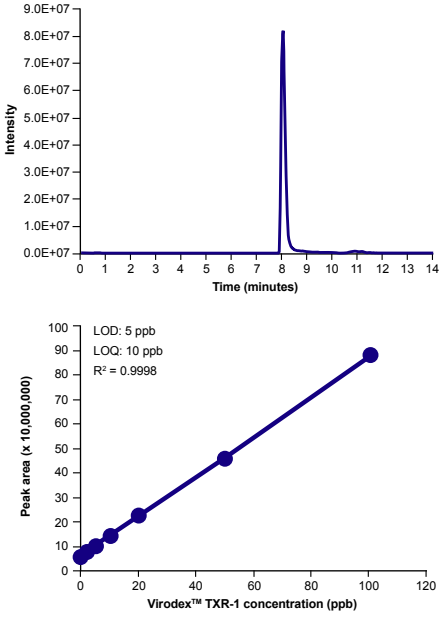

Figure 9. UHPLC-MS detection and quantification of **Virodex™ TXR-1**. A)The total ion chromatogram showing separation of **Virodex™ TXR-1** (1 ppm). B) The standard curve generated by calculating the area under the curve for the characteristic mass (*m/z* 644.4928) of **Virodex™ TXR-1** eluting at 8 min. 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 4 mM ammonium formate in water and 4 mM ammonium formate in 90:10 isopropanol:water was used. The flow rate was 0.25 mL/min and the injection volume was 10 µL.

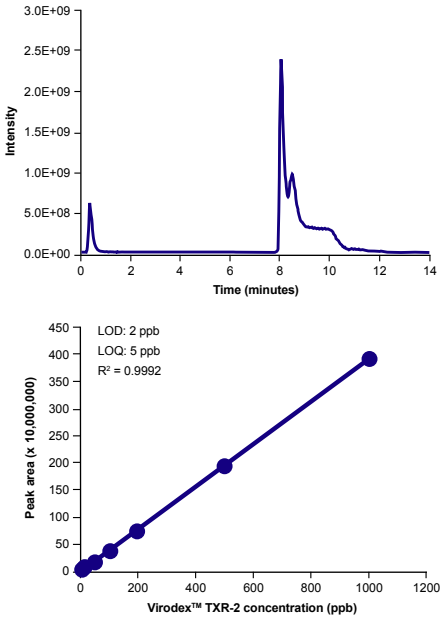

Figure 10. UHPLC-MS detection and quantification of **Virodex™ TXR-2**. A) The total ion chromatogram showing separation of **Virodex™ TXR-2**. B)The standard curve generated by calculating the area under the curve for the characteristic mass (*m/z* +44 from 544.369 to 808.526) of **Virodex™ TXR-2** eluting at 8.0 min. 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 4 mM ammonium formate in water and 4 mM ammonium formate in 90:10 isopropanol:water was used. The flow rate was 0.25 mL/min and the injection volume was 10 µL.

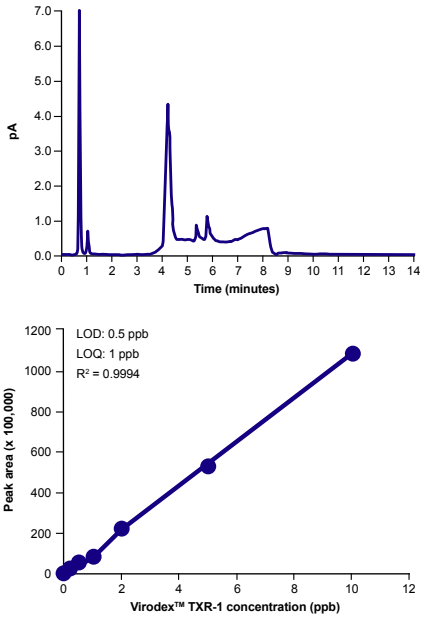

Figure 11. UHPLC-CAD detection and quantification of **Virodex™ TXR-1**. A) CAD chromatogram showing separation of **Virodex™ TXR-1** (5 ppm). B) Standard curve generated by calculating the area under the curve for the **Virodex™ TXR-1** peak indicated in the chromatogram. Chromatography and detection 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 4 mM ammonium formate in water and acetonitrile was used. The flow rate was 0.6 mL/min and the injection volume was 10 µL.

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). A CAD method was chosen over a photodiode array detector because the **Virodex™** detergents lack a chromophore that can be tracked using this common detector. CAD detectors are commonly used in the biopharmaceutical industry and provides sensitive, near-universal, quantitative detection of semi- and non-volatile analytes. This CAD-based 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 11). 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.

Conclusions

Detergents are integral to the DSP of biologicals, plasma-derived products and CGTs, providing several key functions ranging from cell lysis[5] to VI[4,8-12] and endotoxin removal.[14,15] Triton™ X-100 has been used widely in biopharmaceutical, plasma-derived product and CGT manufacturing as this mild, non-ionic detergent doesn't denature protein-based therapeutics or inactivate non-enveloped viral vector therapeutics such as adeno-associated viruses (AAVs), while it effectively lyses cells, inactivates lipid-enveloped viruses and solubilizes endotoxin.[5,10,12, 14,15] The discovery of the ecotoxicological effects of the degradation products of Triton™ X-100 and other octylphenol ethoxylates led to their inclusion on the ECHA candidate list of substances of very high concern (SVHC). Substances on this list are carcinogenic, mutagenic and/or toxic to reproductive systems, and the 4-(1,1,3,3-tetramethylbutyl)phenol breakdown product of Triton™ X-100 is especially harmful to aquatic animals even at low concentrations due to its endocrine disrupting activity and is considered persistent, bioaccumulative and toxic.[16,17] Triton™ X-100 was included in the ECHA's authorization list (Annex XIV) as SVHC in 2017 and is banned in Europe for the manufacture of investigational medical products and new medical products.[8] While the use of Triton™ X-100 is not prohibited in jurisdictions outside the European Union, efforts toward regulatory harmonization makes it likely that regulators in other countries will enact similar restrictions on the use of this detergent.[9]

To identify high-performing replacements for Triton™ X-100 we screened 31 detergents for candidates that met or exceeded the performance characteristics of Triton™ X-100. Two chemistries, **Virodex™ TXR-1** and **TXR-2**, exhibited outstanding performance in several important criteria relevant to DSP in the manufacture of biologics and CGTs. The **Virodex™** chemistries exhibited strong VI activity and rapidly inactivated XmuLV over a range of temperatures commonly encountered in manufacturing processes (15 °C - 28 °C). They also showed broad utility as lysis reagents, effectively lysing two cell lines akin to those used in biomanufacturing processes (HEK-293T and CHO-K1). Both **Virodex™** products are non-ionic, ensuring that they are mild detergents that will not denature proteins. The **Virodex™** products did not negatively affect the enzyme activity of SEAP at concentrations up to 2.5 %, which is well above those typically used for VI.[9]

The results presented here are consistent with the results of Hunter and colleagues, who determined that the chemistries of **Virodex™ TXR-1** and **TXR-2** were as effective as Triton™ X-100 for cell lysis and VI applications.[12] These authors also assessed the impact of these detergents on the yield, structure and function of monoclonal antibodies purified by affinity chromatography and found no detrimental effects, even after extended incubation periods of up to 24 h. Furthermore, they assessed the impact of the **TXR-1** chemistry on AAV yield before and after affinity purification and found equivalent performance to the Triton™ X-100 benchmark.[12] We extended the evaluation of the **Virodex™** detergents to include a comparison to three other products marketed for VI. In all cases, the **Virodex™** products outperformed the competitors under the conditions tested. Collectively, these results demonstrate that the **Virodex™** detergents are versatile and effective high performing Triton™ X-100 alternatives for biologic and CGT manufacturing.

To enable the rapid adoption of the **Virodex™ TXR-1** and **TXR-2** detergents as Triton™ X-100 replacements in highly regulated biologic manufacturing processes, we recognize the importance of product quality. Both **Virodex™** detergents are manufactured to the highest quality standards, and are compendial-grade, cGMP EXCiPACT manufactured. We demonstrated the excellent manufacturing consistency of these products by showing highly reproducible VI activity of three distinct batches of each product. To further aid in the adoption of these products in biomanufacturing process, we developed highly sensitive, quantitative analytical methods capable of detecting and quantifying the detergents at low ppb-levels. The availability of this methodology will enable manufactures to rapidly and accurately quantify the detergents in finished products and waste streams.

From an ease-of-use perspective, Triton™ X-100 replacements should be easy to incorporate into existing processes. The properties of **Virodex™ TXR-1** and **TXR-2** are summarized in Table 2. Both detergents are readily water soluble up to 10 % (w/w) and exhibit lower viscosity than Triton™ X-100.[12] At temperatures below 25 °C **Virodex™ TXR-1** may become a paste, however, this can be easily reversed by heating to 30 °C prior to use. Triton™ X-100 replacements should also be free of sustainability concerns. The **Virodex™** chemistries are not listed as SVHC by the ECHA under the REACH regulations. In addition, **TXR-1** is readily biodegradable and is manufactured at a site with zero scope 2 emissions, while **TXR-2** has a 40 % biobased content.

	Virodex™ TXR-1	Virodex™ TXR-2
Classification	Non-ionic detergent	Non-ionic detergent
Chemistry	Alkyl alcohol ethoxylate	Ethoxylated glyceride
Name(s) and monographs(s)	Macrogol Lauryl Ether 9 (Ph. Eur.) Polyoxyl 9 Lauryl Ether (USP)	Macrogol 6 glycerol caprylocaprate (Ph. Eur.)
CAS number	9002-92-0	361459-38-3
Physical form at room temperature	Pale yellow paste/liquid	Colourless to yellow liquid
Melting point	15-22 °C	Lower than 40 °C
Viscosity (25 °C)	70 mPa·s (liquid at 25 °C)	150 mPa·s
Solubility	Water soluble 0-10 % w/w aq clear solution	Water soluble, with 0.1 % aq slight haziness 1-10 % w/w aq clear solution
Sustainability	Readily biodegradable; zero scope 2 manufacturing emissions	40 % biobased
Regulatory	REACH compliant EXCiPACT cGMP manufactured	REACH compliant EXCiPACT cGMP manufactured
Virus inactivation	Yes - equivalent activity to Triton™ X-100	Yes - equivalent activity to Triton™ X-100
Cell lysis	Yes (CHO-K1 and HEK293T validated)	Yes (CHO-K1 and HEK293T validated)
Protein compatibility	Yes - non denaturing	Yes - non denaturing
Analytical detection	RP-UHPLC-MS: LOD 5 ppb, LOQ 10 ppb RP-UHPLC-CAD: LOD 0.5 ppbm; LOQ 1ppm	RP-UHPLC-MS: LOD 2 ppb, LOQ 5 ppb
Protein A column affinity	No affinity (fully removed)	No affinity (fully removed)
Freeze-thaw stability	10 % w/w - Clear solution, unchanged after 14 cycles 1 % w/w - Clear solution, unchanged after 14 cycles	10 % w/w - Clear solution, unchanged after 14 cycles 1 % w/w - Hazy solution, unchanged after 14 cycles

Table 2. Characteristics of **Virodex™ TXR-1** and **TXR-2**

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